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Genetic and molecular characterization of a novel *iab-8* regulatory domain in the *Abdominal-B* gene of *Drosophila melanogaster*

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SUMMARY

Homeotic (or Hox) genes are key determinants in specifying the anteroposterior axis of most animals. The temporal and spatial expression of these genes requires the presence of large and complex *cis*-regulatory regions. The *Abdominal-B* Hox gene of the bithorax complex of *Drosophila* is regulated by several *infraabdominal* domains, which determine *Abdominal-B* expression in abdominal segments A5 to A9 (parasegments 10 to 14). Some of the *infraabdominal* domains have been characterized, including an *infraabdominal*-8 domain, which has been located 3′ to the *Abdominal-B* transcription unit. We have analyzed the expression and mutant phenotype of a P-*lacZ* element inserted close to the *Abdominal-B m* origin of transcription and of derivatives of this transposon. Some of these derivatives represent a particular class of mutations in the bithorax complex, because they transform the eighth and ninth abdominal segments without affecting more anterior metameres. The analysis of these mutations and of transformants carrying sequences upstream the *Abdominal-B m* transcription unit has allowed us to define a new *infraabdominal*-8 regulatory region, located 5′ to the *Abdominal-B* transcription unit, and has helped to characterize better the complex regulation of the *Abdominal-B* gene.

Key words: *Drosophila*, Hox, *Abdominal-B*, *iab* regulatory domains

INTRODUCTION

Homeotic (Hox) genes determine body structures along the anteroposterior axis in most organisms. In *Drosophila*, the homeotic genes are located in two clusters, the bithorax complex (BX-C) and the Antennapedia complex (ANT-C) (Kaufmann et al., 1990; Lewis, 1978; Sánchez-Herrero et al., 1985), which generate segmental diversity in the embryo and the adult. This segmental specification is achieved by the particular temporal and spatial distribution of Hox proteins. Such a fine regulation requires the presence of large *cis*-regulatory regions (Bender et al., 1983; Karch et al., 1985; Kaufmann et al., 1990).

The *Abdominal-B* (*Abd-B*) Hox gene of the BX-C specifies abdominal segments A5 to A9 [parasegments (PS) 10 to14] (Casanova et al., 1986; Sánchez-Herrero et al., 1985; Tiong et al., 1985). It codes for two proteins that share the C-terminal amino acid sequence, including the homeodomain, but differ in the N-terminal region: Abd-B M (or Abd-B I) protein, which is expressed in PS10-13; and Abd-B R (or Abd-B II) protein, which is present in PS14 (Boulet et al., 1991; Celniker et al., 1989; DeLorenzi and Bienz, 1990; Zavortink and Sakonju, 1989) (see Fig. 1A). Mutations that affect the Abd-B M protein complement those affecting the Abd-B R protein, and both types of mutations fail to complement Abd-B mutations that eliminate the two products (Casanova et al., 1986; Celniker et al., 1990; DeLorenzi and Bienz, 1990). Abd-B M expression starts in PS13 and is later observed, successively, in PS12, PS11 and PS10, with increasing levels in more posterior parasegments (Celniker et al., 1989; DeLorenzi and Bienz, 1990). This indicates that there are regulatory sequences controlling *Abd-B* expression in these parasegments.

The *Abd-B m* regulatory region extends over more than 50 kb 3′ to the *Abd-B* transcription unit, and is genetically subdivided into several *infraabdominal* (*iab*) domains, from *iab-5* to *iab-8* (Barges et al., 2000; Galloni et al., 1993; Gyurkovics et al., 1990; Karch et al., 1985; Lewis, 1978; Zhou et al., 1999). Deletions of these domains cause transformations of the corresponding parasegment into the next, more anterior one (Galloni et al., 1993; Mihaly et al., 1997). DNA elements within these domains, when fused to the *lacZ* gene, direct β-galactosidase expression in the embryo with precise parasegmental anterior limits of expression (Barges et al., 2000; Busturia and Bienz, 1993; Zhou et al., 1999). Similarly, P-*lacZ* elements inserted in a specific *iab* region show β-galactosidase signal restricted to particular parasegments (Barges et al., 2000; Bender and Hudson, 2000; Galloni et al., 1993; McCall et al., 1994; Zhou and Levine, 1999). Therefore, the *iab* domains seem to represent autonomous units that control Abd-B M expression from A5/PS10 to A8/PS13, thus specifying these metameres (Boulet et al., 1991; Celniker et al., 1990; Galloni et al., 1993; Gyurkovics et al., 1990; Mihaly

et al., 1997; Sánchez-Herrero and Akam, 1989; Sánchez-Herrero, 1991).

The *iab* domains are separated from each other by boundary elements that isolate them, thus preventing abnormal activation of *Abd-B* in more anterior segments (Barges et al., 2000; Gyurkovics et al., 1990; Hagstrom et al., 1996; Hagstrom et al., 1997; Mihaly et al., 1997; Zhou et al., 1996; Zhou et al., 1999; Zhou and Levine, 1999). Deletions of these elements cause posteriorward transformations (Barges et al., 2000; Gyurkovics et al., 1990; Mihaly et al., 1997), owing to the inappropriate expression of *Abd-B* in a certain parasegment with the levels and distribution of a more posterior one (Celniker et al., 1990; Galloni et al., 1993; Sánchez-Herrero, 1991). In some cases, it has been shown that boundary regions are physically close to sequences that respond to *trans*-regulators of the *Polycomb* type (*Polycomb* response elements, or PREs), which restrict homeotic gene expression throughout development (Brock and van Lohuizen, 2001). The combined activity of PRE and boundary elements maintains the particular *Abd-B* expression of each parasegment.

Of all the abdominal segments determined by the Abd-B M protein, the A8/PS13 is clearly different from the rest in the set of structures that derive from it, both in the embryo or in the adult, such as the posterior spiracles or the female genitalia (Campos Ortega and Hartenstein, 1985; Nöthiger et al., 1977). An *iab-8* regulatory region, which directs PS13 specific expression in the embryo, has been identified 3′ to the *Abd-B* transcription unit (Barges et al., 2000; Zhou et al., 1999) (Fig. 1A). The *iab-8* domain is separated from the *iab-7* domain, which is located more proximally, by a *Fab-8* boundary that also contains a PRE sequence adjacent to it (Barges et al., 2000; Zhou et al., 1999; Zhou and Levine, 1999). However, there are no known mutations affecting just the A8, and rearrangements that affect the *iab-8* domain do not transform this segment (Gyurkovics et al., 1990; Hendrickson and Sakonju, 1995; Hopmann et al., 1995; Tiong et al., 1987).

We have made use of a P-element insertion in the 5′ region of the *Abd-B* gene to isolate novel mutations that affect the A8 segment and we have characterized a putative *iab-8* region in the 5′ region of *Abd-B*. Our results indicate that *iab-8* sequences 5′ and 3′ to the *Abd-B m* transcription unit contribute to the regulation of *Abd-B* in the A8 segment.

