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The molecular genetics of the bithorax complex of *Drosophila*: *cis*-regulation in the *Abdominal-B* domain

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In *Drosophila* the *Abdominal-B* (*Abd-B*) domain of the bithorax complex (BX-C) spans over 100 kb and is responsible for specifying the identities of adult abdominal segments five (A5) to nine (A9), inclusive, and correspondingly, neuromeres 10-14 of the embryonic central nervous system. The domain consists of a region coding for two proteins, ABD-BI (54 kd) and ABD-BII (36 kd) and *cis*-regulatory regions extending from *infra-abdominal-5* (*iab-5*) to *iab-9*, inclusive. We have used a monoclonal anti-ABD-B antibody to infer that mutants in *iab-8* eliminate the expression of ABD-BI in neuromeres 10-13, inclusive, and that mutants in *iab-9* eliminate expression of ABD-BII in neuromere 14. ABD-B expression is also analyzed in homozygotes for (i) loss-of-function mutants involving the *iab-5*, *iab-6* and *iab-7* regions, (ii) gain-of-function mutants *Miscadastral pigmentation* (*Mcp*) and *Superabdominal* (*Sab*), and (iii) a *trans*-regulator, *Polycomb* (*Pc*). ABD-B expression along the antero-posterior axis is colinear with the chromosomal order of the *cis*-regulatory regions. The behavior of rearrangement-associated *iab-6* and *iab-7* mutants suggests that the enhancer-like region, *iab-5*, and possibly also *iab-6*, may be shared between the *abd-A* and *Abd-B* domains. Such sharing is proposed as a factor that tends to keep gene complexes intact during evolution.

Key words: *cis*-regulation/embryonic development/homeobox/*polycomb*/*trans*-regulation.

Introduction

The Antennapedia complex (ANT-C) (Kaufman *et al.*, 1980; Garber *et al.*, 1983; Scott *et al.*, 1983) and the bithorax complex (BX-C) (for review see Duncan, 1987) control the development of the head, thorax and abdomen of the organism. The entire BX-C encompasses ~300 kb of DNA (Bender *et al.*, 1983; Karch *et al.*, 1985) and is composed of three domains: *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*), and *Abdominal-B* (*Abd-B*) (Sanchez-Herrero *et al.*, 1985a,b; Tiong *et al.*, 1985). The proteins from these domains are designated as UBX, ABD-A and ABD-B, respectively (Beachy *et al.*, 1985; Hogness *et al.*, 1985; Mann and Hogness, 1990).

Control of the BX-C is under negative *trans*-regulation by such genes as *Polycomb* (*Pc*) (Lewis, 1978; Duncan and Lewis, 1982), *extra sex combs* (*esc*) (Struhl, 1981) and the *Pc*-like group genes (Duncan, 1982; Jurgens, 1985) and under positive *trans*-regulation by at least one gene *trx* (also known as *Rg-bx*) (Ingham and Whittle, 1980). Segmentation genes (Nusslein-Volhard and Wieschaus, 1980) of the gap, pair rule and segment polarity classes may in some instances also play a role in *trans*-regulating these genes (Duncan, 1986; Ingham and Martinez-Arias, 1986; Ingham *et al.*, 1986; White and Lehman, 1986). Finally, at least some of the BX-C and ANT-C genes seem to *trans*-regulate each other in a negative and possibly hierarchical fashion based on *in vivo* studies of UBX expression in embryos deleted for *abd-A* and/or *Abd-B* (Struhl and White, 1985) and *in vitro* studies of the effect of UBX on *Antp* transcription (Krasnow *et al.*, 1989).

The sequencing of cDNAs from the BX-C has identified two types of transcripts: (i) those that code for proteins: *Ubx* (Hogness *et al.*, 1985; O'Connor *et al.*, 1988; Kornfeld *et al.*, 1989), *abd-A* (Karch *et al.*, 1990), and *Abd-B* (DeLorenzi *et al.*, 1988; Celniker *et al.*, 1989; Zavortnik and Sakonju, 1989); and (ii) those that do not: *bithoraxoid* (*bxd*) (Lipshitz *et al.*, 1987) and *infra-abdominal-4* (*iab-4*) (Cumberledge *et al.*, 1990). The exons of all the protein-coding transcription units add up to only 15 kb or 5% of the total BX-C DNA. The remaining 95% of the BX-C consists of introns and *cis*-regulatory regions, at least some of which give rise to transcripts that do not code for proteins. In the well characterized *Ubx* domain, a *cis*-regulatory region, bxd, lies 5′ to the protein-coding transcription units (Beachy *et al.*, 1985) and such regions have been found in introns, in the case of *bithorax* and *anterobithorax* (Bender *et al.*, 1983; Peifer and Bender, 1986).

The *Abd-B* domain spans over 100 kb and occupies the distal third of the BX-C. The domain consists of ~5 kb of protein-coding transcription units or roughly 5% of the domain, while the remaining 95% consists of introns and *cis*-regulatory regions.

A consistent terminology for the genetic units of the *Abd-B* domain has not yet been established. We follow the suggestion of Duncan (1987) to designate null mutants with the base symbol *Abd-B* and to designate mutants affecting specific sub-functions with the infra-abdominal nomenclature (Lewis, 1978). We divide his single *iab-8,9* sub-function into two sub-functions, *iab-8* and *iab-9*. The latter have also been referred to as m and r sub-functions (Casanova *et al.*, 1986). We realize that a mutant such as *D14* could be designated as an *Abd-B* allele but designating it as an *iab-8* allele is in keeping with its phenotypic properties, and is therefore in keeping with the usual rules of genetic nomenclature.

Molecular analysis of the *Abd-B* genomic organization has identified four potential *Abd-B* promoters (Celniker and Lewis, 1987; DeLorenzi *et al.*, 1988; Sanchez-Herrero and Crosby, 1988; Kuziora and McGinnis, 1988; Celniker *et al.*, 1989 and Zavortnik and Sakonju, 1989). One promoter produces transcripts that encode a 54 kd protein, ABD-BI. The other three promoters give rise to transcripts that encode a 36 kd protein, ABD-BII. The two proteins share the same homeodomain, a DNA binding motif of 60 amino acids,

(Laughon and Scott, 1984; Shepherd *et al.*, 1984; Desplan *et al.*, 1985) that is conserved amongst all of the known proteins of the ANT-C and BX-C (McGinnis *et al.*, 1984; Scott and Wiener, 1984; Regulski *et al.*, 1985).

We have used a monoclonal antibody, 1A2E9, to examine the effects of mutants located in the *iab* regions on the distribution of the two ABD-B proteins in various embryonic stages. This antibody recognizes an epitope common to both proteins and does not cross-react with other proteins as shown by absence of staining in *Abd-B*$^-$ embryos (Celniker *et al.*, 1989). Using this antibody we have determined that ABD-B is expressed in parasegments 10–15 of the ectoderm, in the visceral mesoderm surrounding the hindgut and in the central nervous system (CNS) in neuromeres 10–14 (Celniker *et al.*, 1989). Using a polyclonal antibody, DeLorenzi and Bienz (1990) have reported a similar pattern of expression. Our studies and theirs have shown that intensity of staining for ABD-B in the CNS increases in discrete steps in going from neuromere 10 to neuromere 14. The wild-type ABD-B distribution directly correlates with the sum of the *Abd-B* transcript distributions (DeLorenzi *et al.*, 1988; Kuziora and McGinnis, 1988; Sanchez-Herrero and Crosby, 1988).

This paper presents a genetic and molecular analysis of *cis*-regulation of the *Abd-B* domain and *trans*-regulation by the *Polycomb* (*Pc*) gene.

