Segmental determination in *Drosophila* central nervous system: analysis of the *abdominal-A* region of the bithorax complex

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ABSTRACT The bithorax complex (BX-C) comprises several genes required for the diversification of posterior segments in *Drosophila*. The BX-C genes control segment differences not only in the epidermis but in other tissues as well, especially in the central nervous system. We have examined the control of one segment-specific neural structure: the lateral dots, a paired structure present in the first abdominal segment of the larval CNS and absent in all following abdominal segments. Our results show that the suppression of lateral dots in segments A3 and A4 requires the presence of two active copies of one of the BX-C genes, *abdominal-A (abd-A)*. We also show that the adjacent BX-C regions, *iab-3* and *iab-4*, can act *in trans* on *abd-A* not only when the two copies of BX-C are paired but also, at least to some extent, when pairing is disturbed.

KEY WORDS: *Drosophila, bithorax complex, trans-vection, central nervous system*

Introduction

Mutations that transform one part of the body into another, and in particular one member of a meristic series into another member of the series, are called «homoeotic» (Bateson, 1894). Homeotic mutations have been described and studied in insects for more than a century, but it took the pioneering work of E.B. Lewis (1963, 1978) to convert what had long seemed an entomological oddity into the means to explore the genetic foundations of development. As a result of this work, the determination of segment identity in the fly was the first developmental operation to be understood in terms of a genetic programme (reviewed in Akam, 1987), and one that turned out to be of general significance (reviewed in Holland, 1988).

In *Drosophila*, the homeotic genes that control the identity of the posterior body segments are clustered in a complex locus, the *bithorax* gene complex (BX-C). The region affected by individual mutations often extends over the posterior part of one segment and the anterior part of the following segment (Lewis, 1951). The limits of these regions coincide with the antero-posterior compartment boundaries (Hayes *et al.*, 1984; Struhl, 1984), indicating that the domains of action of homeotic genes correspond to parasegments rather than to segments. Thus the BX-C genes control the identity of parasegments 5 to 14 (PS5-14).

Flies that lack the BX-C die as late embryos. The epidermis of such embryos reveals that all the parasegments controlled by BX-C become identical to PS4 (Lewis, 1978). Extensive mutational analyses have revealed that loss-of-function mutations in the BX-C fall into two classes. The first class defines three lethal complementation groups, each of which affects several consecutive segments (Sánchez-Herrero *et al.*, 1985; Tiong *et al.*, 1985). The three genes thus defined are *Ultrabithorax (Ubx)*, *abdominal-A (abd-A)* and *Abdominal-B (Abd-B)*. Their domains of action are respectively PS5-6, PS7-9, and PS10-14.

The second class of mutations are homozygous viable and affect mainly one parasegment (Lewis, 1978). For instance *iab-2*, *iab-3* and *iab-4* affect respectively PS7, 8 and 9 (Karch *et al.*, 1985). Viable mutations affecting different parasegments complement each other, but they are not complemented by the corresponding lethal mutations. For example *iab-2/iab-3* heterozygotes are nearly wild type, but *iab-2/abd-A* and *iab-3/abd-A* flies show the mutant phenotype of *iab-2/iab-2* and *iab-3/iab-3* homozygotes. This pattern of complementation suggests that *iab-2* and *iab-3* mutations might alter *cis*-acting control regions that regulate the expression of *abd-A* in PS 7 and 8 respectively (Sánchez-Herrero *et al.*, 1985). Consistent with this view is the observation that the phenotype of embryos homozygous for a *Ubx abd-A Abd-B* triple mutation is identical to that of embryos homozygous for the entire BX-C deletion (Casanova *et al.*, 1987).

Abbreviations used in this paper: *DfA⁻3-4/+, Dp(2;3)P10/+; Df(3R)Ubx*^{109}*/+*; LD, lateral dots; CNS, central nervous system; BX-C, *bithorax* gene complex; *Ubx, Ultrabithorax*; *abd-A, abdominal-A*; *Abd-B, Abdominal-B*; *iab, infra-abdominal*.

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The BX-C has been cloned, and many of its mutations have been localized (Bender *et al.*, 1983; Karch *et al.*, 1985). Three protein coding regions have been discovered, corresponding to the three lethal complementation groups *Ubx*, *abd-A* and *Abd-B*. Each coding region contains a homeobox (McGinnis *et al.*, 1984; Regulski *et al.*, 1985), which is translated into a DNA binding peptide, the homeodomain (Laughon and Scott, 1984; Gehring, 1987). The mutations that are homozygous viable define a set of consecutive regions in the BX-C, each of which is required for the appropriate determination of an individual parasegment. The organization of the *iab-2/abd-A/iab-3/iab-4* region is shown Fig. 1. RNA products are transcribed from the *iab* regions (Cumberledge *et al.*, 1990; Sakonju, personal communication), but they do not seem to code for proteins, and their functions are still obscure.