MATERIALS AND METHODS

Genetics

The mutations used are described elsewhere (FlyBase, 1999). The *Abd-B* mutations employed are: *Abd-B* null mutations; $Abd\text{-}B^{M1}$, $Abd\text{-}B^{D18}$; *Abd-B m* mutations: $Abd\text{-}B^{M5}$, $Abd\text{-}B^{M3}$, $Abd\text{-}B^{D14}$; *Abd-B r* (*iab-9*) mutation: $Abd\text{-}B^{x23\text{-}1}$; mutations in *iab-5* to *iab-7* regulatory sequences: $iab\text{-}7^{Sz}$, $iab\text{-}7^{MX2}$; *Fab-8* mutations: $Fab\text{-}8^{64}$, $Fab\text{-}8^{416}$. We have renamed the $iab\text{-}9^{48}$ and $iab9^{1065}$ (also known as $iab\text{-}7^{65}$) mutations (Celniker et al., 1990; Karch et al., 1985; Kuhn et al., 1992) as $Abd\text{-}B^{48}$ and $Abd\text{-}B^{65}$ mutations, respectively, as they affect the whole *Abd-B* domain (although very mildly in the case of $Abd\text{-}B^{65}$). *DfP9* is a deficiency for the whole bithorax complex and Pc^3 a null mutation at the *Polycomb* (*Pc*) locus (Lewis, 1978). The $Pc^3\ Abd\text{-}B^{lac1}$ double mutant was made by standard recombination.

Constructs and transformation of embryos

The pRCm1 construct (Busturia and Bienz, 1993) was used in the transformation experiments that gave rise to the $Abd\text{-}B^{lac1}$ mutation. This vector carries the *rosy* gene as a transformation marker, a fragment from the *Abd-B* gene and the *lacZ* gene. The *Abd-B* fragment is a 5.6 kb *Pst*I-*Hin*dIII fragment from 50,504 to 44,912 (Martin et al., 1995). This fragment includes the *Abd-B m* promoter region, the initiation site of the *Abd-B m* (or A) transcript, the leader sequence and the sequence encoding for the first five amino acids of the *Abd-B m* protein fused to the bacterial *lacZ* gene. This *Pst*I-*Hin*dIII fragment is referred to in the text as the *Abd-B* proximal promoter, Abd-Bpp (Busturia and Bienz, 1993). The P-element giving rise to the $Abd\text{-}B^{lac1}$ mutation is the construct pRCm 70.2, and contains, in addition, a *Hin*dIII fragment of the *Abd-B* 3′ regulatory region (from 64,580 to 59,073) inserted into *KpnI-XbaI* sites of pRCm1 (see Fig. 1A).

In the analysis of the 5′ *Abd-B* regulatory region, the following constructs were used to transform embryos.

(1) BEAbd-BppX2, which contains the *Eco*RI fragment from 47,992 to 40,968 (X2 fragment) cloned into the Abd-Bppwhite vector (A. Busturia, unpublished). The Abd-Bpp white vector contains the Abd-Bpp sequence (Busturia and Bienz, 1993) cloned into the pwZ50PL vector, kindly provided by T. Gutjahr.

(2) BEAbd-BppL14, which contains the *Eco*RI-*Pst*I fragment from 40,968 to 33,634 (L14 fragment) cloned into the same vector.

(3) BEUbxppB4, which contains the *Hin*dIII-*Eco*RI fragment from 44,912 to 40,968 (B4 fragment) inserted upstream of the *Ubx-lacZ* reporter gene in the CaSpeR *Ubx-lacZ* vector (Qian et al., 1991). This B4 fragment was also cloned into the pwHZ50PL vector, which includes the promoter of the *hsp70* gene, to obtain construct BEpwHZ50PLB4.

(4) pwHZ50PLPS, which contains the *Bam*HI-*Hin*dIII fragment from 48,864 to 44,912 (PS fragment) inserted into the pwHZ50PL vector.

Transformation of embryos was done as described previously (Busturia and Bienz, 1993).

Isolation of P-element derivatives

Derivatives of the P-element insertion in *Abd-B* ($Abd\text{-}B^{lac1}$) were obtained by several methods. In one of them, P [$ry^{+}\ Abd\text{-}B^{lac1}$]/TM3, Δ 2-3 dysgenic males were crossed to ry^{506} females and ry^{-} males obtained from this cross were individually isolated and balanced. A different set of experiments, intended to isolate derivatives independently of their having (or not) the *ry* gene, was carried out as follows: individual dysgenic males of the genotype *st red* $sbd^2\ Abd\text{-}B^{lac1}/TM3$, Δ *2-3* were crossed to $Abd\text{-}B^{M1}/TM6B$ females. The progeny was checked for either the presence of an *Abd-B* phenotype in the $Abd\text{-}B^{lac1}/Abd\text{-}B^{M1}$ progeny, or for the loss of the haploinsufficient *Abd-B* phenotype of the $Abd\text{-}B^{lac1}$ insertion (small seventh tergite and a few bristles in the sixth sternite) in the $Abd\text{-}B^{lac1}/TM6B$ males. In the appropriate cases, individual males were isolated, and stocks were established and further characterized by crosses with different *Abd-B* alleles. To ascertain if these derivatives were ry^{+} or ry^{-}, they were recombined with ry^{506} and crossed to ry^{506} females. The class II (*iab-8,9*) mutants described in this work were isolated by this last method.

In situ hybridization

It was carried out as described by (Casares and Sánchez-Herrero, 1995). The probe for the *m* transcript is a genomic *Bam*HI fragment from 50,702 to 48,864. The probe to detect all the *Abd-B* transcripts, referred to as ‘common probe’, is a genomic *Hin*dIII-*Sal*I fragment from 56,556 to 52,989 that includes the homeobox.

Antibody staining

It was performed as described previously (Casares and Sánchez-Herrero, 1995). The rabbit anti-β-galactosidase antibody (Cappel) was used at a concentration of 1:2000. The Abd-B antibody, used at 1:20 concentration, is monoclonal antibody lA2E9 (kindly provided by S. Celniker), and recognizes both Abd-B M and R proteins (Celniker et

al., 1989). The anti-En monoclonal antibody (Patel et al., 1989) was used at 1:10 concentration, and was kindly provided by T. Kornberg. Staging of the embryos was determined as described previously (Campos-Ortega and Hartenstein, 1985).

Embryonic cuticle analysis

It was carried out as previously described (Wieschaus and Nüsslein-Volhard, 1986).

DNA techniques

The molecular study of the *Abd-B^{lac1}* mutation and of the different derivatives was carried out by Southern blot analysis using the following probes: (1) *Abd-B* DNA not included in the pRCm 70.2 construct; (2) *Abd-B* DNA included in the construct; and (3) DNA from the *rosy* and *lacZ* genes. The *lacZ* probe was obtained from the Bluescript vector. The *rosy* probe is a 7.2 kb *Hin*dIII fragment from the HZ50PL vector (Hiromi and Gehring, 1987). DNA extractions and methods of Southern hybridization were carried out as described previously (Sullivan et al., 2000) with slight modifications.

PCR analysis

Inverse PCR was done as described in FlyBase (http://www.fruitfly.org/methods).

RESULTS

The *Abd-B^{lac1}* insertion

Within a study of the *Abd-B* regulation, a genomic fragment including the *iab-8* regulatory region and the *Fab-8* boundary was subcloned into a vector carrying a 5.6 kb fragment from the *Abd-B* gene (Abd-Bpp) and the *lacZ* gene (Busturia and Bienz, 1993) (see Materials and Methods and Fig. 1A). Transformant lines carrying this construct were isolated and embryos from each line stained with anti-β-galactosidase antibody. Four of the lines showed the basic staining pattern of the Abd-Bpp reported previously (Busturia and Bienz, 1993), and shown in Fig. 1B-C.