Results

For the most part we have used mutants that were selected solely on the basis that they suppress transvection. The screen for their detection involves X-raying wild-type males and mating to females homozygous for the third chromosome, double mutant combination, *Contrabithorax* (*Cbx*) and *Ubx*. These females are also homozygous for an X chromosome carrying a BX-C duplication, *Dp-68*, which covers the recessive lethality of *Ubx* and, at the same time, permits recovery of mutants that might otherwise be dominant steriles or dominant lethals. The majority of the *Cbx Ubx*/+ progeny have weak *Cbx* effects (slightly reduced and spread wings) (Lewis, 1985). With a low frequency, progeny with wild-type wings are produced. These carry a chromosomal rearrangement with a breakpoint between the centromere and the BX-C. Such transvection-suppressing rearrangements (TSRs) evidently reduce pairing of the homologous third chromosomes in the vicinity of the BX-C thereby preventing *Cbx* from activating *Ubx*$^+$ in the wild-type homologue.

TSRs that test positively in the adult male hemizygote (genotype: mutant over a deficiency for the BX-C) for loss-of-function effects fall into several phenotypic classes as follows: breakpoints in the *iab-5* region tend to transform A5 toward A4; those in the *iab-6* region transform A5 and A6 toward A4; those in the *iab-7* region transform A5, A6 and A7 toward A4; those in the *iab-8* region transform A8 toward A7 causing an eighth sternite and tergite to appear; and finally, those in the *iab-9* region transform A9 toward A8, resulting in loss of analia and genitalia. The properties of our new mutants are summarized in Table I. There is a colinear relationship such that the more distal a breakpoint in the *Abd-B* domain (Figure 1A) the more posterior is the region of the body affected.

For our molecular studies we have chosen to avoid hemizygous genotypes for several reasons. The BX-C is haplo-insufficient for many *iab* functions; e.g. *Df-P9*/+ adults show slight transformation of A5, A6 and A8 toward a more anterior segment. At the same time, there are slight gain-of-function effects that indicate that with only one dose of the BX-C there is an imbalance in the functional interdependence of the *iab* regions (Karch *et al.*, 1985). For embryo analyses, homozygous *iab* mutants have been used since even though such homozygotes may carry lethals at other loci there has been no indication that such lethals have an effect at the embryonic stage under study. In cases where the homozygote fails to survive as an adult we have used heteroallelic combinations to generate a homozygous genotype.

Adult phenotypes of homozygous loss-of-function mutants

Adult phenotypes for homozygous *iab* mutants in the *Abd-B* domain have not been previously described in detail and are therefore summarized (Figure 2). Only males are considered since their segmental transformations are more easily demonstrated than are the corresponding ones in females. In the case of *iab-5* mutants the phenotype consists of transformation of A5 toward A4 as in the hemizygote. Remarkably, *iab-6*, *iab-7* and *iab-8* homozygotes differ significantly from the corresponding hemizygotes. Thus *iab-6* mutants have transformation of the sternite and tergite of A6 toward those of A5 but fail to show the dramatic loss

Table I. New mutants of the *Abd-B* domain

Mutants	Source (X-rayed)	Cytology	DNA map	Deduced embryonic CNS protein expression	
				ABD-BI	ABD-BII
Wild-type (C.S.)	–	–	–	PS 10–13	PS 14
iab-5^{994}	C.S.	T(22; 89B-E;90A)	ND[a]	PS 11–13	PS 14
iab-5^{224}	In(3R)1000	Tp(83 to 85;89E)	ND	PS 11–13	PS 14
iab-6^{11}	In(3R)1000	In(72;89E)	+113 to +115 kb	PS 12, 13	PS 14
iab-6^{200}	In(3R)1000	T(29EF-30A;89E)	+120 to +121 kb	PS 12, 13	PS 14
iab-6^{1821}	In(3R)1000	T(30F-31A;89E)	+116 to +118 kb	PS 12, 13	PS 14
iab-7^{770}	In(3R)1000	T(68;89E;91;94)	ND	PS 13	PS 14
iab-7^{164}	In(3R)1000	In(65;89E)	+126 to +128 kb	PS 13	PS 14
iab-7^{3081}	In(3R)1000	T(66;79;89E)	ND	PS 13	PS 14
iab-9^{48}	In(3R)1000	In(89E;100C)	+159 to +165 kb	PS 10–13	none
iab-9^{1392}	In(3R)1000	T(63;89E)	+182 to +187 kb	PS 10–13	weak/none
iab-9^{1645}	In(3R)1000	T(26A;89E)	+182 to +187 kb	PS 10–13	weak/none

[a]ND not determined.

of male-type pigmentation in A5 seen in the hemizygote; indeed, A4 tends to have male-type pigmentation well expressed in every case we have studied (five out of five). Similarly, *iab-7* mutants have a transformation of A7 toward a more anterior segment, leading to a seventh tergite and sternite (normally absent in the male); at the same time, unlike the hemizygote, there is strong male-type pigmentation in A5 and A6 and to a lesser extent in A4 as well. *iab-8* mutants have an eighth as well as seventh tergite and sternite (evidently as the result of A7 and A8 being transformed toward the identity of a more anterior segment), and male-type pigmentation in A5 and A6 and weakly in A4. *iab-9* mutants show only a partial or complete loss of genitalia, evidently as the result of A9 being transformed toward a more anteriorly located segment. Such mutants have the wild-type segmentation pattern with the exception of the 65 allele; this allele has a strong *cis*-overexpression (COE) effect in which A6 is partially reduced presumably by being transformed toward A7 (Lewis, 1985).

ABD-B expression in loss-of-function embryos

We have analyzed embryos homozygous for the null allele, $Abd\text{-}B^{D16}$, for ABD-B expression. They lack antibody staining throughout the embryo (data not shown), confirming our earlier observation (Celniker *et al.*, 1988) that a deletion that includes the entire *iab-8* and *iab-9* regions, Df-C4/Df-P9, lacks such staining.

Four *iab-5* mutants $iab\text{-}5^{301}$, $iab\text{-}5^{843}$, $iab\text{-}5^{994}$, and $iab\text{-}5^{224}$ have been analyzed for alterations in ABD-B distribution in embryonic stages. Each is associated with a chromosomal rearrangement that separates *iab-5* (and more proximal regions of the BX-C) from the *Abd-B* transcription units (Figure 1B). In every case the *iab-5* homozygote has a loss of ABD-B expression in neuromere 10 but no reduction in that expression in neuromeres 11−14 (Figure 3B).

Four *iab-6* mutants, $iab\text{-}6^{11}$, $iab\text{-}6^{200}$, $iab\text{-}6^{C7}$ and $iab\text{-}6^{1821}$ have been similarly analyzed. With the exception of $iab\text{-}6^{C7}$, each of the *iab-6* mutants is associated with a chromosomal rearrangement that separates *iab-6* (and more proximal regions) from the *Abd-B* transcription units. In homozygous *iab-6* embryos, we detect a loss of ABD-B expression in neuromeres 10 and 11 but no reduction in that expression in neuromeres *12−14* (Figure 3C). The $iab\text{-}6^{C7}$ mutant is associated with an insertion of DNA from region 90E of chromosome 3R into the complex at +124 (Karch *et al.*, 1985), the *iab-6*/*iab-7* border. Apparently this insertion does not affect *iab-7* function as we detect ABD-B labeling in neuromere 12.

