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P element homing to the *Drosophila* bithorax complex

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SUMMARY

P elements containing a 7 kb DNA fragment from the middle of the *Drosophila* bithorax complex insert preferentially into the bithorax complex or into the adjacent chromosome regions. This 'homing' property is similar to that reported for the *engrailed* promoter (Hama, C., Ali, Z. and Kornberg, T. B. (1990) *Genes Dev.* 4, 1079-1093). The 7 kb fragment does not contain any known promoter, but it acts as a boundary element separating adjacent segmental domains. An enhancer-trap P element was constructed with the homing fragment and the selectable marker flanked by FRT sites. P insertions can be trimmed down by Flp-mediated recombination to just the *lacZ* reporter, so that the β-galactosidase pattern is not influenced by sequences inside the P element. Twenty insertions into the bithorax complex express β-galactosidase in segmentally limited patterns, reflecting the segmental domains of the bithorax complex where the elements reside. The mapping of segmental domains has now been revised, with enlargement of the *abx/bx*, *bxd/pbx*, and the *iab-3* domains. The FRT sites in the P elements permit recombination between pairs of elements on opposite chromosomes, to generate duplications or deletions of the DNA between the two insertion sites. Using this technique, the length of the *Ultrabithorax* transcription unit was varied from 37 to 138 kb, but there was surprisingly little effect on *Ultrabithorax* function.

Key words: Homing, bithorax, *Drosophila*, *Gene mapping*

INTRODUCTION

The most striking feature of the *Drosophila* homeotic complexes is that mutations are aligned on the chromosome in the order of the segments that they affect (Lewis, 1978, 1996; Kaufman et al., 1980). This rule of colinearity has been observed through evolution since the divergence of insects and mammals, but the molecular mechanism that so constrains the order is still unknown. The regulatory domains of the bithorax complex (BX-C) that control successive segments have been located on the molecular map by defining the mutant lesions associated with different transformation phenotypes (Duncan, 1987). These assignments may be imprecise for several reasons. In some cases, segmental transformations are difficult to recognize, because adjacent segments are nearly identical (like the third and fourth abdominal segments). Other segments are difficult to see in the larva or adult (like the 9th and 10th abdominal segments). Mutations used for mapping often affect more than one segmental domain. This is particularly true for rearrangement breaks, which constitute the largest class of alleles. Such breaks often separate more than one segmental domain from the relevant homeotic promoter, and so multiple parasegments are transformed. If a rearrangement interrupts a transcription unit, then all regulatory regions controlling that transcript lose their function. Rearrangements also introduce foreign sequences next to the sequences of the BX-C, and so position effects must be considered. The second largest class of alleles are insertions of mobile elements, whose interactions with nearby enhancers and promoters are difficult to predict. Small deletions, which are the most useful alleles in the study of regulatory regions, are rare.

Enhancer traps offer an independent way to map domains in the BX-C, since their expression is limited by the segmental domain in which they reside (McCall et al., 1994, and this report). Insertions in the *bxd* domain, for example, which regulates Ultrabithorax (Ubx) expression in parasegments 6 through 12, show *lacZ* expression restricted to PS6-12. The difficulty has been in finding enough examples of enhancer traps in the BX-C to construct a complete map. The homing property of the P element described here removes that obstacle.

Insertions of P element transgenes are generally assumed to be random throughout the euchromatic portions of the genome, but a few examples of non-random targeting have been described. The first example involved P elements carrying the promoter region of the *engrailed* locus; 35% of transformants were found to be in 47F or 48A, the cytological location of *engrailed* (Hama et al., 1990). Some of these P insertions mapped to the *engrailed* promoter, but others were spread over region of more than 100 kb around the *engrailed* gene. Hama et al. (1990) called this phenomenon 'homing'. Taillebourg and Dura (1999) have recently reported a more precise example of homing; P elements carrying the *linotte* promoter inserted into the endogenous *linotte* promoter in approx. 20% of cases. The targeted insertions were tightly clustered, all within a 600 bp region, and most within a 36 bp segment just upstream of the *linotte* RNA start site.

There have been sporadic cases of P elements carrying pieces from the Antennapedia complex (ANT-C) or BX-C inserted back into the homeotic complexes (Engström et al., 1992; Castelli-Gair et al., 1992; Galloni et al., 1993). However, most of the promoters of the BX-C and ANT-C have been studied in P element constructs, and none have shown homing in the fashion of the *engrailed* or *linotte* promoter regions. We describe here a DNA fragment from the bithorax complex that effects homing to the chromosomal region of the BX-C with a frequency of approx. 30%. The fragment is far from any known promoter on the BX-C, but it appears to contain a boundary element separating segmental domains. We have used the targeted insertions to refine the mapping of the segmental regulatory domains. Our insertions can also be used, pairwise, to create duplication or deletion mutations.