1986). Larvae where one copy of *abd-A* is inactivated, however, have LD in A2 but not in the following segments, suggesting that other BX-C functions play a role in the suppression of LD in A3 and A4. Here we further investigate this question, and the nature of the relation between *abd-A*, *iab-3* and *iab-4*.

Results

LD suppression in segments A2-A5

The pattern revealed by the monoclonal antibody 16F12 in the CNS of a wild type third instar larva is shown Fig. 2A (Ghysen *et al.*, 1985). This antibody reveals the presence in A1, but not in more posterior segments, of a paired structure called «lateral dots» (LD). When only one copy of the BX-C is present (Fig. 2B) LD appear in all

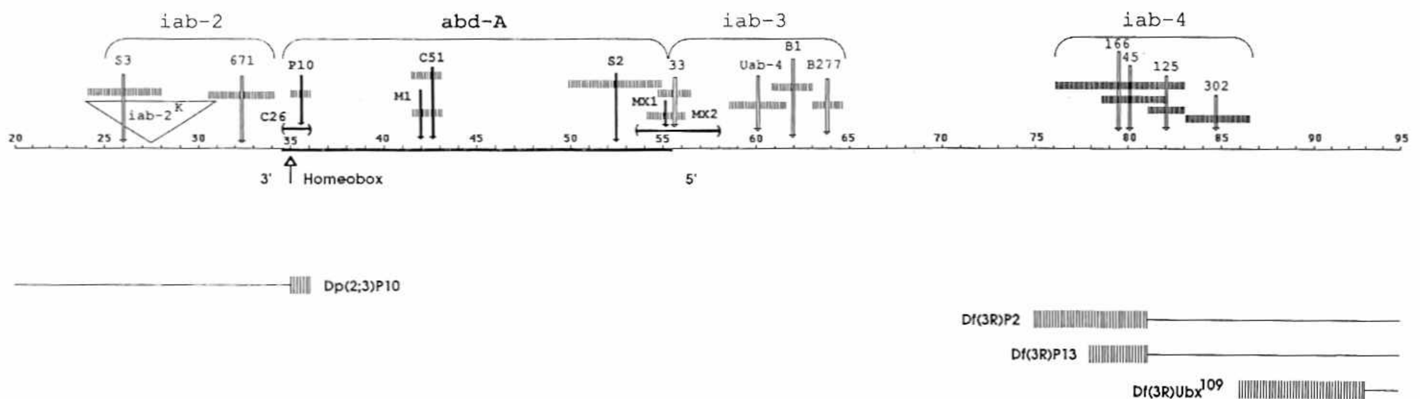

Fig. 1. Synoptic view of the *abdominal-A* region. *The horizontal line represents the genomic DNA of the* abdominal-A *region, marked in kilobases:* iab-2, abd-A, iab-3 *and* iab-4. *The* abd-A *transcription unit is shown by a thicker line, the 5' and 3' ends and the homeobox are also indicated. The mutations localized in that region are indicated above the DNA line. Triangles, vertical arrows, and horizontal lines between brackets stand respectively for insertions, chromosomal break-points and deletions. Homozygous lethal mutations are indicated in bold face and black lines. They are all localized in the* abd-A *transcription unit and affect to various extents the* abd-A *proteins. Homozygous viable mutations are indicated in normal type and open lines. They all map outside the* abd-A *transcription unit and define the three DNA regions* iab-2, iab-3 *and* iab-4. *According to phenotypic observations these regions affect respectively PS 7, 8 and 9. Below the DNA line, the duplication and the deficiencies used in this work are also drawn. The lines indicate the extent of DNA still present in these mutants. For all the mutations, hatched horizontal lines indicate the limits of mapping uncertainty.*

These genetic and molecular data are consistent with a functional model of BX-C where the complex would comprise three genes, each of which is controlled by a very large *cis* regulatory region comprising several parasegment-specific subregions. The regulatory regions would control the pattern of expression of the protein in each segment, thus defining segmental identity (Peifer *et al.*, 1987).