Five *iab-7* mutants, $iab\text{-}7^{MX1}$, $iab\text{-}7^{164}$, $iab\text{-}7^{MX2}$, $iab\text{-}7^{770}$

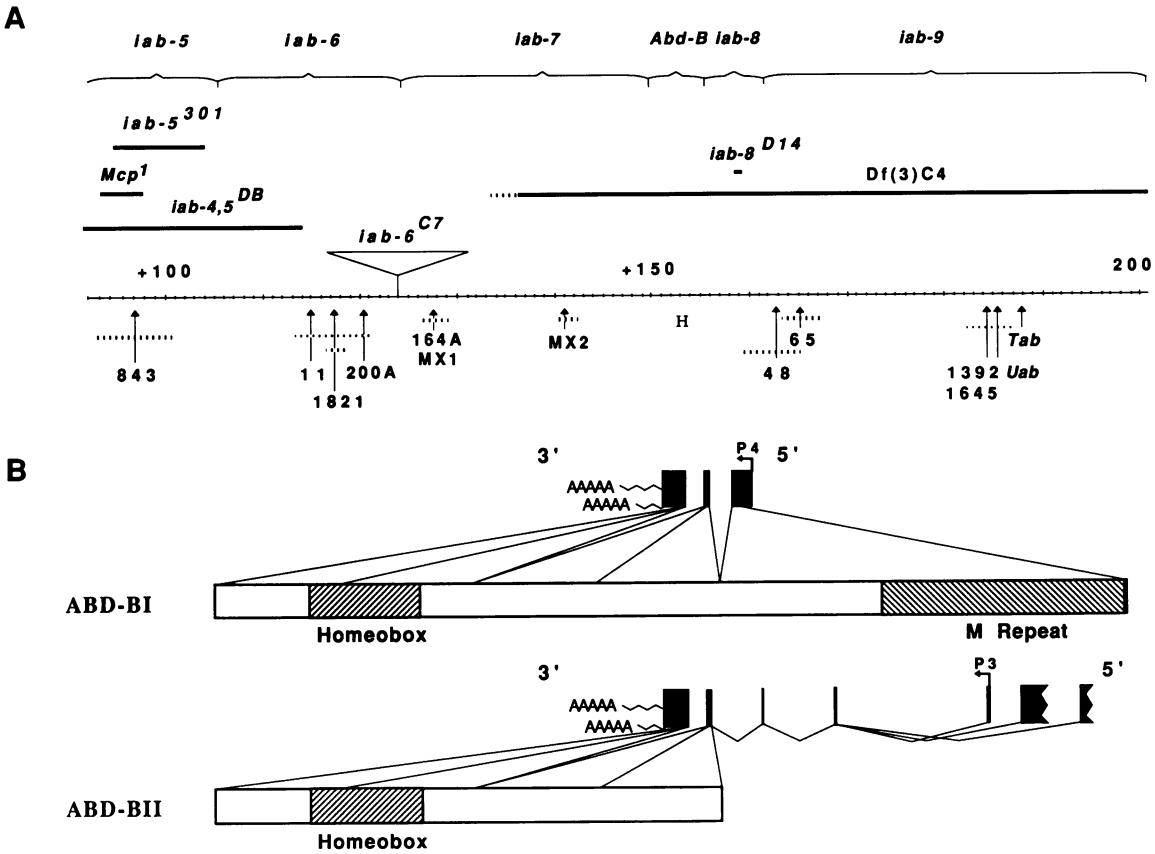

Fig. 1. Organization and structure of genomic DNA and representative homeobox-containing cDNAs from the *Abd-B* gene. (**A**) The thin line represents the genomic DNA, the scale is marked in kilobases, and the numbers refer to the BX-C map position according to Karch *et al.* (1985).The *iab-5* to *Abd-B* regions delineated by brackets are indicated according to Duncan (1987), the *iab-8* and *iab-9* regions have been separated for reasons described in the text. The thick lines represent the sequences deleted in various deficiencies. The vertical arrows indicate the location of mapped chromosomal rearrangements, hatching indicates the limits of uncertainty in the positions of these breaks mapped by Southern analysis. Triangles indicate DNA insertions. (**B**) Transcription map based on intron−exon mapping and cDNA analysis. All of the intron−exon boundaries were determined by sequencing both genomic and cDNA clones. Polyadenylation at two sites differing by 269 bp was determined by cDNA sequencing. Evidence for exons 1a and 1b comes from data presented in Kuziora and McGinnis (1988) and DeLorenzi *et al.* (1988). The starts of transcription from P3 and P4 were determined by Zavortnik and Sakonju (1989).

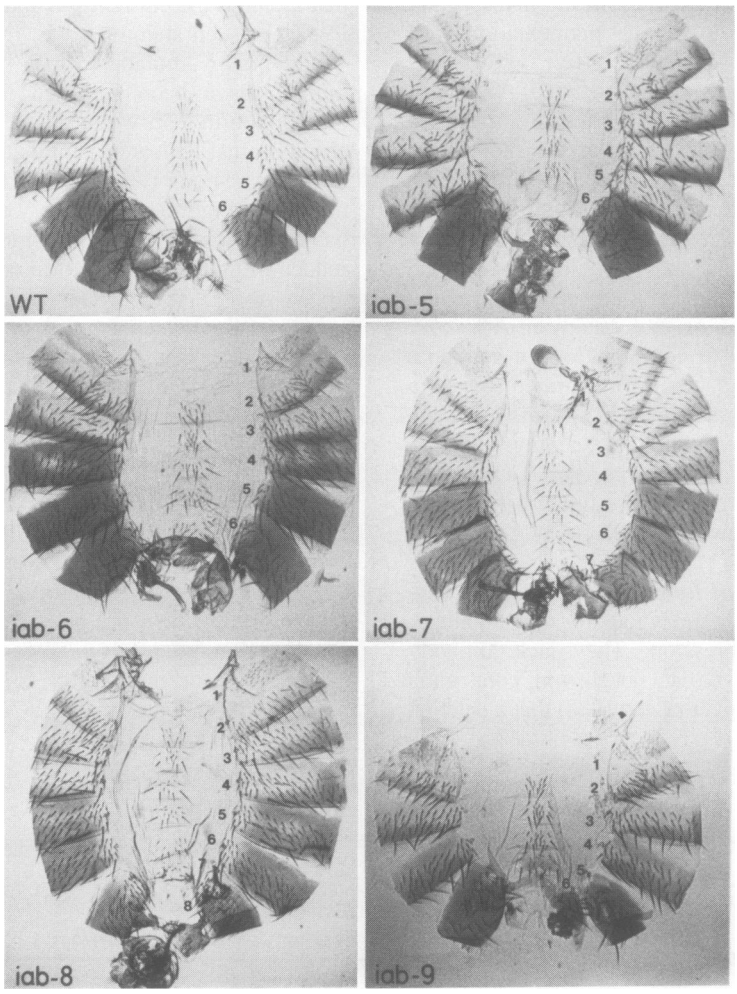

Fig. 2. Adult male phenotypes of homo- or heteroallelic *iab* mutants. Shown are whole mounts of adult male abdominal cuticle photographed using bright field optics. Abdomens were cut dorsally along the midline, mounted on a glass slide and prepared as described in Materials and methods. The abdominal segments are numbered. To the right of the number is the tergite. Immediately to the left are the sternites. WT: Canton S. The wild-type male has six sternites and six tergites. The fifth and sixth tergites are completely pigmented black. *iab5: iab* $-5^{301}/iab\text{-}5^{301}$ shows loss of pigment in A5. *iab6: iab-*$6^{11}/iab\text{-}6^{11}$. Extra bristles on the A6 sternite. A5 type pigmentation on A4. *iab-7: iab-*$7^{164}/iab7^{MX\text{-}2}$. Extra A7 tergite and A6 and A7 sternites with bristles. A5 type pigmentation on A4−A7. *iab-8: iab-*$8^{D6}/iab\text{-}8^{D14}$. Extra A7 and A8 tergites and sternites with bristles on A6 and A7. *iab-9: iab-*$9^{1065}/iab\text{-}9^{1065}$. Loss of genitalia and analia. Reduction in A6 tergite.

and *iab-*7^{3081} have been analyzed. Each is associated with a chromosomal rearrangement that separates *iab-7* (and more proximal regions) from the *Abd-B* transcription units. In every case the *iab-7* homozygous embryo has a loss of ABD-B protein expression in neuromeres 10, 11 and 12 but has essentially wild-type ABD-B expression in neuromeres 13 and 14 (Figure 3D). The transcript distribution has been reported for homozygous *iab-*7^{MX2} embryos (Sanchez-Herrero and Akam, 1989) and is identical to the protein distribution we detect in our *iab-7* mutants.