MATERIALS AND METHODS

Construction of the 'homing pigeon'

The 'homing pigeon' was derived from the PZ enhancer trap (Mlodzik and Hiromi, 1992), kindly supplied by Y. Hiromi. First, we cloned both an FRT site (as a *Bam*HI/*Eco*RI fragment from the plasmid pJFs36; Senecoff et al., 1985) and the homing fragment (a 6.7 kb *Eco*RI/*Sal*I fragment from the BX-C) together into the Bluescript II-KS vector. A *Not*I/*Sal*I fragment from this plasmid was used to replace the *Not*I/*Sal*I segment of PZ containing the kanamycin resistance gene and bacterial origin. Next, the *Hin*dIII site near the 3′ end of the P element was replaced with a linker containing *Xho*I and *Sac*II sites. Finally, a second FRT site, cloned into Bluescript, was recovered as an *Xho*I/ *Sac*II fragment and inserted into the 3′ linker site. The final P element retains the *lacZ* gene driven from the P element promoter, as well as the *rosy* transformation marker.

P element insertion and removal

Most transformants were recovered by injection of *cn ry* embryos with plasmids containing the homing pigeon and the pπ25.7wc transposase source (Spradling, 1986). Initial transformants were jumped to new chromosome locations either by crossing to the X chromosome transposase source (*Dp(3:1) Δ2-3*, from Ed Grell; a translocation of 97F-100, including the 99B Δ2-3 (Robertson et al., 1988), onto the tip of the X) or by injection of embryos with pπ25.7wc. P elements were removed by crossing to the 99B Δ2-3 transposase source, recombined onto a chromosome deficient for most of the BX-C (*Df(3R)Ubx*^{109}), in order to preclude DNA repair from the homolog (Engels et al., 1990).

FLP recombination

Recombination between FRT sites was induced by crossing in an X chromosome heat shock/Flp source (FLP 122; Kopp et al., 1997). Males were heat shocked for 1 hour at 37°C while they were first instar larvae, and their sons were scored for recombination.

Inverse PCR

Sequences flanking P element insertions were recovered by inverse PCR (Ochman et al., 1988), using divergent primer pairs from either the 5′ or the 3′ end of the P element. The PCR products were sequenced manually using the Thermosequenase Radiolabeled Terminator Cycle Sequencing Kit (USB).

lacZ Immunohistochemistry

Embryos were fixed, stained, and dissected as described by Karch et al. (1990), using a mouse monoclonal antibody to β-galactosidase (Promega), followed by an HRP-conjugated goat anti-mouse secondary antibody (Biorad), both at a 1/1000 dilution.

RESULTS

In prior studies, we tested DNA fragments from much of the bithorax complex, looking for segment-specific enhancers (Simon et al., 1990) or sites of Polycomb regulation (Chiang et al., 1995). Of the more than 200 P element insertions generated, we noticed that a few had inserted into the bithorax complex. These were usually recognized because the expression patterns of their *lacZ* reporter genes were strictly limited to particular parasegments, and some insertions also showed recessive segmental transformation phenotypes (McCall et al., 1994). A total of eight such insertions were found; five of these carried a particular fragment within the P element. This was a 7 kb *Sal*I fragment from the region between the *bxd* (PS6) and the *iab-2* (PS7) regulatory regions, spanning +23.5 to +30.5 on the traditional map coordinates (Bender et al., 1983) or bases 186,335 to 178,824 on the DNA sequence map of the bithorax complex (SEQ89E, Martin et al., 1995). We call this the 'homing fragment'; its map position is illustrated in Fig. 1, along with the positions of the 5 fortuitous P element insertions that carried this fragment.