We have re-examined the relationship between *abd-A* and its associated *iab* regions in the case of the larval CNS. The CNS is a very sensitive system to assess BX-C function, since in several cases segmental transformations are observed when one copy of the BX-C is deleted (Teugels and Ghysen, 1985; Ghysen and Lewis, 1986). An example is the LD, a pair of dot-like structures visualized in the CNS of the third instar larvae by a monoclonal antibody, 16F12, isolated by Y.N. Jan and L.Y. Jan (Ghysen *et al.*, 1985). This structure is normally present in A1 but not in more posterior segmental ganglia. Larvae where one of the two copies of BX-C is deleted have LD in all abdominal ganglia up to A7, showing that the suppression of LD requires two doses of BX-C (Ghysen and Lewis, 1986). Larvae where one copy of *abd-A* is inactivated, however, have LD in A2 but not in the following segments, suggesting that other BX-C functions play a role in the suppression of LD in A3 and A4.

abdominal segments, indicating that the suppression of LD in segments A2 to A7 requires two doses of the BX-C. A deletion of the *abd-A* transcription unit and of the adjacent *iab-3* and *iab-4* regions, hereafter called *DfA·3·4·* (see Materials and Methods for a full description of the mutations used) leads to the formation of LD in segments A2 to A5 (Fig. 2C). If *iab-2*, *iab-3* and *iab-4* were *cis*-acting control regions regulating the expression of *abd-A* for the determination of PS 7-9, one would expect mutations in *abd-A* to have an effect similar to that of the deficiency of *abd-A* and the surrounding *iab* regions. The analysis of larvae where one copy of *abd-A* is mutated by a rearrangement which breaks the *abd-A* homeobox, *abd-A*^{P10}, shows however that additional LD appear only in A2 (Fig. 2D). This result was interpreted as an indication that other functions besides *abd-A* are able to contribute to LD suppression in A3-A5 (Ghysen and Lewis, 1986).

An alternative interpretation is that, even though *abd-A*^{P10} breaks the *abd-A* homeobox, this mutant retains some *abd-A* function such that the residual activity is sufficient to completely suppress the LD