Three *iab-8* mutants, *iab-*8^{D14}, *iab-*8^{D6} and *iab-*8^{D15} have been analyzed. They were induced with EMS by Ian Duncan and one, *iab-*8^{D14}, has now been sequenced (Zavortnik and Sakonju, 1989; S.E.Celniker and D.J.Keelan, unpublished results) and shown to be a 396 bp deletion spanning the transcription start site of the P4 promoter region (see Figure 1B). In homozygous *iab-8* embryos we detect a loss of ABD-B protein expression in neuromeres 10, 11, 12 and 13 but essentially wild-type ABD-B expression in neuromere 14 (Figure 3E). It has been reported that the mutants, *Abd-*B^{M2}, *Abd-*B^{M3}, *Abd-*B^{M4}, *Abd-*B^{M5}, and *Abd-*B^{M7} show an identical ABD-B expression pattern (DeLorenzi and Bienz, 1990) to the *iab-8* ABD-B expression pattern.

Of the five *iab-9* mutants, *iab-*9^{48}, *iab-*9^{65}, *iab-*9^{1645}, *iab-*9^{1392}, and *iab-*9^{Uab1}, which we have analyzed, each is associated with a chromosomal rearrangement. In homozygous *iab-9* embryos ABD-B expression is reduced, relative to wild-type, in neuromere 14 (Figure 3F). Within the *iab-9* group it may be significant that map location of the rearrangement breakpoint correlates with the degree of ABD-B expression. Thus, among the five mutants, *iab-*9^{48}, at map position +160, shows the greatest reduction in ABD-B expression, not only in neuromere 14 but also in the level of expression in neuromeres 10−13. *iab-*9^{65}, at map position +165, shows less reduction in expression of ABD-B in neuromere 14 than does *iab-*9^{48} and virtually wild-type expression in neuromeres 10−13, inclusive. *iab-*9^{1645}, *iab-*9^{1392}, and *iab-*9^{Uab1} with chromosomal rearrangement breakpoints from +182.5 to +187.5, show ABD-B expression in neuromere 14 similar to that in neuromere 13 of wild-type and wild-type expression in neuromeres 10−13, inclusive.

In wild-type embryos at Stage 16, in addition to ABD-B expression in the CNS, there is also strong ABD-B expression in the visceral mesoderm surrounding the hindgut (Figure 4A). *iab-5*, *iab-6*, *iab-7* (data not shown) and *iab-8* (Figure 4B) homozygous mutant embryos show wild-type ABD-B expression in the visceral mesoderm surrounding the hindgut. This is also true for $iab\text{-}9^{1645}$, $iab\text{-}9^{1392}$, and $iab\text{-}9^{Uab1}$ (data not shown). However, in two *iab-9* mutants, $iab\text{-}9^{48}$ and $iab\text{-}9^{65}$, we do not detect any ABD-B expression in this tissue (Figure 4C).

Wild-type ABD-B protein expression can be detected by germ band extension (Stages 8−12). By Stage 12, ABD-B expression is abundant in the ectoderm of parasegments 13 and 14 with marginal expression detectable in parasegments 11 and 12 (Figure 4D). Since *iab-8* and *iab-9* mutants affect ABD-B expression in parasegments 13 and 14, late in development, we analyzed such mutants to determine if we could detect a change from the wild-type pattern as early as germ band extension. We were able to detect altered ABD-B expression in both classes of mutants (Figure 4E and F). In homozygous *iab-8* embryos we detect ABD-B expression solely in parasegment 14 (Figure 4E). Unlike the expression in the CNS, *iab-9* embryos show an expansion of the distribution and elevated levels of ABD-B protein expression in parasegments 11 and 12, a nearly normal distribution and level of expression in parasegment 13 and reduced expression, relative to wild-type, in parasegment 14 (Figure 4F).

Adult phenotypes of gain-of-function mutants

We have analyzed ABD-B expression in two dominant gain-of-function mutants *Mcp* (Lewis, 1978) and *Sab* (Sakonju *et al.*, 1984) and the double mutant combination (Figure 6). *Mcp* results in the adult A4 being transformed toward A5. The *Sab* homozygote and heterozygote show male-type pigmentation in a variable pattern in A3 and A4; surprisingly, such pigmentation is slightly more pronounced in A3 than it is in A4. [*Sab* was induced with EMS on an *Mcp* chromosome and selected on the basis that it extended the male-type pigmentation found in A4 of *Mcp* males into A3. *Sab* was separated from the *Mcp Sab* chromosome by crossing over and was found to lie slightly distal to *Mcp* (based on three crossovers from *ss Mcp Sab Mc*/+ + females mated to *ss* males).]

The double mutant, *Mcp Sab*, has much stronger male-type pigmentation of A3 and A4 than does *Sab* alone. However, A4 is consistently more heavily pigmented than A3, unlike the *Sab* homozygote or heterozygote.

ABD-B expression in gain-of-function iab mutants

In homozygous and heterozygous *Mcp* embryos (Stage 16) we detect an extension of ABD-B expression in the CNS into neuromere 9. In neuromeres 10−14, the pattern of expression is similar to that of wild-type except that expression in neuromere 10, at least, may be slightly more intense than that of wild-type (Figure 5B).

Homozygotes for *Sab* (without *Mcp*) show very weak staining for ABD-B in neuromere 8 but no detectable staining in neuromere 9 (data not shown). Homozygous *Mcp Sab* embryos (Stage 16) show extension of ABD-B expression into neuromeres 8 and 9 with expression in neuromeres 10−14 perhaps slightly more intense than in wild-type (Figure 5C).

Effect of the trans-regulator, Polycomb, on ABD-B expression

In embryos homozygous for the *Polycomb* mutant and a duplication for the BX-C, Dp(3;3)P5, ABD-B expression is observed in the ectoderm and CNS of all the body segments more or less to the same extent (Celniker *et al.*, 1989 and Figure 5D). To dissect this expression we have examined embryos homozygous for the double mutant combination, $Pc^3 iab\text{-}8^{D14}$. In such embryos, ABD-B expression is detected in the mesoderm as early as germ band extension. By early germ band retraction, ABD-B expression is detected in the ectoderm of virtually all the segments and by the completion of germ band retraction, into all the neuromeres of the CNS (data not shown). Since in these $Pc\ iab\text{-}8^{D14}$ embryos, the $iab\text{-}8^{D14}$ mutant likely eliminates ABD-BI (see Figure 3E and Discussion), it is ABD-BII that is being ectopically expressed.