The fifth line, however, showed a restricted β-galactosidase expression, confined to the posterior abdominal segments and resembling the *Abd-B* pattern of expression. Males heterozygous for this insert presented a typical *Abd-B* haploinsufficient phenotype (some bristles in the sixth sternite and a small seventh tergite). Therefore, we decided to characterize this line further. The insertion point of the P-element was located by inverse PCR at position 48,957 (Martin et al., 1995), 242 bp upstream the *Abd-B m* transcription start site (Zavortink and Sakonju, 1989). Genetic tests with *Abd-B m* and *Abd-B r* mutations indicated that the insertion is an amorph for the *Abd-B r* function and a strong hypomorph for *Abd-B m* function. Staining of homozygous embryos with an anti-Abd-B antibody showed almost absence of expression in PS10-14. These observations indicate that the P-element has generated a mutation in *Abd-B* that we have called *Abd-B^{lac1}*.

The *lacZ* expression of *Abd-B^{lac1}*embryos differs from that of *Abd-B* in wild-type embryos in several respects (Fig. 2). In *Abd-B^{lac1}* embryos, the *lacZ* expression starts simultaneously in PS12-14 at stage 9, and this signal is maintained at stage 10 (Fig. 2A) and thereafter (Fig. 2C). Expression in PS10-11 is low or absent and, at late stages, there is a strong PS12 signal in the ventral cord (Fig. 2E). By contrast, Abd-B products are observed only in PS13 and PS14 in stage 10 wild-type embryos (Fig. 2B). Later on, *Abd-B* expression decreases from PS14 to PS10 both in the epidermis and the ventral cord (Fig. 2D,F). The particular strong *lacZ* expression in the ventral cord of *Abd-B^{lac1}*embryos disappears in *Pc^3 Abd-B^{lac1}* double mutant embryos (Fig. 2G).

We hypothesize that the *lacZ* expression in *Abd-B^{lac1}* embryos may have some characteristics derived from the place of insertion and others due to the *Abd-B* regulatory region included within the P-element. To delimit each contribution, and to obtain mutations in the region of the *Abd-B* promoter, we mobilized the P element (see Materials and Methods).

Some *Abd-B^{lac1}* derivatives constitute a distinct class of mutations in *Abd-B*

We obtained different types of derivatives when mobilizing

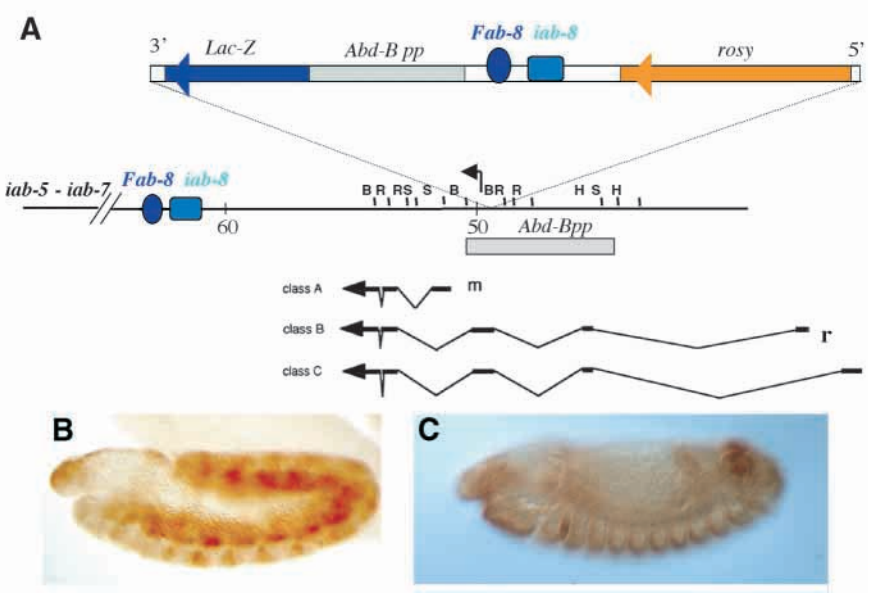

Fig. 1. Insertion of the P-element, which causes the *Abd-B^{lac1}* mutation, and pattern of expression of the same P-element inserted outside the BX-C. (A) Scheme of the *Abd-B^{lac1}* insertion, showing the P-element and the place of integration in the *Abd-B* DNA. The *Abd-B* 3′ regulatory DNA included in the P-element is marked in white. The oval and the square within this region represent the *Fab-8* boundary and 3′ *iab-8* regulatory sequences, respectively. The *Abd-B* promoter region (Abd-Bpp) included in the same transposon is marked in gray, the *lacZ* gene in blue and the *rosy* gene in orange. The arrow in the genomic DNA represents the transcription start site of the *Abd-B m* RNA. *iab-5* to *iab-8* and *Fab-8* endogenous sequences are represented to the left of the insertion. The numbers refer to the coordinates in kb (Martin et al., 1995). Below the line representing the genomic DNA, we show the *Abd*-B transcription unit, indicating the *Abd-B m* (class A) and the *Abd-B r* (class B, class C) transcripts. There is also an *Abd-B* transcript, expressed in PS15 (Kuziora and McGinnis, 1988), not represented in the figure. B, *Bam*HI; R, *Eco*RI; H, *Hin*dIII; S, *Sal*I. (B,C) Expression of the *lacZ* gene when the P-element is inserted outside the BX-C in a stage 11 (B) and a stage 14 embryo (C).

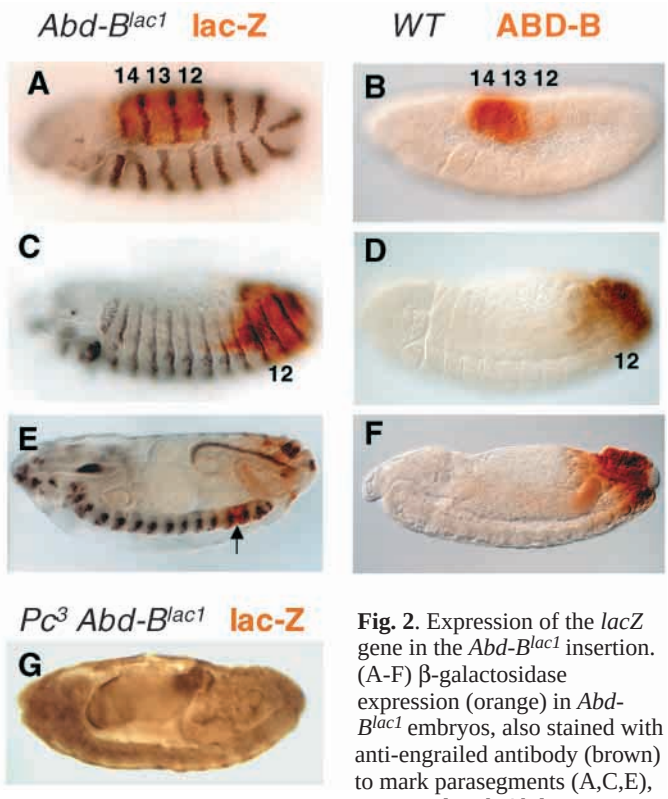

Fig. 2. Expression of the *lacZ* gene in the *Abd-B*^{lac1} insertion. (A-F) β-galactosidase expression (orange) in *Abd-B*^{lac1} embryos, also stained with anti-engrailed antibody (brown) to mark parasegments (A,C,E), compared with Abd-B protein expression in wild-type embryos (B,D,F). (A,B) Stage 11 embryos; (C,D) stage 13 embryos; (E) a stage 16 embryo; (F) a stage 14 embryo. Numbers indicate parasegments. Note that β-galactosidase expression in the stage 11 *Abd-B*^{lac1} embryo extends from PS12 to PS14 (A), whereas in wild-type embryos at this stage there is no Abd-B protein expression in PS12 (B). Note also strong β-galactosidase expression in the ventral cord of the stage 16 *Abd-B*^{lac1} embryo (E, arrow). (G) β-galactosidase expression in a stage 16 *Pc*^{3} *Abd-B*^{lac1} embryo, showing the absence of PS12 specific signal.