P element construct

It seemed likely that the 7 kb homing fragment was causing preferential insertion of the P elements into the neighborhood of the bithorax complex, by analogy with the homing properties of the *engrailed* promoter fragment (Hama et al., 1990). We took advantage of the homing property with a modified enhancer trap P element called the 'homing pigeon'. Its map is illustrated in Fig. 2. The element is derived from the PZ element of Mlodzik and Hiromi (1992); it uses the P promoter to drive the bacterial *lacZ* gene. The element carries a slightly shortened copy of the homing fragment (*Eco*RI at 185,492 to *Sal*I at 178,824), as well as a 7.2 kb *Hin*dIII fragment carrying the *rosy* gene. *rosy* was used as the selectable marker because it is non-autonomous; the cell-autonomous *white* gene cannot be detected when inserted into the BX-C, because it is repressed in segments of the head (McCall et al., 1994). At most insertion sites, the homing fragment drives *lacZ* expression in parasegments 6-12, and it is possible that the *rosy* regulatory region might influence the P promoter as well. Therefore, we flanked both the homing fragment and the *rosy* gene with FRT sites, so that these fragments could be deleted by recombination (Golic and Lindquist, 1989) after the P element had inserted into the chromosome. Fig. 2 shows how an insertion of this construct near the *Abdominal-B* (*Abd-B*) promoter (insertion HCJ199, see below) initially gave a complicated pattern, due to enhancers both internal and external to the P element. Deletion of all but the *lacZ* gene by recombination greatly simplified the pattern, which then resembled the expression pattern of *Abd-B*.

Homing frequencies

The frequency of homing is dramatically affected by the source of the donor P element. We first injected the homing pigeon construct into *rosy*$^{-}$ embryos and recovered *rosy*$^{+}$ transformants in the progeny of the injectees (Spradling, 1986). Each *rosy*$^{+}$ insertion was mapped by meiotic recombination relative to a dominant mutation (*Fab*) in the BX-C. Embryos from each line were also fixed and stained with antibody to β-galactosidase. Three quarters of the insertions were on the third