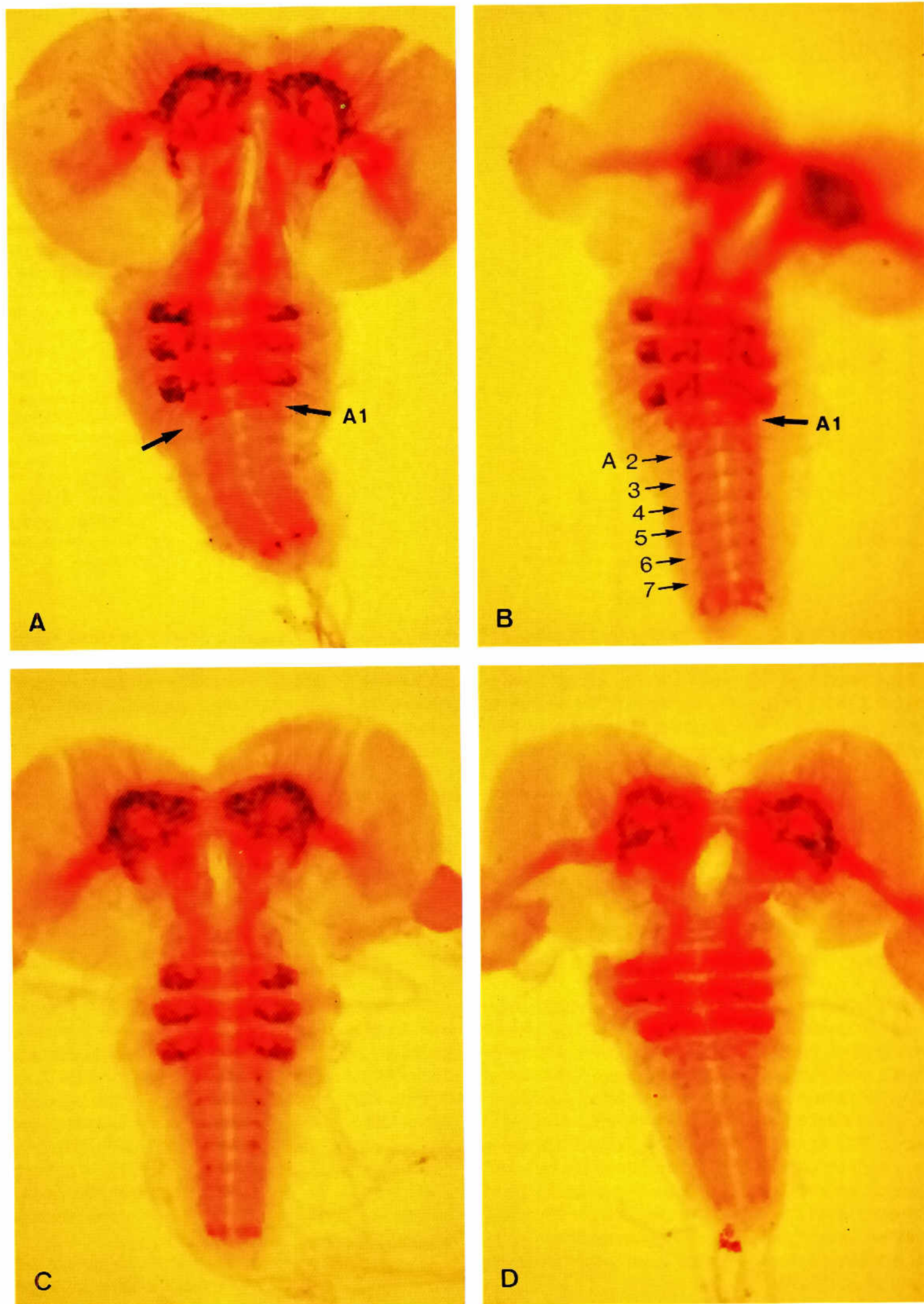

Fig. 2. Presence of additional LD in BX-C mutants. (A) *The larval CNS in the wild-type, showing the normal LD in A1 (arrows).* **(B)** *In* Df(3R)P115/+, *where one copy of the BX-C is deleted, additional LD are formed in segments A2 to A7 (thin arrows).* **(C)** *In* $DfA^-3^-4^-$/+, *additional LD appear up to A6.* **(D)** *In* abd-A^{P10}/+, *where one copy of* abd-A *is mutated, additional LD appear only in A2.*

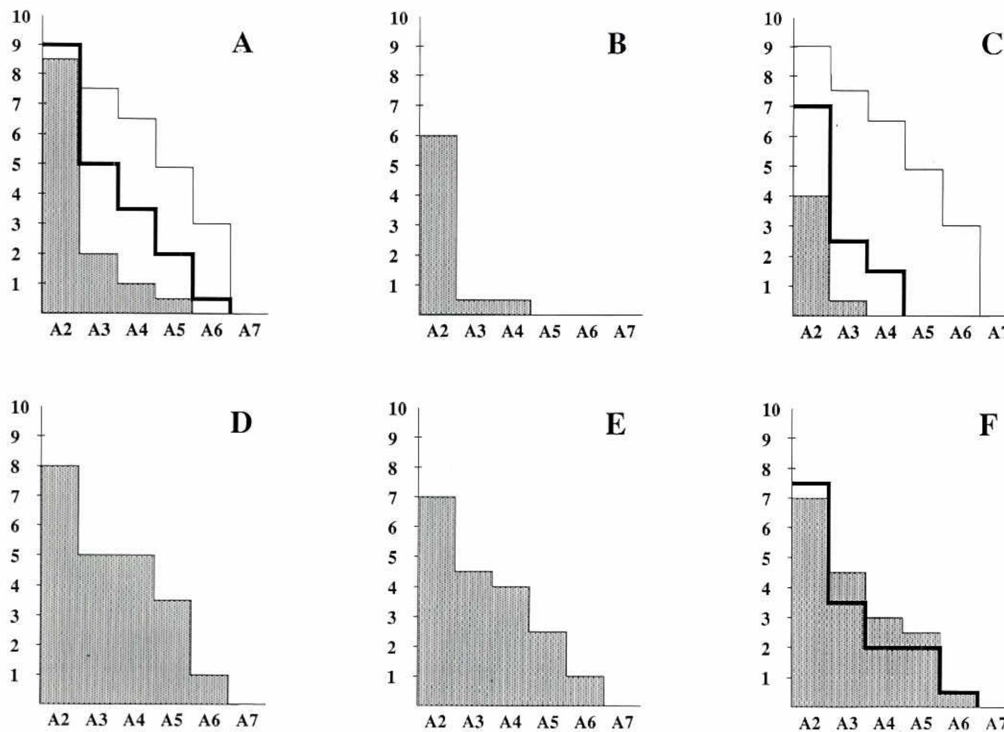

Fig. 3. The two classes of *abd-A* mutants and the test of trans-vection. *Each phenotype is expressed as a histogram of the size of LD in segments A2 to A7 (see scoring, in Materials and Methods). The* abd-A *alleles tested are shown by shaded histograms.* **(A)** abd-A^{M1}/+; **(B)** abd-A^{MX2}/+; **(C)** abd-A^{P10}/+; **(D)** abd-A^{D24}/+; **(E)** abd-A^{MX1}/+, *and* **(F)** abd-A^{C26}/+. *In panels A, C and F, the thick lines represent the phenotypes of* abd-A *mutants where homologous BX-C pairing has been prevented by the translocation of the intact BX-C copy to the X chromosome. Genotypes are:* (A) Dp (1;3) P115/+; abd-A^{M1}/Df (3R) P115, (C) Dp (1;3) P115/+; abd-A^{P10}/Df(3R) PI115, (F) Dp (1;3) P115/+; abd-A^{C26}/Df (3R)P115. *In panels A and C, the thin line represents the phenotype of* DfA˙3˙4˙/+.