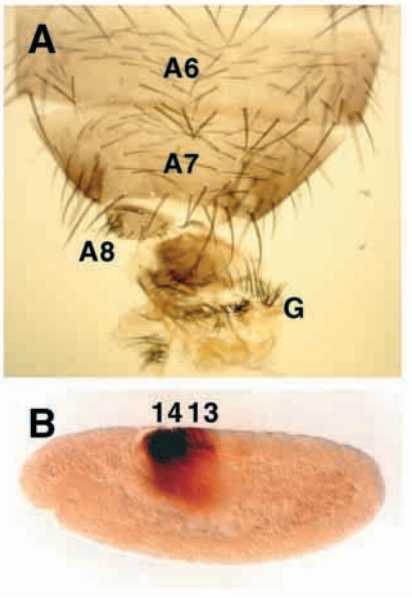

Fig. 3. Phenotype and *Abd-B* and *lacZ* expression of class I derivatives. (A) Posterior abdominal segments of an *Abd-B*^{T2N}/*DfP9* male (*Abd-B*^{T2N} is a class I derivative), showing the transformation of the A7 and A8 segments. Note the large size of the A7 (absent in wild-type males) and the small A8. G, male genitalia. (B) Abd-B protein expression (violet/black) and β-galactosidase expression (orange) in a stage 10 *Abd-B*^{T2N} homozygous embryo. While *Abd-B* expression is confined to PS14 (Abd-B R protein), β-galactosidase signal is strong in PS13. Numbers indicate PS.

Abd-B^{lac1}, but most of them could be classified into two different groups, defined by their phenotype, *Abd-B* expression and molecular structure.

Derivatives of the first group (class I; nine alleles isolated) are *ry*$^{-}$ but retain the *lacZ* gene. They present in heterozygosis an *Abd-B* haploinsufficient phenotype and complement *Abd-B r* (*iab-9*) mutations. In hemizygosis they show a transformation of segments A5-A8 (PS10 to PS13) into A4 (PS9) (Fig. 3A), characteristic of *Abd-B m* mutations (Casanova et al., 1986). Accordingly, in class I homozygous embryos Abd-B protein or RNA ('common' probe) expression is detected normally in PS14 but very weakly in PS10-13 (Fig. 3B). β-galactosidase staining of these embryos shows a strong signal in PS13 (Fig. 3B). The phenotype and staining pattern of these mutations resemble the derivatives of a P-*lacZ* element, *iab-8*$^{Plac(+159)}$, inserted very close to *Abd-B*^{lac1} (McCall et al., 1994).

Derivatives of the second group (class II; six alleles isolated) are *ry*$^{+}$, have lost the *lacZ* gene, and show no haploinsufficient phenotype. Homozygous adults are not recovered because of a second-site lethal mutation present in the original *Abd-B*^{lac1} chromosome. In hemizygous condition, these mutants show a distinct phenotype. In females, the dorsal A8 (eighth tergite) is covered with bristles and trichomes characteristic of an A6/A7 segment (Fig. 4B, compare with *DfP9*/+ females in Fig. 4A) and the genitalia is variably transformed into a sternite (Fig. 4F; compare with wild-type females in Fig. 4E). The males show an eighth tergite bearing trichomes (Fig. 4D, compare with *DfP9*/+ males in Fig. 4C) and a small eighth sternite with one to six bristles (Fig. 4H, compare with wild-type males in Fig. 4G). These phenotypes indicate there is a partial transformation of A8 to A6/A7. In both sexes, the A5-A7 segments of class II hemizygous adults only show the haploinsufficient phenotype that is due to the *DfP9*. These mutants lack genitalia and analia, and show occasionally a small ninth segment (Fig. 4I). Class II mutations do not affect the dominant phenotype of the *Fab-8*^{64} and *Fab-8*^{416} mutations, which delete the *Fab-8* boundary and transform PS12 into PS13 (Barges et al., 2000). They also complement mutations like *iab-7*^{Sz} and *iab-7*^{MX2}, which affect A7 and A5-A7 segments, respectively (Galloni et al., 1983; Sánchez-Herrero et al., 1985), but fail to complement *Abd-B r* (*iab-9*) mutations. In hemizygous embryos, the A8 segment is transformed into a more anterior one (Fig. 4K, compare with the wild type in Fig. 4J). In the view of the embryonic and adult phenotypes, we have named these derivatives *iab-8,9* mutations. This is a distinct class of BX-C mutations that transform the A8 and the A9 but not more anterior segments. The *Tab*^{R96} mutation (Celniker and Lewis, 1987) also affects both segments, although the effect in the A8 is very weak.

The *iab-8,9* phenotype correlates with the Abd-B protein distribution in *iab-8,9* homozygous embryos: *Abd-B* expression is normal in PS10 and 11, reduced in PS13 and almost absent in PS14 (Fig. 5B,D, compare with wild-type

expression in Fig. 5A,C). In situ hybridization with either a probe specific for the *m* transcript or with the 'common' probe also shows reduced signal in PS13. In some of these embryos, especially in the germband retracted stage, *Abd-B* expression in PS12 ectoderm is slightly increased compared with wild-type embryos (Fig. 5D, compare with Fig. 5C). These data suggest that *iab-8,9* mutations affect the expression of *Abd-B r* transcripts in A9/PS14 and of the *Abd-B m* RNA mainly in the A8/PS13.

We have characterized the molecular structure of 3 class I and 2 class II derivatives by Southern blot analysis (see Materials and Methods). The data indicate that both types of mutations are the result of recombination events between the 'exogenous' promoter (the Abd-Bpp included in the P element) and the corresponding endogenous sequences. In class I derivatives, the *ry*, *Fab-8* and *iab-8* sequences of the P element are lost as a result of this recombination. In *iab-8,9* alleles, the *lacZ* gene is lost (see Fig. 6). We have also defined, by inverse PCR, the location of the defective P-element of the *iab-8,9*^{T1J} mutation. This location is precisely in the same position as in the *Abd-B*^{lac1} mutation.