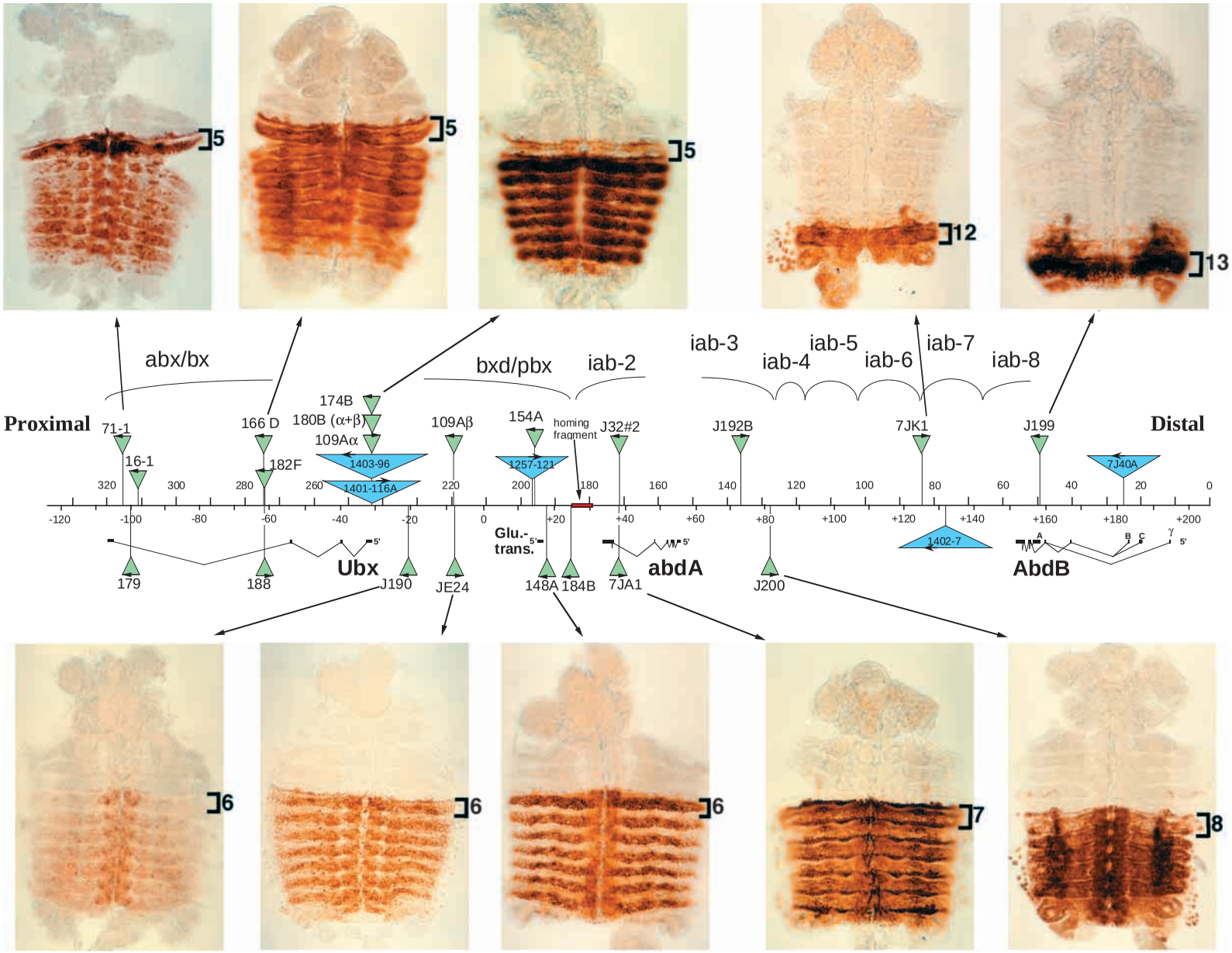

Fig. 1. Homing pigeon insertions in the BX-C, with representative *lacZ* patterns. The central horizontal line represents approx. 330 kb of DNA covering the BX-C, marked in kb according to the DNA sequence (above the line), or according to the traditional coordinates (below the line). Transcription units are marked for the three homeotic products, *Ubx*, *abd-A* and *Abd-B*, and the position of the predicted glucose transporter homolog is also shown. The small red segment in the middle of the DNA line shows the position of the homing fragment. Blue triangles indicate five P insertions containing the homing fragment, which were discovered in prior studies. Green triangles indicate the positions of homing pigeon insertions generated in this study; the flipped-out derivatives of each insertion are drawn to scale. The isolate numbers are indicated, with the HC prefix omitted. The arrow on the horizontal side of each triangle shows the direction of transcription of the *lacZ* reporter. Above and below the map are shown ten pelts of stage 14 embryos. The numbered bracket on each pelt indicates the most anterior parasegment with prominent β-galactosidase expression.

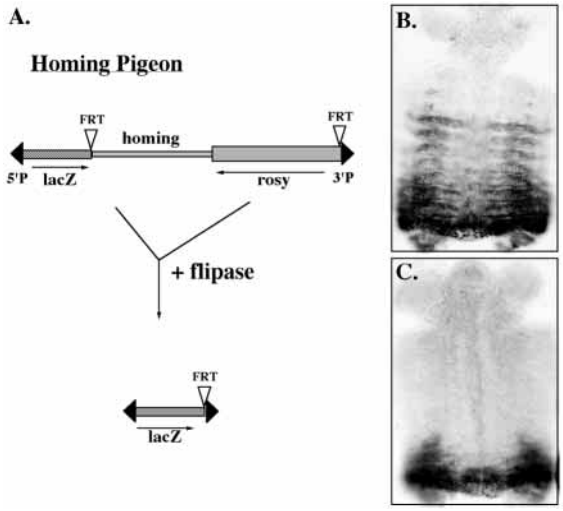

Fig. 2. The homing pigeon P element. (A) Diagram of the full length P element, with the 'flipped-out' version given below. The small triangles indicate the FRT sites used to recombine out the bulk of the P element. The segment marked 'homing' is the 7 kb fragment from the middle of the BX-C, at the *bxd-iab2* border. (B) A pelt of a stage 14 embryo with the full length HCJ199 insertion, which lies just downstream of the RNA start site for the major *Abd-B* RNA. Note the β-galactosidase staining beginning in parasegment 6, which is due to enhancers on the homing fragment within the P element. (C) A comparable pelt from an embryo with the flipped-out version of HCJ199, with staining only in parasegments 10-14, driven by the regulatory sequences of *Abd-B* outside the P element.

chromosome, and 32% mapped within approx. 1 cM of the *Fab* marker (Table 1). Half of these (18% overall) gave *lacZ* patterns with a strict anterior segmental limit maintained throughout embryogenesis. Insertions in the latter group were subsequently shown to lie within the BX-C (see below).

We hoped to recover many additional P element insertions into the BX-C by mobilizing existing P elements. It is most convenient to mobilize an insertion on the X or second chromosome if the third chromosome is the target, because the preferred source of transposase is also on the third chromosome (at 99B; Robertson et al., 1988). We mobilized four different insertions on the second chromosome to generate 44 new insertion sites, but, surprisingly, none of these were within the BX-C.