in A3-A5, but not in A2. Consistent with the idea that the *abd-A*^{P10} breakpoint might not completely inactivate the gene, we observe that even in A2 the phenotype of *abd-A*^{P10}*/+* (Fig. 3C, shaded) is weaker than that of *DfA˙3˙4˙/+* (Fig 3C, thin line). In the epidermis also, the phenotype of *abd-A*^{P10} is less severe than that of *DfA˙3˙4˙* homozygous embryos (Morata *et al.*, 1983), or other *abd-A* mutants like *abd-A*^{M1}, *abd-A*^{C51}, *abd-A*^{D24} (Busturia *et al.*, 1989).

abd-A *phenotypes and trans-vection*

In order to assess the role *of abd-A* in LD suppression, we tested five other *abd-A* alleles and examined whether heterozygous larvae showed a CNS phenotype similar to that of *abd-A*^{P10}*/+* or similar to that of *DfA˙3˙4˙/+*. The results (Fig. 3, shaded) indicate that some *abd-A* alleles result in the formation of LD mostly or only in A2 (Fig.3 A-C), while others result in the formation of LD in several segments (Fig.3 D-F).

These two phenotypes can be explained in (at least) three different ways. One interpretation is that the differences in phenotype might reflect differences in the level of inactivation of *abd-A* by the different alleles, such that relatively weak alleles would show an effect only in A2 while more extreme alleles would affect A3, A4 and A5 as well. This explanation appears unlikely in the case of *abd-A*^{M1} for several reasons: first, its effect in A2 is stronger than that of any other allele, and nearly indistinguishable from that of the deficient combination; second, no *abd-A* product can be immunodetected in embryos homozygous for this mutation (Macias *et al.*, 1990); and third, the epidermal phenotype of homozygous embryos is as extreme as that of embryos deleted for the entire *abd-A iab-3 iab-4* region (Busturia *et al.*, 1989).

The second explanation is that functions other than *abd-A* are capable of suppressing the LD in A3-A5. In this view the alleles *abd-A*^{M1}, *abd-A*^{MX2}, and *abd-A*^{P10} would specifically affect *abd-A*, while the alleles *abd-A*^{D24}, *abd-A*^{MX1} and *abd-A*^{C26} would inactivate to various extents the other functions as well as *abd-A*, resulting in the formation of additional LD in segments posterior to A2. This second explanation is difficult to reconcile with the molecular analysis of the middle region of the BX-C which suggests that this region comprises only one coding gene, *abd-A*.

The third interpretation is that the phenotype of the A2-specific *abd-A* alleles reflects an interaction between the mutant and the normal copies of the BX-C. The analysis of heterozygous phenotypes in the BX-C has demonstrated that *trans*-heterozygotes for certain pairs of mutations have a less severe phenotype when the two complexes are adjacent than when pairing of homologous chromosomes is prevented by some chromosome rearrangement (Lewis, 1954). This phenomenon, called trans-vection, suggests that short-range interactions can take place between the two paired copies of BX-C such that the control regions of one particular gene can have an effect *in trans* on the homologous gene of the adjacent complex.

We assessed the possible involvement of short-range interactions by examining the phenotypes of several *abd-A* alleles in larvae where the pairing of the two BX-C is prevented by a translocation to the X chromosome of the intact copy of the BX-C, *T(1;3)P115* (Fig. 3, thick lines). For the *abd-A* mutant of the second group that has been tested, there is no significant increase in phenotype when the normal copy of the BX-C is translocated (Fig. 3F). On the other hand, preventing pairing increases the phenotypes of the two tested *abd-A* mutants of the first group, such that the phenotype of these combinations now resembles those of the *abd-A* mutants of the second group (Fig. 3A and C).