ABD-B protein expression in Ubx and abd-A mutants

To examine the possibility of *trans*-regulation of *Abd-B* by proteins from the *Ubx* and *abd-A* domains, we have examined embryos lacking one or the other, or both, of these two classes of proteins. Embryos homozygous for Ubx^1 lack detectable amounts of *Ubx* proteins when assayed with antibodies to an epitope common to all of such proteins (Beachy *et al.*, 1985; Hogness *et al.*, 1985). Homozygous Ubx^1 embryos show the wild-type pattern of ABD-B expression when stained with our 1A2E9 antibody (data not shown). Embryos homozygous for $abd\text{-}A^{MX1}$ lack ABD-A proteins detectable by anti-*abd-A* antibody (Karch *et al.*, 1990). In such embryos, there is also no change in the wild-type pattern of ABD-B expression (data not shown). Finally, embryos homozygous for $Df(3)Ubx^{109}$, which deletes the *Ubx* and most of the *abd-A* domain (Lewis, 1978) and which lacks the *Ubx* and *abd-A* transcription units, are also wild-type with respect to ABD-B expression (data not shown).

Discussion

The molecular organization of the *Abd-B* domain has proved to be quite unlike that of the other domains of the BX-C in that there are three regions, *iab-5*, *iab-6*, and *iab-7*, that are clearly *cis*-regulatory and two regions, *iab-8* and *iab-9*, that not only give rise to multiple transcripts coding for at least two proteins, ABD-BI and ABD-BII, but contain presumptive *cis*-regulatory elements as well.

In order to dissect *cis*-regulation within the *Abd-B* domain we have used an anti-ABD-B antibody to deduce the distribution of ABD-BI and ABD-BII in the embryonic CNS. Our interpretation of the behavior of loss-of-function and gain-of-function mutants on the expression of ABD-BI and ABD-BII proteins is summarized in Figure 7.

ADB-B expression in loss-of function iab mutants

To infer the parasegmental patterns of expressions of ABD-BI and ABD-BII respectively, we have made use of loss-of-function mutants in the *iab-8* and *iab-9* regions, inclusive, and studied their effects, especially on the CNS (Table II). The results indicate that ABD-BI is expressed in neuromeres 10−13, inclusive. ABD-BII, on the other hand, appears to be expressed only in neuromere 14. The $iab\text{-}8^{D14}$ mutant has proven especially useful in that it maps to the first exon of the transcription unit coding for ABD-BI and is a small

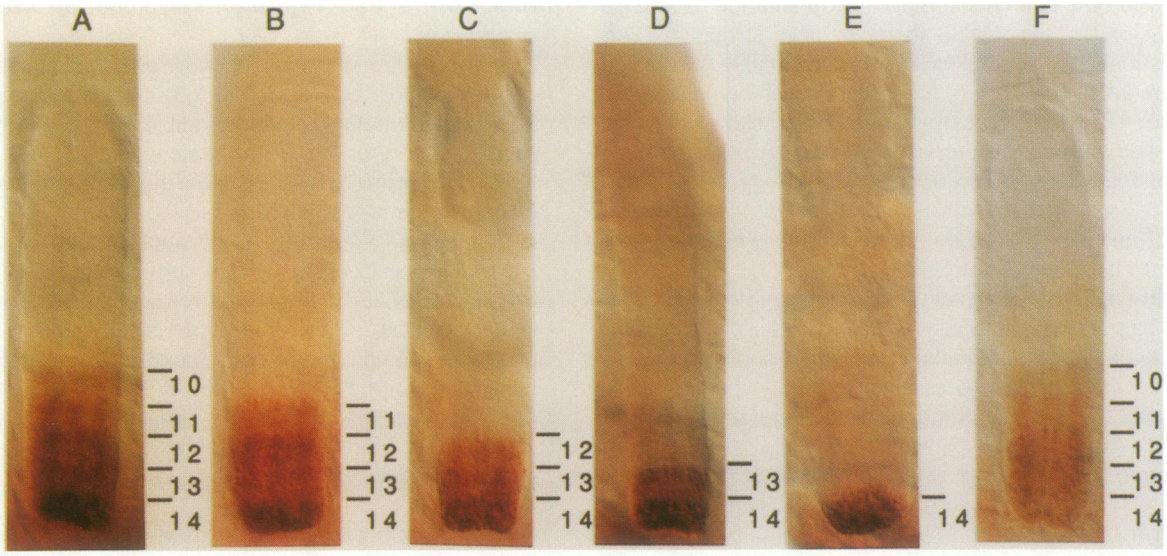

Fig. 3.

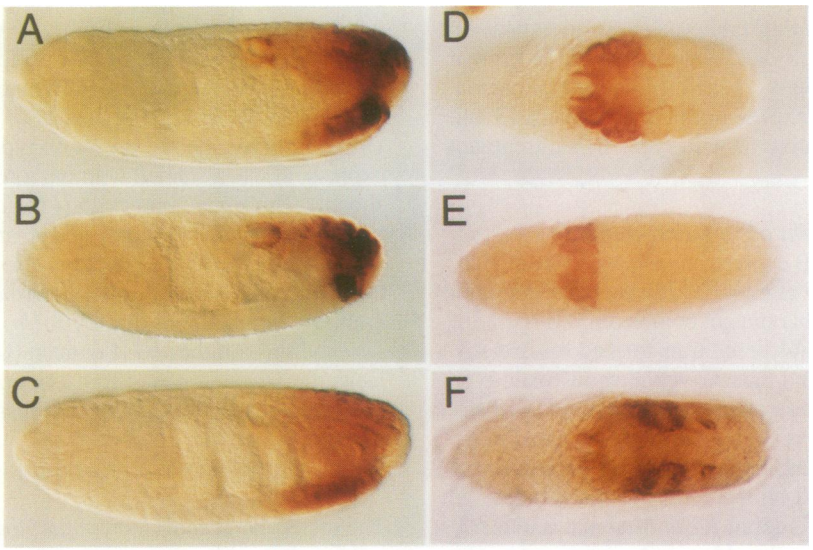

Fig. 4.

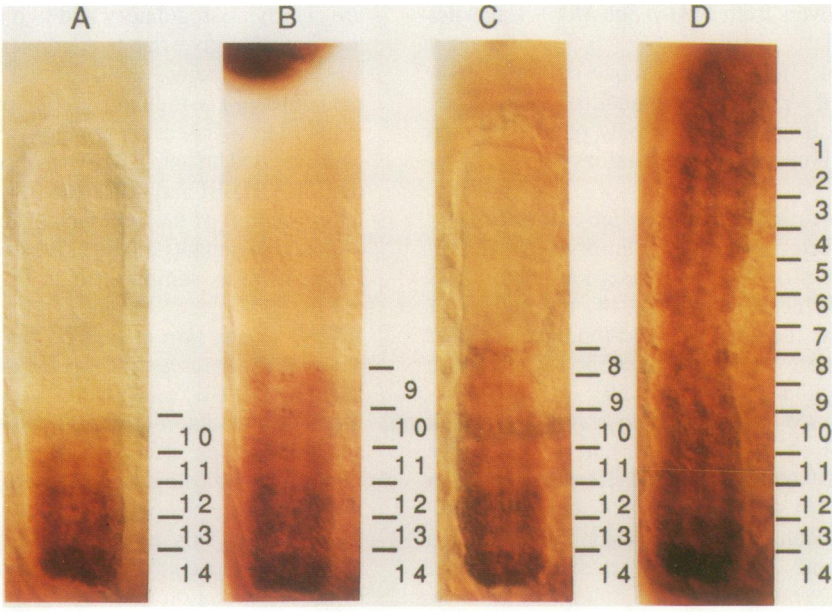

Fig. 5.

deletion of 396 bp. Hence, *iab-8*^{D14} homozygotes are expected to lack the ABD-BI protein. With respect to ABD-BII, *iab-8*^{D14} maps to the third intron of its transcription unit. Our results suggest that the deletion does not interfere with splicing and allows functional ABD-BII to be produced. Hence we attribute the observed anti-ABD-B staining in neuromere 14 to ABD-BII and the observed lack of such staining in neuromeres 10−13 to absence of ABD-BI. Consistent with this interpretation are the reports of Sanchez-Herrero and Crosby (1988) and Kuziora and McGinnis (1988) that the majority of transcripts expressed in neuromere 14 of wild-type embryos are those encoding ABD-BII. Trace amounts of transcripts encoding ABD-BI, have been detected in neuromere 14 but these have been attributed to unspliced precursors (Sanchez-Herrero and Crosby, 1988).