The second chromosome insertions used for the above experiment were themselves generated by mobilization, made at a time when all of our homing pigeon insertions were on the third chromosome. The donor homing pigeon (HC7) was inserted close to the BX-C in distal 89E (subsequently localized within the *Daughters against dpp* (*Dad*) gene, approx. 83 kb distal to the most distal *Abd-B* promoter); the other third chromosome was a balancer (MKRS), and the source of transposase was on the X chromosome. We recovered the desired second chromosome insertions, but we also found two insertions into the BX-C on the MKRS balancer. The MKRS balancer has only one transposition, and the 89E region of the BX-C is nearly always paired in the salivary chromosomes of MKRS/+ heterozygotes. It seemed likely that hopping between paired third chromosomes might be a more efficient way to recover BX-C insertions, and so we initiated

Table 1. Homing frequencies

P insertion method	No. of lines	Map near BX-C	Into BX-C
1. Direct injection	68	22	12
		32%	18%
2. Jumps from Chrom. II	44	1	0
		2%	0%
3. Jumps from BX-C on homolog	16	7	6
		44%	38%

jumps back from MKRS. Among 16 new jumps off the MKRS chromosome, 6 fell within the BX-C (Table 1). The new insertions in the BX-C were somewhat clustered within the complex, with each group close to the map position of the homing pigeon on the donor chromosome (Fig. 3).

Patterns and phenotypes

To map insertions within the BX-C, we used inverse PCR reactions to recover DNA adjacent to the insertion sites. The sequences of these PCR fragments were aligned with the published sequence of the 89E region (Martin et al., 1995). Since the inverse PCR reaction uses primers specific for one end of the P element, a sequence alignment reveals the orientation, as well as the position, of each insertion (listed in Table 2). Of the 20 lines analyzed by inverse PCR, two (HC109 and HC180) gave a pair of bands, and in each case the sequences matched two distinct sites within the BX-C. It is possible that additional lines contain multiple insertions, but the inverse PCR reaction failed to give a DNA product for each insert.

All of the homing pigeon insertions in the BX-C were treated with flipase (Flp), as shown in Fig. 2, to remove almost everything but the *lacZ* reporter. Fig. 1 illustrates the positions of the insertions in the complex, and shows representative examples of *lacZ* expression patterns for the trimmed-down P elements. Each *lacZ* pattern shows an anterior limit of expression consistent with its location in a segmental regulatory region of the BX-C. Table 2 lists the phenotypes of each insertion; additional comments on some of the lines are given below, in chromosomal order.

bx inserts

The three insertions near the proximal limit of the Ubx transcription unit (HC71-1, HC179, and HC16-1) all show very similar *lacZ* patterns, like that reported for the $bx^{Plac(-61)}$ enhancer trap in the *bx* region (McCall et al., 1994). Only the HC16-1 homozygotes had a clear *bx* phenotype, although it was weaker than previously tested *bx* alleles (Peifer and Bender, 1986). The HC16-1 insertion is complex; it has a full copy of the homing pigeon P element plus an adjacent partial copy.

Three other insertions (HC166D, HC182F, and HC188) fell near positions of known *bx* alleles; they map in a tight cluster within 400 base pairs (Table 1). The cluster is only 110 base pairs from the site of the $bx^{Plac(-61)}$ enhancer trap (at 274,127), mentioned above. HC182F makes no β-galactosidase; it is probably mutated in its *lacZ* copy. The other two show *lacZ* patterns like that of the $Ubx^{Plac(-61)}$ insertion described previously (McCall et al., 1994).

McCall et al. (1994) suggested that *Ubx* transcription across enhancer traps might interfere with *lacZ* transcription, if both are transcribed from the same strand. This model might account for the reduced *lacZ* levels in PS6-11 seen in the three more proximal HC insertions. However, the model is