This result demonstrates that if interactions between the two copies of the BX-C are prevented, the different *abd-A* mutants have qualitatively similar effects in LD suppression. Therefore we conclude that (1) two copies of *abd-A* are required for the complete suppression of LD in segments A3 and A4 as well as A2, and (2) in some but not all *abd-A* alleles, other elements of the BX-C, presum-

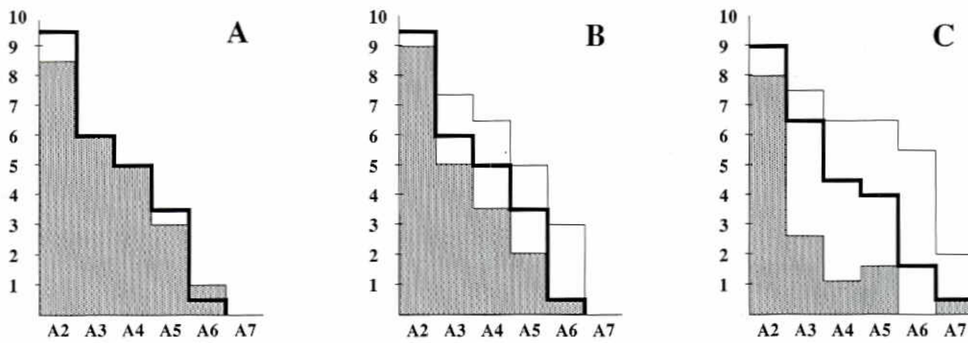

Fig. 4. *Trans* activation of *abd-A* by *iab-3* and *iab-4*. Panel A, *shaded,* Dp(2;3) P10/+; Df(3R)P13/+, *thick line:* Dp (2;3) P10/+; Df(3R) P2/+. **Panel B,** *shaded,* Dp(1;3) P115/+; abd-A^{M1} /Df(3R) P115, *thick line:* Dp(2;3)P10/+; Df(3R)P2/+, *thin line:* $DfA^-3^-4^-$/+. **Panel C,** *shaded,* abd-A^{M1} Abd-B^{M8}/+, *thick line:* Dp(1;3)P115/+; abd-A^{M1} Abd-B^{M8}/Df(3R)P115, *thin line:* Df 3R)P115/+.

ably the *iab-3* and *iab-4* regions upstream of the mutated *abd-A* gene, may act *in trans* on the *abd-A*$^+$ gene of the adjacent complex to increase its rate of transcription or processing in A3 and A4. This would result in a higher level of LD suppression in the corresponding segments.

Can iab-3 *and* iab-4 *trans-activate* abd-A*?*

A comparison of the different *abd-A*$^-$/+ phenotypes (Fig. 3, shaded) to that of *DfA*$^-$*3*$^-$*4*$^-$ (Fig. 3A,C, thin line) reveals that when homologous pairing is prevented, the phenotype of the deficiency is always significantly more severe in segments A3-A5 than that of even the strongest *abd-A* mutant. In order to determine whether this difference is due to some peculiarity of *DfA*$^-$*3*$^-$*4*$^-$, we examined two smaller deficiencies, *Dp(2;3)P10/+; Df(3R)P13/+* and *Dp(2;3)P10/+; Df(3R)P2/+*, each of which deletes the *abd-A* coding region and the *iab-3* control region, but not the *iab-4* region. We observed that both deficiencies give very similar results in heterozygous larvae (Fig. 4A): the phenotype is more extreme than that of the strongest *abd-A* allele (Fig. 4B, shaded), but weaker than that of the larger *DfA*$^-$*3*$^-$*4*$^-$ (Fig. 4B, thin line). This suggests that the reason why *Dp(2;3)P10/+; Df(3R)P13/+* and *Dp(2;3)P10/+; Df(3R)P2/+* are more extreme than any *abd-A* allele is that they remove *iab-3* in addition to *abd-A*, while *DfA*$^-$*3*$^-$*4*$^-$ is more extreme than *Dp(2;3)P10/+; Df(3R)P13/+* and *Dp(2;3)P10/+; Df(3R)P2/+* because it removes *iab-4* as well. It follows that *iab-3* and *iab-4* have an effect on LD suppression even if there is no functional *abd-A* gene *in cis*.

We also examined whether the effect of the *iab-3* and *iab-4* regions could occur through the *cis* activation of the next coding gene, *Abd-B*, by assaying the phenotype of larvae heterozygous for a double mutant *abd-A Abd-B*. When homozygous, this combination results in the development of embryos that are indistinguishable from embryos deleted for the entire BX-C complex in segments A2-A8. The CNS of heterozygous larvae, however, presents a phenotype (Fig. 4C, shaded) that is much milder than that observed with the deletion of the entire locus (Fig. 4C, thin line), even when the wild type copy of BX-C is translocated to the X chromosome (Fig. 4C, thick line). This suggests that *iab-3* and *iab-4* can act *in trans* on *abd-A*, even when the pairing of the two copies of BX-C is disturbed.