Two *iab-9* mutants, *iab-9*^{48} and *iab-9*^{65}, are associated with chromosomal rearrangments having breakpoints in the third intron of the transcripts encoding ABD-BII. Hence, homozygotes for these mutants are not expected to produce ABD-BII. Since the breakpoints of these mutants fall outside the transcription unit coding for ABD-BI, the homozygotes are expected to produce functional ABD-BI. Reports of Sanchez-Herrero and Crosby (1988) and Kuziora and McGinnis (1988) are again consistent with these assumptions in that the only ABD-B transcripts expressed in neuromeres 10−13 of wild-type embryos are those encoding ABD-BI.

Expression of ABD-B in neuromeres 10, 11 and 12 is under the control of a large *cis*-regulatory region spanning 58 kb that resides 3′ to the *Abd-B* transcription unit. In each of three separable regions, *iab-5*, *iab-6* and *iab-7*, are presumably enhancer-like elements that are required for proper ABD-B expression. Thus, loss-of-function *iab-5* mutants selectively eliminate ABD-B expression in neuromere 10, *iab-6* mutants in neuromeres 10 and 11, and *iab-7* mutants in neuromeres 10, 11 and 12. The *iab-5*, *iab-6* and *iab-7* mutants do not seem to affect ABD-B expression detectably in neuromeres 13 and 14. By applying the above interpretation of ABD-B expression in *iab-8* and *iab-9* mutants to these results we infer from the mutant analysis that, in wild-type, the *iab-5*, *-6* and *-7* regions are primarily involved in *cis*-regulating expression of ABD-BI as opposed to ABD-BII.

In the wild-type CNS, levels of ABD-B expression increase in discrete steps commencing in neuromere 10 and continuing through 14. In an *iab-5* loss-of-function mutant the increase is still observed in neuromeres 11−14, inclusive, at levels comparable with those in wild-type. In *iab-6* and *iab-7* loss-of-function mutants, the corresponding increases are observed commencing in neuromeres 12 and 13, respectively. Nor is the stepwise increase in neuromeres 10−14 altered in the gain-of-function mutants, *Mcp* and *Sab*.

The stepwise increase in ABD-B expression in neuromeres 10−14, inclusive, might result from a number of factors: (i) the concentration of *trans*-regulatory molecules may vary incrementally (decreasing for negative, or increasing for positive, regulators); and/or (ii) affinities of the *iab-5* to *iab-9* regions, for the *trans*-regulatory molecules may vary incrementally; and (iii) an increasing number of cell types may begin to express ABD-B commencing with a subset of such types activated by the *iab-5* region, another subset by *iab-6*, and correspondingly for the remaining *iab* regions. Since in *iab-5* mutants the increments in ABD-B expression starting in neuromere 11 and proceeding to 14 are not obviously different from the corresponding increments in wild-type (Figure 3B), factors (i) and (ii) would have to predominate over factor (iii). Moreover, the faint staining of neuromere 10 in wild-type seems uniformly distributed rather than being confined to a small subset of cells, and therefore factor (iii) appears not to play a primary role in the observed pattern of ABD-B expression.

Colinearity of ABD-B expression and chromosomal order of cis-elements

The *cis*-regulatory regions *iab-5* to *iab-7*, inclusive, are arranged consecutively in a proximo−distal order that is colinear with the expression patterns of ABD-B as revealed by the analysis of mutants in each of these regions. Sanchez-Herrero and Akam (1989) show spatially ordered transcription of *iab* regions that presumably results in non-coding, *bxd*-like, transcripts. Thus, their analysis confirms colinearity between gene order and gene transcription along the antero−posterior axis of the organism. Presumably, as Lipshitz *et al.* (1987) have suggested, such transcription reflects activation of enhancer regions that in turn regulate transcription of the protein-coding regions.

Fig. 3. Localization of ABD-B proteins in the CNS of wild-type and homozygous *iab* mutant embryos (Stage 16 as defined by Campos-Ortega and Hartenstein, 1985). Embryos were dechorionated, the vitelline membrane removed, fixed and incubated with anti-ABD-B antibody (1A2E9) and proteins were visualized by use of horseradish peroxidase-conjugated secondary antibodies. The neuromere boundaries are indicated. (**A**) Wild-type. ABD-B proteins are expressed in a concentration gradient increasing from weak expression in neuromere 10 to strong expression in neuromere 14. (**B**) *iab-5: iab-5*^{224} the labeling is wild-type except for a loss of expression in neuromere 10. (**C**) *iab-6: iab-6*^{11} the labeling is wild-type except for a loss of expression in neuromeres 10 and 11. (**D**) *iab-7: iab-7*^{164} the labeling is wild-type except for a loss of expression in neuromeres 10−12 inclusive. (**E**) *iab-8: iab-8*^{D14} normal labeling is seen in neuromere 14 but no labeling is detectable in the neuromeres 10−13 inclusive. (**F**) *iab-9: iab-9*^{65} compared with wild-type, neuromeres 10−13 show a slight reduction of labeling, while neuromere 14 shows a significant reduction of labeling.

Fig. 4. Localization of ABD-B proteins in the visceral mesoderm of wild-type and *iab* mutant Stage 16 embryos and in the ectoderm of wild-type and *iab* mutant Stage 12 embryos. Embryos were prepared and stained by the method described in Figure 3. The anterior end of the embryos is always to the left. (**A**−**C**) Lateral views (Stage 16) and (**D**−**F**) dorsal views (Stage 12) of optical sections using Nomarski optics. (**A**) Wild-type (**B**) *iab-8: iab-8*^{D14} normal ABD-B protein expression in the visceral mesoderm surrounding the hindgut. (**C**) *iab-9: iab-9*^{65} absence of detectable ABD-B protein expression in the visceral mesoderm surrounding the hindgut. (**D**) Wild-type (**E**) *iab-8: iab-8*^{D14} Labeling is detectable only in the ectoderm of PS14 and PS15. (**F**) *iab-9: iab-9*^{65} Labeling is detectable in a novel pattern in the ectoderm of PS11−14 inclusive. Relative to wild-type labeling is elevated in PS 11 and 12 and reduced in PS 14 and 15.

Fig. 5. Localization of ABD-B proteins in the CNS of wild-type and over-expression mutant stage 16 embryos. Embryos were treated and labeled as described in Figure 2. (**A**) Wild-type. The ABD-B proteins as described in Figure 2 are expressed in a concentration gradient increasing from weak expression in neuromere 10 to strong expression in neuromere 14. (**B**) *Mcp/+* the labeling is wild-type except for a gain of expression in neuromere 9. (**C**) *McpSab/+* the labeling is wild-type except for a gain of expression in neuromeres 9 and 10. (**D**) *Pc*^{3}*P5/Pc*^{3}*P5* in addition to the apparently normal labeling of ABD-B proteins in neuromeres 13 and 14 all the anterior neuromeres of the developing CNS show labeling.