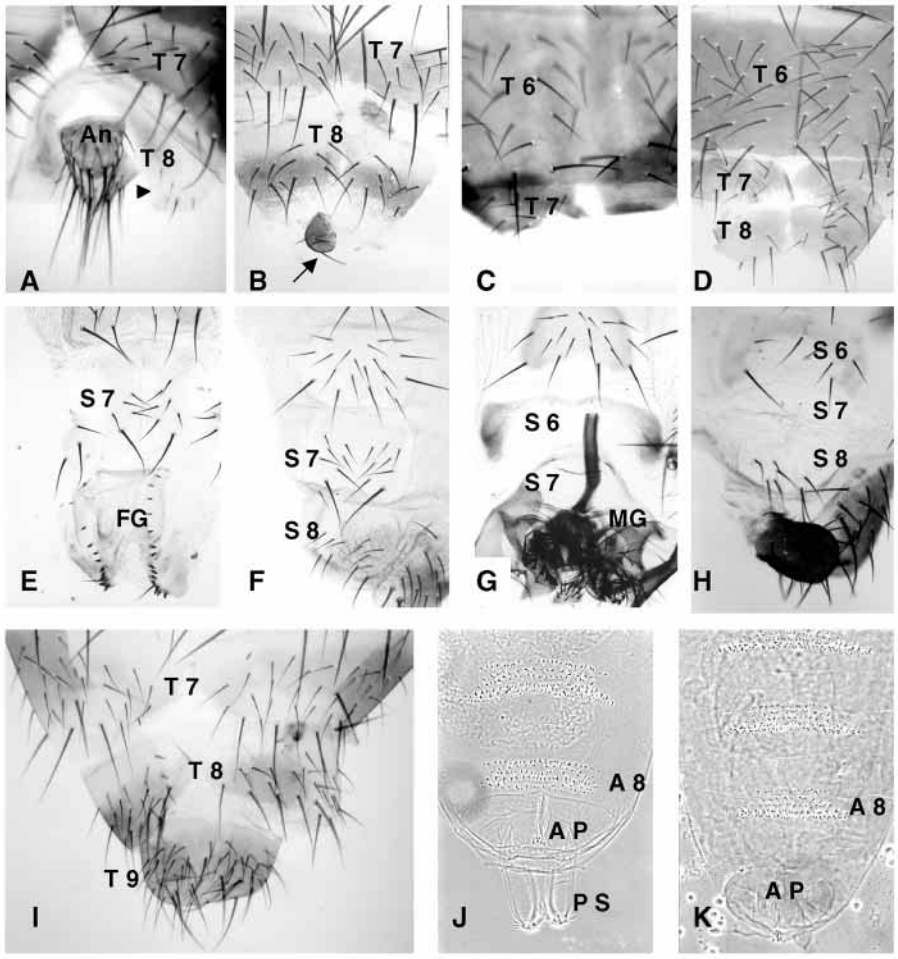

Fig. 4. Embryonic and adult phenotype of *iab-8,9* mutations. T indicates tergite (dorsal part of an abdominal segment) and S indicates sternite (ventral part of an abdominal segment). Numbers after T or S refer to the abdominal segment. The posterior dorsal abdomen of *DfP9*/+ females (A; An stands for analia) and males (C), and the ventral abdomen and genitalia of adult wild-type females (E) and males (G) are compared with those of *iab-8,9*^{T1J}/*DfP9* females (B, dorsal; F, ventral) and males (D, dorsal; H, ventral). The genitalia of *DfP9*/+ males and females are similar to those of wild-type animals, except that male genitalia of *DfP9*/+ flies are rotated. Three main differences can be observed in *iab-8,9* mutations with respect to wild-type or *DfP9*/+ adults: (1) they have an A8 (T8 in B and D), which is much reduced and with just a few bristles in *DfP9*/+ females (A; the arrowhead indicates the T8 bristles) and absent in *DfP9*/+ males; (2) wild-type female genitalia (FG in E) are replaced by a sternite in these mutations (S8 in F); and (3) there is an incomplete eighth sternite in the male of *iab-8,9* mutations (S8 in H), which is absent in wild-type (G) or *DfP9*/+ males. Male genitalia (MG in G) are replaced in *iab-8,9* mutations by an unidentified tissue (H). In *iab-8,9* adults, segments anterior to A8 show only the haploinsufficient phenotype of *DfP9*: a small T7 (C,D) and some bristles in the S6 (H). The arrow in B indicates a small A9 segment. (I) Posterior abdomen of an *iab-8,9*^{T1J}/*DfP9* female, showing an A9 segment. (J) Posterior cuticle of a wild-type embryo. (K) Posterior cuticle of an *iab-8,9*^{T1J}/*DfP9* embryo, showing transformation of the A8 into a more anterior segment, revealed by the trapezoidal form of the A8 denticle band (rectangular in the wild type), the space between the A8 denticle belt and the anal pads (AP) and the absence of posterior spiracles (PS). Occasionally, a small A9 can be observed in these embryos.

Characterization of *iab-8* regulatory sequences in the 5′ region of the *Abd-B* transcription unit

The genetic and molecular characterization of *iab-8,9* mutations suggested that an *iab-8* regulatory region could be located upstream the *Abd-B m* transcription unit (see Fig. 6). To test this prediction, we fused either a 7 kb genomic fragment from 47,992 to 40,968 (X2 fragment) or a 7.3 kb fragment from 40,968 to 33,634 (L14 fragment) to the basal construct containing the Abd-Bpp, obtaining the BEAbd-BppX2 and BEAbd-BppL14 constructs, respectively (Fig. 7A and Materials and Methods). We stained embryos of five independent lines transformed with each construct with an anti-β-galactosidase antibody. The BEAbd-BppX2 construct shows, in four out of five lines, an increase in staining in lateral epidermal cells of PS13 (A8) compared with embryos transformed with the basal construct (Fig. 7C; compare with Fig. 7B), suggesting the presence of a PS13 regulatory region in the construct. In late stages of embryogenesis the PS13 signal is not observed, but the embryos show a strong expression in posterior and anterior spiracles (Fig. 7F). The BEAbd-BppL14 construct does not give a particular pattern in any segment of the transformed embryos (not shown).

The Abd-Bpp shows, by itself, a strong basal staining that could obscure some particular signal coming from the tested fragments. To overcome this problem, and to better define the

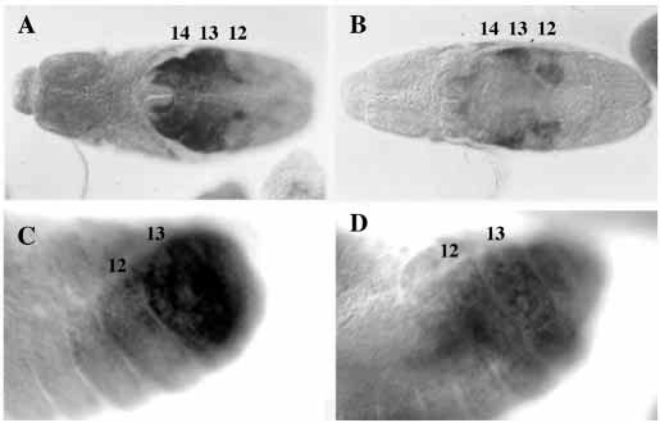

Fig. 5. Expression of Abd-B protein in *iab-8,9* mutations. Numbers refer to parasegments. (A) Wild-type Abd-B protein expression (dorsal view of a stage 12 embryo). (B) The same stage and view in an *iab-8,9* homozygous embryo. The expression in PS14 and PS13 is clearly reduced but expression in PS12 does not change (slightly out of focus in A). (C) *Abd-B* expression in the posterior region of a stage 14 wild-type embryo. (D) Similar region of an *iab-8,9* homozygous embryo. Note that Abd-B expression is reduced in PS13 (A8) and slightly increased in PS12 (A7) when compared with that of a wild-type embryo (C).