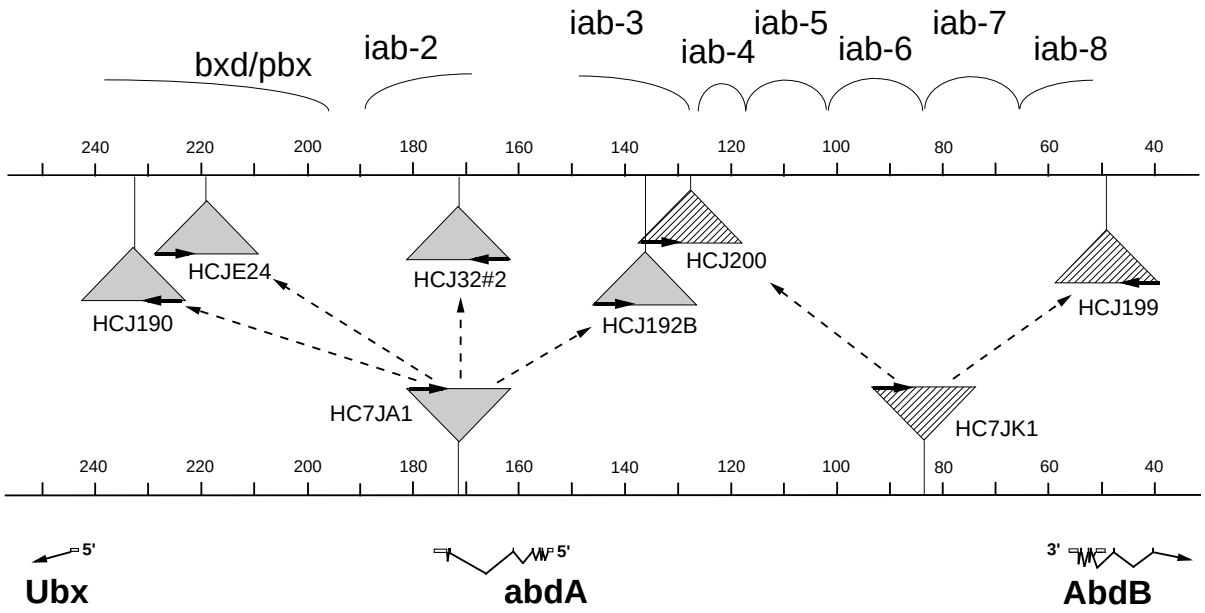

Fig. 3. Jumping the homing pigeon from the homolog. The bottom line represents the BX-C map on the MKRS balancer chromosome, showing two insertions of the homing pigeon. Each was used separately as a donor element to generate new jumps; those that landed in the BX-C on the homolog are shown on the upper line.

Table 2. Insertions

Insert no.	Position*	Orientation‡	lacZ§	ry$^+$ 19 kb phenotype¶	ry$^-$ 5 kb phenotype¶	Comments
bx:						
HC71-1	315,379	**3′-5′**	PS5s, 6-12m	approx. WT	approx. WT	
HC179	312,868	**3′-5′**	PS5m,6-12w	approx. WT	approx. WT	
HC16-1	310,815	**3′-5′**	PS5m,6-11w,12vw	w. *bx*	approx. WT	complex insert
HC166D	274,528	**3′-5′**	PS5-11s,12m	pharate lethal, s. *bx*	m. *bx* (/Δ)	
HC188	274.395	**5′-3′**	PS5-11s,12m	lethal	m. *bx* (/Δ)	
HC182F	274,236	**3′-5′**	none	lethal	approx. WT	no β-gal made
Ubx:						
HC109Aα	242,847	5′-3′	ND	ND	ND	not separated from HC109Aβ
HC174B	242,806	**3′-5′**	PS5m,6-12s, 13w	ph. lethal, s. *bxd*, m. *bx*	ph. lethal, s. *bxd*, m. *bx*	
HC180Bα	242,806	**3′-5′**	(α+β examined)	ph. lethal, m.*bxd*, w. *bx*	ph. lethal, m. *bx*, m. *bxd*	α and β not separated
β	242,758	5′-**3′**	PS5w,6s,7-12m			
bxd:						
HCJ190	232,727	3′-**5′**	PS5vw, 6-12w	m. *bxd* (/Δ)	approx. WT	
HC109Aβ	219,737	**3′-5′**	PS6-12s	s. *bxd* (/Δ)	wk. *bxd*	separated from HC109α
HCJE24	219,228	**5′-3′**	PS6-12s	s. *bxd*	str. *bxd*	
HC154A	195,830	**3′-5′**	PS6-12m,13w	w. *bxd*	approx. WT	
HC148A	192,677	**3′-5′**	PS6-12s,13w	w. *bxd*	v. wk. *bxd*	
HC184B	185,505	**3′-5′**	PS3-5w, 6-12s, 13w	approx. WT	wk. *bxd*	insert next to homing fragment
iab-2:						
HC7JA1	171,382	5′-**3′**	PS7-13s	lethal (abdA$^-$)	lethal	insertion onto MKRS balancer
HCJ32#2	171,185	**3′-5′**	PS7-13m	lethal	approx. WT	
iab-3:						
HCJ192B	135,875	**5′-3′**	PS7vw, 8-13s	lack gonads	WT (fertile)	
HCJ200	127,367	**5′-3′**	PS8-13s	lack gonads	WT (fertile)	
iab-7:						
HC7JK1	85,577	5′-**3′**	PS12-13s	*iab-7*	*iab-7*	insertion onto MKRS balancer
AbdB:						
HCJ199	49,335	**3′-5′**	PS11w, 12m, 13s,14m	lethal	dom. *iab-7*, recess. lethal	