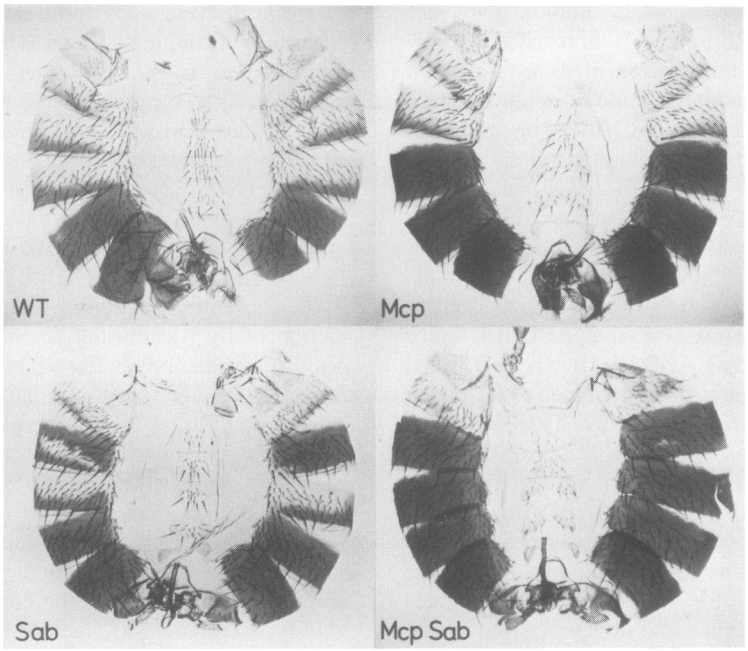

Fig. 6. Adult male phenotypes of homozygous gain-of-function mutants. Photographs of whole mounts prepared as described as in Figure 2. WT: Canton S. *Mcp*: A4 has an A5 type of pigmentation. *Sab:* A3 has an A5 type of pigmentation. A4 has a weak A5 type of pigmentation. *Mcp Sab* Double Mutant: A3 and A4 have an A5 type of pigmentation.

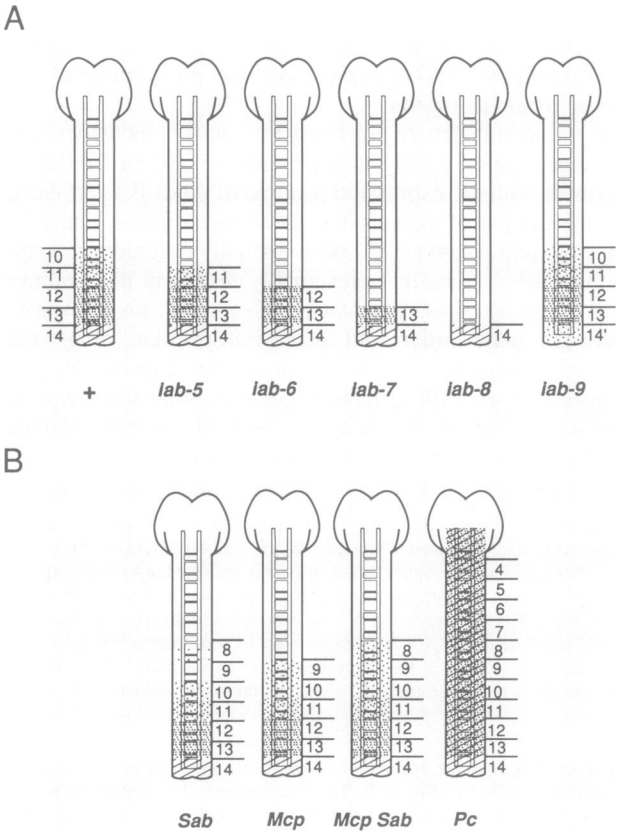

Fig. 7. Model for the distribution of the large and small ABD-B proteins in the CNS of wild-type and mutant embryos. The parasegmental boundaries are indicated on the side. The small protein distribution is represented by hatching, large protein distribution is represented by dots. The concentration of the large protein distribution labeling is represented by the density of the dots. (**A**) *iab* loss-of-function mutants. (**B**) *iab* gain-of-function mutants.

Table II. Inferred ABD-B expression in the CNS of wild-type (WT) and in mutants of the *Abd-B* domain

Genotype	ABD-BI	ABD-BII
WT	+	+
iab-8^{D14}	–	+
iab-9^{1065}	+	–
DfC4[a]	–	–

[a]Data from Celniker *et al.* (1989).

Possible sharing of cis-regulation of ABD-A and ABD-B expression by iab-5 and iab-6 regions

ABD-B expression in the embryonic CNS does not correlate with the adult phenotypes of homozygotes for mutants having breakpoints in the *iab-6* and *iab-7* regions. In both of these mutant categories male-type pigmentation is still present in A5 and A6 even though we do not detect ABD-B expression in the corresponding embryonic neuromeres (9 and 10, respectively). We interpret these adult phenotypes as indicating that, in the *iab-6* rearrangment mutant, the wild-type *iab-5* region, being far removed from the remainder of the *Abd-B* domain, now *cis*-regulates ABD-A function. Presumably therefore, *iab-5* contains DNA elements that directly or indirectly effect male-type pigmentation in A5 of wild-type males. Further, we assume that these elements would be able to direct such pigmentation using either the ABD-A or ABD-B protein. Similarly, in the case of *iab-7* rearrangements, the *iab-5* region is again far removed (along with *iab-6*) from the rest of the *Abd-B* domain and therefore can now *cis*-regulate ABD-A expression. In other words in wild-type, the *iab-5* region is assumed to direct ABD-B expression in cells destined to produce male-type pigmentation in A5; in *iab-6* and *iab-7* mutants, when *iab-5* is removed from the *Abd-B* domain it directs ABD-A

expression in such cells. Implicit in this interpretation is the assumption that ABD-A or ABD-B can activate downstream genes for male-type pigmentation. We note that Duncan has also proposed that the *abd-A* and *Abd-B* domains may not function independently of one another (Duncan, 1987).

The finding that homozygotes for *iab-6* and *iab-7* show male-type pigmentation in A5, A6, and to some extent in A4; whereas, the hemizygotes tend to lack such pigmentation in A4, A5, and A6, can be attributed to such pigmentation being very sensitive to the concentration of ABD-A in those segments. Thus, with only one dose of the rearrangement in the hemizygote there would be insufficient ABD-A to provide pigmentation in A5, or A6, while with two doses in the homozygote, the levels would be sufficient to produce male-type pigmentation partially in A4 and nearly completely in A5 and A6. A similar argument applies to the behavior of ABD-B expression. Hemizygotes for a deficiency for the BX-C, or for just the *Abd-B* domain, have considerably weakened pigmentation in A5, which in some backgounds can result in complete lack of male pigmentation. Thus, dosage of wild-type copies of the *Abd-B* domain, as already implied for the *abd-A* domain in *iab-6* mutants, is clearly a factor in determining the extent of male-type pigmentation in A5.

We expect that sharing of the *iab-5* region between the *abd-A* and *Abd-B* domains should also operate in, and carry a selective advantage to, wild-type. As a result such sharing might explain the tendency for gene complexes of the bithorax type to remain intact during evolution. Another example would be the discovery by Enver *et al.* (1990) of a demonstrably advantageous sharing of a *cis*-regulatory locus activating region (LAR) between the fetal and adult β-hemoglobin genes of human beings. Such sharing might again be a factor in keeping the β-hemoglobin complex intact during evolution.