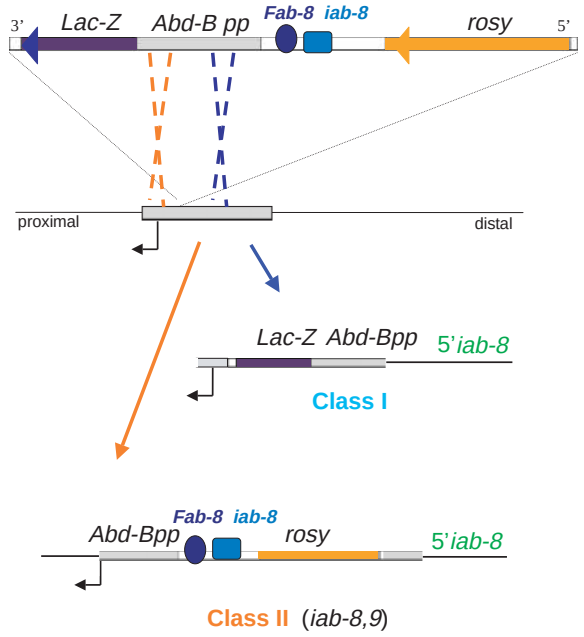

Fig. 6. Scheme of the recombination events in the Abd-Bpp region that were probably the origin of class I (*Abd-B* m^-) and class II (*iab-8,9*) derivatives of *Abd-B*^{lac1}. The different DNA regions included in the P-element are marked as in Fig. 1. We indicate in the distal part of both derivatives the postulated 5′ *iab-8* domain.

putative PS13 regulatory region, we fused the *Hin*dIII-*Eco*RI fragment from 44,912 to 40,968 (B4 fragment) upstream two different basal promoters: The *Ubx* minimal promoter (Qian et al., 1991), generating the construct BEUbxppB4, and the *hsp70* minimal promoter (Hiromi and Gehring, 1987), obtaining the construct BEpwHZ50PLB4 (Fig. 7A). Neither the *hsp70* minimal promoter nor the *Ubx* minimal promoter on their own gives any segment-specific staining pattern in embryos (Hiromi and Gehring, 1987; Qian et al., 1991). We stained six lines of embryos transformed with the BEUbxppB4 construct and seven of those transformed with the BEpwHZ50PLB4 one. None of the two constructs presents a stronger expression in PS13, although both show a clear signal in both anterior and posterior spiracles in late embryonic stages (Fig. 7D,G and data not shown). This suggests that the putative *iab-8* sequences require the presence of an intact *Abd-B* promoter to direct a PS13 specific expression.

Regulation of expression in the spiracles

The construct containing only the Abd-Bpp shows a strong expression in anterior and posterior spiracles (Busturia and Bienz, 1993) (Fig. 1C, Fig. 7E). This suggests that the *Abd-B* promoter is specifically activated in both anterior and posterior spiracles, although gap genes repress *Abd-B* expression in anterior spiracles (Harding and Levine, 1988; Reinitz and Levine, 1990). The same expression in the posterior and anterior spiracles is observed in embryos transformed with a construct carrying a *Bam*HI-*Hin*dIIII fragment (PS) included within the Abd-Bpp (see Fig. 7). We note, however, that the B4 fragment also directs expression in these organs (Fig. 7G). This suggests that there is more than a single *Abd-B* 5′ region that could direct expression in the spiracles.

DISCUSSION

Several *iab* domains in the *Abd-B* 3′ regulatory region control its expression in PS10-13. An *iab-8* domain is located within this region, between 59,500 and 62,541 (Barges et al., 2000; Zhou et al., 1999), and we refer to it as the 3′ *iab-8* domain. However, mutants in which this domain is deleted (*Fab-7*^{R59} deletion) or separated from the *Abd-B* promoter (*iab-7*^{S10} translocation) show no effect in the A8 segment (Gyurkovics et al., 1990; Hendrickson and Sakonju, 1995; Hopmann et al., 1995; Tiong et al., 1987) (see Fig. 8), suggesting there are other *iab-8* regulatory sequences in *Abd-B*. We have obtained genetic and molecular evidence indicating the presence of such sequences 5′ to the *Abd-B m* transcription unit.

This conclusion stems in part from the study of a P-element with *Abd-B* regulatory sequences inserted in the *Abd-B* gene (*Abd-B*^{lac1}). This phenomenon, in which a P-element containing regulatory sequences of a gene is inserted in the same gene is called ‘homing’ (Hama et al., 1990) and is particularly frequent in the BX-C (Bender and Hudson, 2000; Fitzgerald and Bender, 2001; McCall et al., 1994). The analysis of the *Abd-B*^{lac1} mutation and of some of its derivatives has helped to better understand *Abd-B* regulation.

Class I and Class II (*iab-8,9*) mutations

Some derivatives from the *Abd-B*^{lac1} insertion (*iab-8,9* mutations) transform segments A8 and A9 but not more anterior segments. Curiously, the transformation of the A8 segment is not to A7 but, partially, to A6. This suggests that the *iab-7* regulatory sequences are not fully active in the A8 segment, not even when *iab-8* activity is reduced. We explain below the effect on *Abd-B* expression that causes these transformations.

In *iab-8,9* mutations, the defective P-element responsible for the phenotype retains the *ry* gene, the 3′ *iab-8* region and the *Fab-8* exogenous boundary, and is inserted in an intron of the B and C transcripts (see Fig. 1A and Fig. 6). We propose that the *ry* gene included in the transposon interrupts this transcription, preventing *Abd-B* expression in A9/PS14. Such effect on transcription, which also occurs in *Abd-B*^{lac1}, has been previously documented for other P elements inserted in introns of different genes (Casares et al., 1997; Horowitz and Berg, 1995; McCall et al., 1994). By contrast, in *iab-8,9* embryos there is no general effect in the transcription of the *Abd-B m* transcript. The *Abd-B m* promoter is normal, and, accordingly, *Abd-B* expression and function is wild type in PS10-12 (except for a slight increase in PS12 *Abd-B* expression). Therefore, the transformation of PS13 is due to the specific inactivation of *iab-8* regulatory sequences. To explain this phenotype, we propose the existence of novel *iab-8* regulatory sequences located upstream the *Abd-B m* transcription unit and named by us the 5′ *iab-8* domain. These sequences would be inactive in *iab-8,9* mutations because of the presence of the exogenous *Fab-8* boundary, placed between them and the *Abd-B m* promoter (see Figs 6, 8).

The absence of *Abd-B* transforms female (A8) and male (A9) genitalia into leg tissue (Estrada and Sánchez-Herrero, 2001). In *iab-8,9* mutations, however, the presence of low levels of *Abd-B* expression in these two metameres is sufficient to transform them, instead, to abdomen. This transformation prevents a normal development of the genital disc and indirectly produces the absence of analia, which derive from the A10 segment and do not require *Abd-B* function (Sánchez-Herrero et al., 1985; Tiong et al., 1985).