Discussion

The current view of the organization of the bithorax complex is that it comprises three coding genes, each of which would be required for the correct determination of several consecutive segments, and an array of *cis*-acting regulatory sequences that would control the pattern of expression of the complex in the different segments. In particular, the determination of the abdominal segments A2 to A4 would depend on the expression of the gene *abd-A* under the control of three regions, *iab-2, iab-3* and *iab-4* corresponding respectively to parasegments PS7, PS8 and PS9. It was reported, however, that in the larval CNS an alteration of one of the three genes, *abd-A*, shows an effect that is restricted to segment A2, as if other functions could substitute for *abd-A* in the determination of segments A3 and A4 (Ghysen and Lewis, 1986). This work was based on the analysis of the lateral dots (LD), a structure present in the CNS of segment A1 but not in segments A2-A7.

There are several differences between the segmental control of LD and previously studied phenotypes that might account for this apparent discrepancy. One major difference between the LD system and other phenotypes is that the appearance of additional LD is a haploinsufficiency phenotype which is assessed in heterozygous individuals, while all previous studies were done in homozygous or hemizygous embryos or cell clones.

Another important difference is that phenotypic analyses of *abd-A* mutants have so far been mostly (Karch *et al.*, 1985; Busturia *et al.*, 1989) or entirely (Sánchez-Herrero *et al.*, 1985; Tiong *et al.*, 1985) confined to the analysis of epidermal phenotypes. BX-C genes control segment differences in the CNS as well as in epidermis (Green, 1981; Jiménez and Campos-Ortega, 1981; Teugels and Ghysen, 1983; Thomas and Wyman, 1984) but there is tissue-specificity both in the pattern of expression (Akam, 1983), and in mutant phenotypes, suggesting that different products might act in the epidermis and in CNS (Ghysen *et al.*, 1983; Weinzierl *et al.*, 1987, Mann and Hogness, 1990).

The present work was undertaken to elucidate the relative role of *abd-A, iab-3* and *iab-4* in LD suppression. We show that, provided pairing between the two copies of BX-C is disturbed, all *abd-A* mutations affect A3 and A4 as well as A2. This suggests that two active copies of *abd-A* are required in all these segments, and therefore that no other BX-C function can substitute for *abd-A* in these segments. This conclusion is in complete agreement with the 3-gene model. Our results also show that, as already amply documented in other BX-C mutant combinations, the phenotype is sensitive to trans-vection, that is, the mutant phenotype of the heterozygote is enhanced if pairing between the two copies of the BX-C is disrupted. Trans-vection is usually considered as indicating that a regulatory region on one chromosome can somehow activate its target gene present on the other chromosome provided the two homologs are adjacent.

While it is clear that the phenotype of several *abd-A* mutations can be enhanced by disrupting the pairing between the two copies of BX-C, this phenotype never reaches that of a deficiency that

removes simultaneously *abd-A* and its control regions *iab-3* and *iab-4*. This is true even for the strongest *abd-A* mutation available, *abd-A*^{M1}, which is nearly as extreme as the deficiency *DfA*3̄4̄ in segment A2. The difference between mutants and deficiency in segments A3-A5 could be due to several factors: (1) some low level of pairing might subsist between the two copies of the BX-C, in spite of the fact that one copy has been translocated to another chromosome; (2) some residual activity might subsist even in the strongest *abd-A* alleles, (3) products of the *iab-3*$^{+}$ and *iab-4*$^{+}$ region could move over to the homologous *abd-A*$^{+}$ gene even when pairing is disrupted, implying that these products can diffuse and act *in trans* on a distant *abd-A*$^{+}$ target, albeit less efficiently than if the two copies of BX-C were adjacent.

At the moment there is no simple way to distinguish between these or other possibilities. We consider, however, that the first two possibilities are somewhat unlikely for the following reasons. Breakpoints anywhere around the BX-C appear sufficient to disturb pairing as measured by assaying the trans-vection effect, and therefore it seems unlikely that a copy translocated to the X chromosome would still be able to pair, even at a low level, with the copy remaining on the third chromosome. On the other hand it must be mentioned that the *abd-A*^{P10} and *abd-A*^{M1} mutations, being themselves breakpoints, would be expected to prevent pairing, and yet the phenotype of both mutations is enhanced when the homologous copy is translocated. The second possibility would require that even *abd-A*^{M1}, a breakpoint at the middle of the *abd-A* transcription which completely removes all *abd-A* antigenic material and presents in the epidermis a phenotype as extreme as that of the deficiency *DfA*3̄4̄, nevertheless retains some level of activity in the CNS. This seems not very likely. However, the idea that a broken homeobox could still support some activity appeared unlikely in the case of *abd-A*^{P10}, and yet this clearly happens, suggesting that the possibility of residual activity in the *abd-A* alleles can not be ruled out.