ABD-B expression in cis-acting gain-of-function mutants

Correlated with the transformation in *Mcp* homozygotes and heterozygotes of A4 of the adult toward A5, is our finding that in these genotypes ABD-B expression extends anteriorly into neuromere 9. Although it is not certain whether *Mcp* lies in *iab-4* or *iab-5* or crosses the boundary between these regions, we interpret the gain-of-function in this mutant to result from deletion of repressor-binding sites located in the *iab-5* region. In wild-type embryos such sites would normally prevent ABD-B expression in neuromere 9. In *Mcp* animals, the absence, or reduction in number, of such sites would lead *iab-5* to *cis*-regulate expression of ABD-B in neuromere 9 as well as more posterior neuromeres.

The greater phenotypic effect of *Sab* on A3 than A4, correlates with our molecular analysis; we can detect, albeit very weakly, ABD-B expression in neuromere 8 and lack thereof in neuromere 9. The observed expression in neuromere 8 can be explained if *Sab* is assumed to delete repressor-binding sites in *iab-5* that are distinct from those deleted by *Mcp*. Such sites would have to be distal to those deleted in *Mcp*, since *Sab* has been mapped genetically just distal to *Mcp*. The resultant lowered affinity of the *iab-5* region for repressor(s) might then allow that region to *cis*-regulate ABD-B protein expression in neuromere 8 and 9 of the embryo and presumably in A3 and A4 of the adult. The observed lack of expression in neuromere 9 versus that in 8, and the stronger phenotypic expression in A3 than in A4, is therefore, not readily explained. One possibility is that the wild-type *iab-4* region normally suppresses male-type pigmentation in A4; in that case, since *iab-4* is not operative in A3 but is operative in A4, it would reduce male-type pigmentation in A4 but not A3.

In *Mcp Sab* double mutants, the resultant deletion of both sets of repressor elements in the *iab-5* region would then allow ABD-B protein expression in neuromere 8 and 9 of the embryo and presumably in A3 and A4 of the adult, since these are both strongly pigmented.

Regulation of ABD-B expression by trans-regulatory locus, Polycomb

In animals homozygous for Pc^3, *Abd-B* transcripts encoding ABD-BI and ABD-BII are known to be expressed throughout the embryo (Kuziora and McGinnis, 1988). We have previously described expression of ABD-B in embryos homozygous for Pc^3 and a duplication for the BX-C, Dp(3;3)P5 (Celniker *et al.*, 1989). Such double homozygotes tend to show a much more extreme *Pc* phenotype than those homozygous only for the Pc^3 mutant (Duncan and Lewis, 1982).

In Pc^3 $iab\text{-}8^{D14}$ double mutant homozygotes, we see strong ectopic expression of ABD-B throughout all of the neuromeres of the CNS. Since Pc^3 $iab\text{-}8^{D14}$ embryos should lack ABD-BI we infer that it is ABD-BII that is being ectopically expressed in all of the neuromeres of the CNS. This expression is seen at germ band extension and is slightly earlier than the expression of ABD-BII transcripts (Kuziora and McGinnis, 1988).

Conclusions

(i) In several respects *cis*-regulation of ABD-B proteins appears to be unique when compared with that of other homeobox proteins of the BX-C and ANT-C domains. The *Abd-B* transcription units have multiple promoters and produce at least two proteins, ABD-BI and ABD-BII, that differ at their amino termini. *Cis*-regulatory regions extend over 50 kb 3′ to the *Abd-B* transcription units as well as an undetermined number of kb 5′. Such regions are colinear in their order in the chromosome with their order of control of ABD-B expression along the body axis of the organism. (ii) There is suggestive evidence based on the phenotypes of homozygotes for breakpoints in the *iab-6* and *iab-7* regions that the *iab-5* region can *cis*-regulate expression of *abd-A*, as well as that of *Abd-B*. To the extent that this sharing occurs also in wild-type and confers a selective advantage, it would constitute a powerful force keeping gene complexes intact during evolution.

Materials and methods

Mutant descriptions

The hemizygous adult phenotypes of the *infra-abdominal* mutants $iab\text{-}5^{301}$, $iab\text{-}5^{843}$, $iab\text{-}7^{MX2}$, $iab\text{-}8^{D14}$, $iab\text{-}8^{D15}$, $iab\text{-}8^{D6}$, $iab\text{-}9^{1065}$ and the heterozygous adult phenotypes of the dominant mutants *Mcp* and *McpSab* have been previously described in Karch *et al.* (1985). $iab\text{-}5^{994}$ was obtained from the 31616 screen described below and is associated with breaks in 22, 89B-E, and 90A. The remaining *iab* mutants were obtained from the 38000 screen described below with the exception of $iab\text{-}9^{48}$, an inversion In(3R)89E;100C induced upon In(3R)35250 with breaks in 81 and 92. $iab\text{-}5^{224}$ Tp(3R)83-85;89E, $iab\text{-}6^{11}$ In(3LR)72;89E, $iab\text{-}6^{200}$

T(2;3)29EF or 30A;89E, *iab-6*^{1821} T(2;3)30F or 31A;89E, *iab-7*^{770} complex with breaks in 68, 91, and 94, *iab-7*^{164} In(3LR)65;89E, *iab-7*^{3081} complex with breaks in 66, 79, and 89, *iab-9*^{1392} In(3LR)63A;89E, *iab-9*^{1645} T(2;3)26A;89E.

Induction and recovery of new infra-abdominal mutants

Genetic screens, based on the suppression of transvection, have yielded chromosomal rearrangements that are initially unselected for their effects on the BX-C. The first screen, numbered 31616, has been previously described (Lewis, 1985). The second screen, numbered 38000, uses a third chromosome inversion, In(3R)1000, with breaks in 81F and 90C so that the BX-C is only 10 bands from the centromere. The close proximity of the BX-C to the centromere results in an order of magnitude greater frequency of breaks within the BX-C.

Nucleic acid preparation and DNA sequence analysis

Seven new chromosomal rearrangement breakpoints have been localized by genomic Southern analysis: *iab-5*^{843} (W.Bender, personal communication), *iab-6*^{11} (W.Bender, personal communication), *iab-6*^{200} (W.Bender, personal communication, *iab-6*^{1821} (M.Charles, this laboratory), *iab-7*^{164} (L.Crosby, personal communication), *iab-9*^{1392} (G.Hong, this laboratory) and *iab-9*^{1645} (G.Hong, this laboratory). Purification of fly DNA followed the procedure of Bender *et al.* (1983). Digoxigenin-labeled DNA probes were synthesized and the hybridization reactions for whole-genome Southern blots were carried out according to the Boehringer Mannheim Nonradioactive DNA Labeling and Detection Kit. Double-strand sequencing of cDNAs, p11-15 and p11-24, and *Abd-B* genomic subclones was done using the dideoxy chain termination method of Sanger *et al.* (1977) using a modified T7 DNA polymerase (Sequenase, US Biochemicals) following the manufacturer's reaction conditions.

Preparation of adults for cuticular analysis

Adult flies were preserved, cleared and mounted as described by Duncan (1982).

Immunolocalization methods

Embryos were prepared and stained with the primary mouse monoclonal antibody 1A2E9 as previously described (Celniker *et al.*, 1989). The stocks and the fraction of embryos showing the mutant phenotypes illustrated in Figure 2 were *iab-5*^{224}*/SbDpP5*, 7/31; *iab-6*^{11}*/SbDpP5*, 9/35; *iab-7*^{164}*/TM3p*^{p}*SbSer*, 7/30; *iab-8*^{D14}*/TM1*, 10/37; *iab-9*^{65}*/SbDpP5*, 6/34. Homozygous *Ubx*^{1} and *Ubx*^{109}, embryos were identified by the absence of anti-*Ubx* antibody labeling and the genotype confirmed in Stage 17 embryos by the absence of specific midgut constrictions (Tremml and Bienz, 1989). *abd-A*^{MX1} embryos were identified in Stage 17 embryos by the absence of the second and third midgut constrictions.

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