*Position of base immediately proximal to P insertion (i.e. most distal base of 8 bp repeat). Sequence numbering from SEQ89E (Martin et al., 1995).
‡Proximal to distal orientation of P element. The edge of the P element actually recovered by inverse PCR is shown in bold type.
§Pattern of 5 kb element; staining in designated parasegments (PS) noted as strong (s), medium (m), weak (w), or very weak (vw).
¶Tested as homozygote (unless noted; /Δ=tested over a deficiency), WT, wild type; ph., pharate; s., strong; m., medium; w., weak; dom., dominant; recess., recessive.

apparently contradicted by the HC166D example, which is transcribed in parallel to *Ubx*, but which shows little reduction in the *lacZ* pattern in PS6-11.

Ubx inserts

The 200 base region immediately upstream of the *Ubx* RNA start site is a pronounced hot spot for P element insertions. McCall et al. (1994) reported three insertions in this promoter region, and our present collection of homing pigeon insertions includes three more events. The HC180B line contains two insertions, 56 base pairs apart, in head to head orientation (Table 2). The HC174B line, which was clearly generated in an independent event, inserted at the exact position of one of the HC180B inserts, and in the same orientation. The HC109A line also had two insertions, one in the *bxd* region (see below), and one in the *Ubx* promoter at the same position and orientation as the $Ubx^{Plac(-31)}$ insertion mapped by McCall et al. (1994). The insertions at the *Ubx* promoter are lethal, even in the flipped-out forms, but the homozygotes die as pharate adults, that can be examined for segmental transformations. The effects on PS6 function are more severe than on PS5.

bxd inserts

The initial double-insert HC109A line was crossed to a source of P element transposase to remove the insertion at the *Ubx* promoter. The resulting single insert line is called HC109Aβ. It and another insert (HCJE24) map close to each other, within the minimal Polycomb Response Element (PRE) fragment defined by Chan et al. (1994). Two more insertions (HC154A and HC148A) flank the glucose transporter-like coding region identified from the DNA sequence analysis of the BX-C (Martin et al., 1995). The flipped out forms of these four *bxd* insertions (HC109Aβ, HCJE24, HC154A, and HC148A) have very similar *lacZ* patterns (Fig. 1). The most proximal *bxd* insertion, HCJ190, has surprisingly weak *lacZ* expression in PS6-12. It also shows very weak expression in PS5, in a pattern similar to the PS5 pattern of *Ubx*. The staining pattern of the most distal *bxd* insertion, HC184B, is peculiar; it is described separately below. The phenotypes of the three most distal *bxd* inserts are very weak, with only a partial reduction in the size of the first abdominal tergite. It is surprising that they show any phenotype at all, because they lie further from *Ubx* than a series of rearrangement breakpoints which show little or no adult transformation (Bender et al., 1985; E.B. Lewis and W. B., unpublished results). Perhaps the P promoter, or some other sequence in the insertion, interferes with the normal enhancer-promoter interaction of *Ubx*.

iab-2 inserts

The HC7JA1 and HCJ32#2 insertions lie in the largest intron of the *abdominal-A* (*abd-A)* transcription unit, within 200 bp of each other, but in opposite orientations. Both show similar staining in PS7-13 (Fig. 1), but HCJ32#2 gives a weaker pattern.

iab-3 inserts

Two insertions lie upstream of the *abd-A* promoter in the regulatory regions previously designated *iab-3* (HCJ192B) and *iab-4* (HCJ200) (Karch et al., 1985). Both full size elements cause recessive sterility due to lack of gonads; neither cause transformation of the third abdominal sternite to the character of the second abdominal sternite. By these phenotypic criteria, both would be deemed *iab-4* alleles (Karch et al., 1985). However, the flipped-out versions of both elements give β-galactosidase expression beginning in PS8 (Fig. 1), a pattern expected of the *iab-3* regulatory region. We believe the *lacZ* pattern is a more reliable indication of the regulatory region, and call both insertions *iab-3* mutants (see Discussion). The PS8 anterior limit of *lacZ* expression is not absolute in either line. There is expression in a few cells in the posterior compartment of the first abdominal segment (PS7), and occasional cells stain weakly in PS6.