If the third possibility is correct, it suggests that the *iab* control regions, or part of them, act through transcripts that can diffuse over some distance. Quite understandably the shorter the distance the better, and therefore one would imagine that the activating effect is strongest on the *cis*-adjacent copy of *abd-A*, somewhat less effective on the trans-adjacent copy, even less effective if the pairing is disturbed by a breakpoint in the BX-C, and the least effective if the homologous copy is completely translocated to another chromosome. Even then, however, there may still be some *trans*-activation, which would explain the difference between this combination (*abdA*^{M1}*/T(1;3)P115*, Fig. 4B, shaded) and the entire removal of *iab-3* and *iab-4*, together with *abd-A* (in *DfA*3̄4̄, Fig. 4B, thin line).

Materials and Methods

Fly strains

Flies were reared on standard cornmeal-yeast-agar medium at 25°. Canton S was used as wild-type. The BX-C locus is completely deleted by *Df(3R)P115*. The localization of all other mutations used is described in Karch *et al.* (1985), or in Casanova *et al.* (1987) for *abd-A*^{M1} and the double mutant *abd-A*^{M1}*Abd-B*^{M8}. The epidermic phenotype of these mutations and some of their combinations are mainly described in Lewis (1978), Sánchez-Herrero *et al.* (1985), Karch *et al.* (1985), Casanova *et al.* (1987), and Busturia *et al.* (1989).

In this paper, we have followed Duncan's proposal in naming the mutations (Duncan, 1987). Thus, we have distinguished between mutations affecting the whole *abd-A* domain (named *abd-A*), and those affecting a single parasegment (named *iab*).

Fly crosses

Mutant chromosomes were balanced with *TM6B* which carries the mutation *Tb (Tubby)*. Mutant combinations were easily distinguished, since they lacked the tubby body-shape phenotype displayed by *TM6B* heterozygous larvae.

The genotypes *Dp(2;3)P10/+; Df/+*, where *Df* is *Df(3R)P2, Df(3R)P13*, or *DfA*3̄4̄, were obtained by crossing *y/y; Dp(2;3)P10 Dp(1;2)y*$^{+}$*/+; +/+* with *y/Y; +/+; Df/+* (obtained by crossing *y/y; +/+* with *+/Y; Df/TM6B*). The non-yellow larvae were dissected and half of them were genotypically *Dp(2;3)P10 Dp(1;2)y*$^{+}$*/+; Df/+*. The second half carried a wild-type third chromosome instead of the *Df* chromosome and were normal with respect to their LD phenotype.

Immunoperoxidase staining of larval CNS:

Late third instar larvae are dissected in phosphate buffer (13 mM NaH_2PO_4, 87 mM Na_2HPO_4, pH 7.6), their CNS are fixed for 30 min in Carnoy fixative (ethanol: chloroform: acetic acid, 6:3:1) and rinsed in phosphate buffer. The following steps are performed in phosphate buffer containing 0.3% sodium deoxycholate and 0.3% triton (PDT buffer). The ganglia are incubated for 3 h with normal horse serum (1:25) and the mouse monoclonal antibody 16F12 (1:25), isolated and kindly provided by Y.N. Jan and L.Y. Jan; and then rinsed for I h. The presence of the antibody is revealed by the biotin-avidin-peroxidase method using the Vectastain ABC kit of Vector Laboratories (PK-4002). The ganglia are incubated for 3 h with the second antibody (biotinylated antimouse IgG antiserum, 7:1000), rinsed for I h, incubated for 3 h with avidin and biotinylated peroxidase (14:14:1000), rinsed for I h with Tris buffer (0.12 M, pH 7.6), incubated for 30 min with a freshly prepared di-amino-benzidine (DAB) solution (2 mg/ 5 ml in Tris buffer); then, 10 to 20 µl of a 1% H_2O_2 solution are added. The reaction is performed for 5 to 10 min until a brownish coloration appears. The ganglia are dehydrated in successive alcohol baths (75%, 90% and 100% twice), rinsed in xylene and mounted in D.P.X. mountant (BDH Chemicals). Twelve to 24 larvae were examined for each genotype.

Scoring

The size of LD in each of the hemisegments was assigned a value ranging from 0 (no LD) to 10 (fully developed LD). Since the intensity of the staining varies between experiments, we used the normal LD in A1 as an internal reference. The reproducibility of the scoring was assessed by rescoring one set of ganglia after a three month interval. The difference between the two sets of results was inferior to 0.5 in each segment.

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