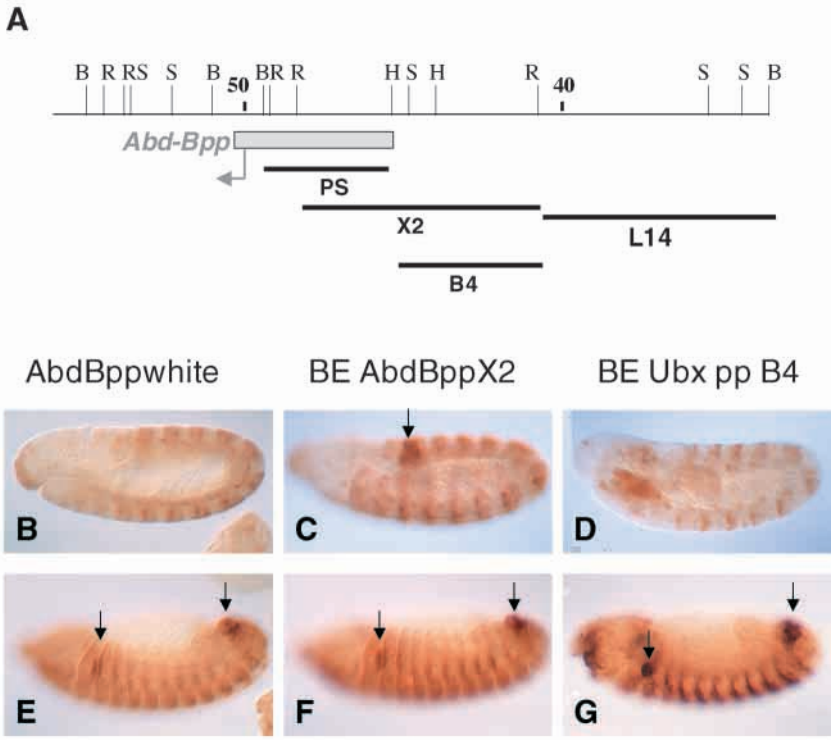

Fig. 7. Expression patterns of transgenes including sequences in the *Abd-B m* 5′ regulatory region. (A) The *Abd-B* upstream regulatory region and the different genomic fragments used to make the constructs. Coordinates, symbols of restriction sites and transcription start site as in Fig. 1. (B-H) Embryos stained with a anti-β-galactosidase antibody at the germ band extended (B-D) and germ band retracted (E-G) stages. (B) Embryo transformed with a construct containing the Abd-Bpp fragment and the *lacZ* gene (Abd-Bpp white) showing the basal expression pattern. (C) Embryos with the BEAbd-BppX2 construct. There is strong expression of β-galactosidase in PS13 at the germband extended stage (arrow). (D) Embryos transformed with the BEUbxppB4 construct. Note that now there is no strong expression in PS13. After germband retraction, the three constructs show similar strong expression in anterior and posterior spiracles (E-G, arrows).

In class I derivatives, the exogenous *Fab-8* and 3′ *iab-8* sequences, as well as the *ry* gene, are not present. The absence of the exogenous *Fab-8* boundary allows for the activity of endogenous *iab-8* sequences, and strong *lacZ* expression in PS13 results. *Abd-B* expression is reduced in PS10-PS13, as it is in *Abd-B*^{lac1} embryos, because the P-element interrupts the *Abd-B* promoter region. However, as the *ry* gene is absent, there is complete transcription of *Abd-B r* RNAs and normal *Abd-B* expression in PS14 (see Fig. 3B, Fig. 6).

The 5′ *iab-8* regulatory domain of *Abd-B*

The existence of the 5′ *iab-8* domain is supported by the analysis of transformants carrying fragments of this region. The PS13 *lacZ* expression of embryos with constructs containing the X2 fragment is enhanced. Sequences from the distal part of this region (B4 fragment, not included in the Abd-Bpp), however, do not reproduce this particular expression when fused to heterologous minimal promoters (*hsp70* or *Ubx* promoters), suggesting that they require the *Abd-B* promoter for their proper activity. The need of enhancers to have their own promoter for a wild-type activity has been previously reported in *Drosophila* (Li and Noll, 1994; Merli et al., 1996; Ohtsuki et al., 1998; Schier and Gehring, 1992).

The preceding data, combined with previous results from the literature, help to delimit the putative 5′ *iab-8* domain. The mutation *tuh-3* maps around 34,000 and affects the A9 but not the A8 segment (Casanova et al., 1986; Mack et al., 1997) (see Fig. 8), thus setting the distal limit of the putative *iab-8* region. In the more proximal region, the *Abd-B*^{48} mutation, located at about 45,700, causes a strong reduction of *Abd-B* expression in PS10-PS13 (Celniker et al., 1990; Karch et al., 1985; Kuhn et al., 1992) (see Fig. 8), indicating that this breakpoint interrupts the *Abd-B* promoter region required for correct PS10-13 expression (Sipos et al., 1998). By contrast, the *Abd-B*^{65} mutation, which breaks at about 43,700, only mildly affects PS10-PS12 (Boulet et al., 1991; Celniker et al., 1990) (B. E., F. C., A. B. and E. S.-H., unpublished), but affects a bit more strongly PS13 (data not shown), suggesting the mutation breaks near the region that separates the *Abd-B m* upstream regulatory region common for PS10-PS13 from the 5′ *iab-8* domain (see Fig. 8). All

these results confine the 5′ *iab-8* domain to the region indicated in Fig. 8A.

Models to explain the PS12 *lacZ* expression in *Abd-B^{lac1}* embryos

The *lacZ* gene in *Abd-B^{lac1}* shows an early and strong signal in PS12 when compared with the *Abd-B* wild-type expression. The 3′ *iab-8* sequences included in the P-element are unlikely to be responsible for this particular staining for three reasons: first, they are separated from the *lacZ* gene by the *Fab-8* boundary, which should prevent or attenuate the *lacZ* transcription directed by these sequences (Barges et al., 2000; Zhou et al., 1999; Zhou and Levine, 1999) (see Figs 1, 8). Second, the 3′ *iab-8* sequences direct expression in PS13 and not in PS12 (Barges et al., 2000; Zhou et al., 1999). Finally, embryos carrying this transposon outside the *Abd-B* gene or in the *Ultrabithorax* region of the BX-C do not show this PS12 expression (Fig. 1B,C and data not shown). Rather, we think that the *Fab-8* and PRE exogenous sequences included in the P-element are the cause of this particular staining. We have observed that the strong PS12 β-galactosidase expression in the ventral cord of *Abd-B^{lac1}* embryos disappears in a *Pc* mutant background. This suggests that PREs are instrumental in maintaining this PS12 signal.

A PRE is found distal to the *Fab-8* boundary (Barges et al., 2000; Zhou et al., 1999), and therefore included in the *Abd-B* 3′ regulatory region of the P-element (see Fig. 8A). As PREs frequently pair (Fauvarque and Dura, 1993; Kassis, 1994; Muller et al., 1999; Sigrist and Pirrotta, 1997), it is possible that the exogenous and endogenous PREs may pair in *Abd-B^{lac1}*, creating a loop in the DNA. The *Fab-8* boundaries may also contribute to this pairing (Hogga et al., 2001), as is the case with other types of boundary regions [such as the *su* (*Hw*) insulators (Cai and Shen, 2001; Muravyova et al., 2001)]. Based on this pairing mechanism, we propose two models to explain the early and strong signal in PS12 of *Abd-B^{lac1}* embryos.

In the first model (Fig. 8B), the pairing takes place between the endogenous and exogenous *Fab-8*/PRE sequences (*iab-8* sequences could also contribute to it). This would both approximate the *iab-7* sequences (which direct expression in PS12) to the *lacZ* gene and stabilize the looping of these sequences, resulting in a strong expression in PS12. Supporting this model, P-lacZ elements inserted in the *iab-7* domain show a similar strong β-galactosidase expression in PS12 in the ventral cord (Barges et al., 2000; Galloni et al., 1993). This suggests that the *iab-7* sequences are driving *lacZ* expression in PS12 of *Abd-B^{lac1}* embryos. This model could also explain the early *lacZ* expression in PS12 for the following reason. In the wild type, the *m* transcript is detected consecutively and with decreasing amount of expression in PS13, PS12, PS11 and PS10 (Boulet et al., 1991; Kuziora and McGinnis, 1988; Sánchez-Herrero and Crosby, 1988). The outcome is that stage of development in which transcription is detected and the amount of expression are related (Crosby et al., 1993). The increased *lacZ* expression in PS12 of *Abd-B^{lac1}* embryos may result in an ‘earlier’ β-galactosidase expression in PS12. A similar case has been described for a P-*lacZ* insertion in the *iab-7* domain: in this *bluetail* mutation, *lacZ* expression starts in PS12 at stage 8, before *Abd-B* expression is detected in PS12 of wild-type embryos (Galloni et al., 1993). The model requires the *iab-7*

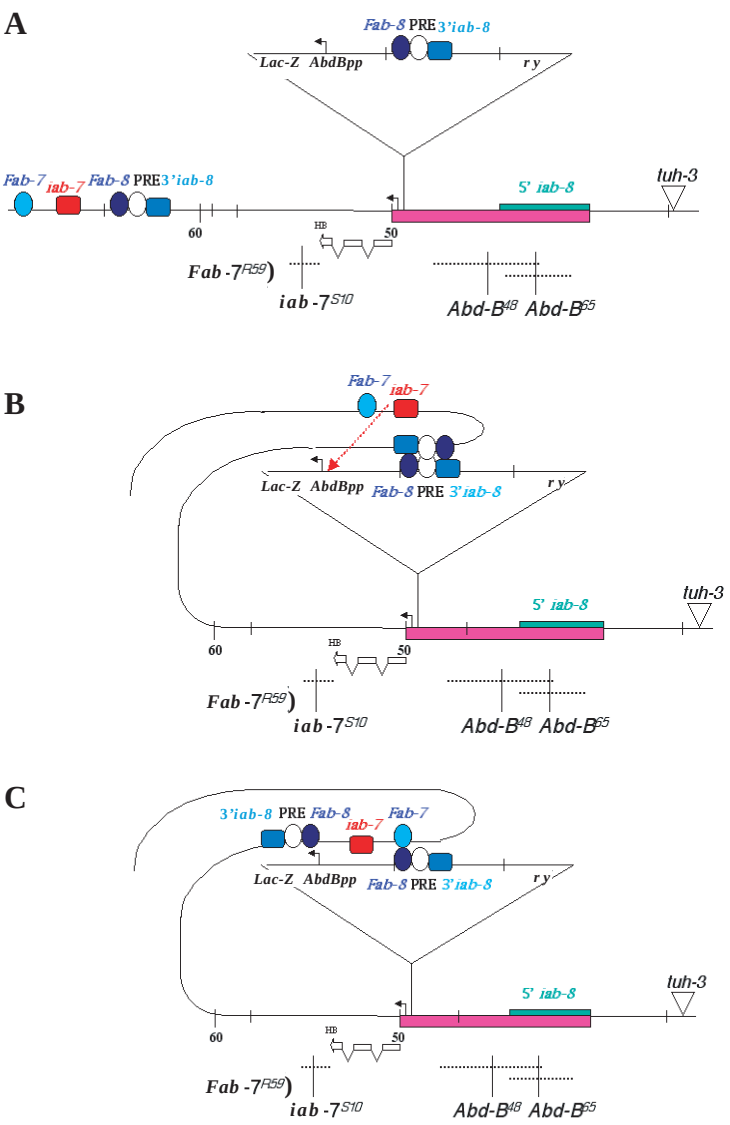

Fig. 8. 5′ *Abd-B* regulatory region, 5′ *iab-8* domain and models of regulation in the *Abd-B^{lac1}* mutation. (A) The *Abd-B m* transcription unit (white boxes; HB, Homeobox), the upstream and downstream regulatory regions and the breakpoints of some *Abd-B* mutations are shown. Different DNA regions are marked as in Fig. 1. The *iab-7* regulatory region is marked in red and the *Fab-7* boundary as a light blue oval. The region in pink indicates the fragment that drives expression in PS13, and the region in green the possible location of the 5′ *iab-8* region. (B) Model 1. Scheme of the pairing between the exogenous and endogenous *Fab-8* /PRE/*iab-8* elements in the *Abd-B^{lac1}* mutation. This pairing may facilitate the interaction between *iab-7* sequences and the *lacZ* gene and hinder the interaction of the 3′ endogenous *iab-8* sequences and the *Abd-B* endogenous promoter. (C) Model 2. The *Fab-7* boundary pairs with the exogenous *Fab-8* boundary. To simplify the figure, the possible pairing of PRE sequences has not been indicated (there is a PRE adjoining the *Fab-7* boundary) (Mihaly et al., 1997). The *iab-7* and endogenous *3′iab-8* sequences are now in the same domain, and active in PS12.

sequences to bypass the *Fab-8* boundary, although this also takes place in the wild type.

The second model (Fig. 8C) proposes that *Fab-7*/PRE sequences pair with the exogenous *Fab-8*/PRE DNA. Pairing between different *Fab* sequences have been previously proposed to occur in normal development (Hogga et al., 2001). We assume there is a competition between endogenous and exogenous *Fab-8*/PRE sequences to pair with *Fab-7*/PRE and that the pairing with the exogenous ones is somehow preferred. As a result, the 3′ *iab-8* sequences would be within the same domain as the *iab-7* DNA and both would activate the *lacZ* gene in PS12. This model would explain the similar PS12 and PS13 expression in *Abd-B*^{lac1} embryos. Supporting this model, the *lacZ* signal in germ band extended *Abd-B*^{lac1} embryos is similar to that of *Fab-8*^{64} embryos. This mutation is a derivative of a P-*lacZ* element inserted in the *iab-7* domain, which retains the *lacZ* gene; in this mutant, the *Fab-8* boundary is deleted and, as a result, both the *iab-7* and *iab-8* regulatory sequences activate the *lacZ* gene (Barges et al., 2000). However, the model does not explain the absence of strong PS13 *lacZ* expression in the nervous system of *Abd-B*^{lac1} embryos. It is possible that the pairing between *Fab*/*PRE* sequences changes throughout development. If so, the *lacZ* pattern of *Abd-B*^{lac1} embryos could be explained by a combination of both models.

The two models predict an increase of Abd-B protein expression in PS12 of *Abd-B*^{lac1} and *iab-8,9* embryos. In the first model, *iab-7* interaction with the *Abd-B m* promoter is reinforced. In the second one, 3′ *iab-8* sequences could now be active in PS12. In *Abd-B*^{lac1} embryos, this effect could be difficult to detect, as *Abd-B* transcription is strongly reduced. However, it should be visible in *iab-8,9* embryos, because the exogenous and endogenous *Fab-8*/*PRE* sequences could also pair and the *Abd-B* promoter is intact. We have observed a slight increase in *Abd-B* expression in PS12 of *iab-8,9* homozygous embryos, but this effect is insufficient to cause a clear phenotype in either *iab-8,9* homozygous embryos or *iab-8,9* heterozygous females.

Abd-B regulation in PS13

Our data, and previous results (Barges et al., 2000; Zhou et al., 1999), support the existence of both 3′ and 5′ *iab-8* domains. The 3′ domain seems dispensable for a wild-type A8/PS13 development if the 5′ domain is intact, as in the *Fab-7*^{R59} and *iab-7*^{S10} mutations, but it is not clear if the opposite is true. In *iab-8,9* mutations, the 5′ *iab-8* domain is inactive (determined by the presence of the exogenous *Fab-8* boundary), but, according to the first model, the pairing of exogenous and endogenous 3′ *iab-8* sequences may also reduce the activity of the 3′ *iab-8* domain. Thus, in these mutations the activity of both 5′ and 3′ *iab-8* sequences could be compromised. This interpretation suggests that either the 3′ or the 5′ *iab-8* domains could determine Abd-B protein distribution in A8/PS13. Alternatively, both domains should be necessary for A8/PS13 development, but the 5′ domain alone could direct A8/PS13 development if the 3′ domain is absent. In any case, these results underline the complexity of *Abd-B* regulation.

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