iab-7 and *iab-8* inserts

HC7JK1 lies in the *iab-7* region, just distal to the *Fab7* boundary region between *iab-6* and *iab-7* (Gyurkovics et al., 1990), and about 200 base pairs distal to the site of the 'bluetail' P element insertion (Karch et al., 1994). Both the full length and the flipped-out elements show a moderate *iab-7* phenotype. The full length (19 kb) element, but not the flipped-out form, also shows a posterior transformation when hemizygous; spots of black pigment appear on the tergites of the 2nd, 3rd, and 4th abdominal segments of the male. The HCJ199 insertion lies 145 base pairs downstream of the start site of the *Abd-B* class A RNA (Zavortink and Sakonju, 1989). Both large and flipped-out forms are homozygous lethal, but fertile as heterozygotes, and so they are not completely null for *Abd-B* function. The embryonic cuticles resemble those of $iab\text{-}7^{D14}$ (Karch et al., 1985), which is a small deletion removing the class A promoter (Zavortink and Sakonju, 1989). Presumably, the B,C, and γ forms of *Abd-B* RNA are still produced. The *lacZ* pattern (Figs 1 and 2) mimics that of the *Abd-B* 'm' or 'form I' protein encoded by the class A RNA (Boulet et al., 1991).

Insertions in the distal region of the BX-C are less common than those in the proximal half (Fig. 1). It is possible that some distal insertions were missed, either because enhancers in the homing fragment drove deleterious misexpression of *Abd-B*, or because the *rosy* transformation marker was repressed to a level below our detection threshold.

Near misses

From our various attempts to insert the homing pigeon into the complex, we accumulated 13 insertions that map within approx. 1 cM of the BX-C, but they fail to show *lacZ* expression with a tight segmental restriction. One of these, HC184B, maps within the BX-C; it is discussed below. The remaining 12, which we call near misses, were mapped by in situ hybridization (4 lines) and/or by sequencing inverse PCR products (11 lines). All 11 sequences matched partially completed BAC sequences posted by the Berkeley *Drosophila* Genome Project. Fig. 4A shows the chromosomal locations of these insertions.

Insertions of the full length homing pigeon into the BX-C show a weak *lacZ* pattern in PS6-12, in addition to the stronger pattern dictated by its position in the BX-C (Fig. 2). The PS6-12 staining, due to enhancers within the homing fragment, is well maintained throughout embryogenesis. Insertions outside the BX-C usually show a weak PS6-12 pattern, sometimes with a tissue-specific 'enhancer trap' patterns superimposed, but the anterior PS6 boundary is usually not maintained beyond approx. 9 hours. However, a few lines show a PS6-12 partial restriction of *lacZ* expression at late embryonic stages. The majority of these insertions mapped close to the BX-C. Fig. 4B shows late embryos from HC164A and HC111A, insertions on

either side of the BX-C. HC164A maps approx. 34 kb proximal to the *Ubx* homeobox, 38 bp upstream of the RNA initiation site of the *Fasciclin 1* (*Fas1*) gene (McAllister et al., 1992). On the distal side, a correlation of our chromosome walk with a sequenced contig (AC013925) places HC7, HC111A and HCJ97A at positions 69, 77, and 131 kb distal to the distal end of SEQ89E, respectively. HC111A maps within the *Dad* transcription unit (Tsuneizumi et al., 1997); inverse PCR gives two products spaced 90 bp apart. The improved temporal maintenance of the PS6-12 pattern of HC164A and HC111A may reflect long distance interaction between the homing construct and *Polycomb* response elements in the BX-C. In any case, the partially restricted PS6-12 pattern is useful for finding the near-miss insertions.

Flp duplications and deletions

The FRT sites on the homing pigeon were designed for flipping out of sequences internal to the P element, but they also permit recombination between different insertions. Golic and Golic (1996) demonstrated that site-specific recombination can occur between FRT sites on different chromosomes. We have used this feature to make deletions and duplications within the BX-C between homing pigeons that share the same orientation. Recombination can occur at either of the two FRT sites on each homing pigeon; Fig. 5 diagrams the possible recombination products. The P element remaining at the site of the crossover can be either 33 kb, 19 kb, or 5 kb. The 5 kb form lacks the *rosy* marker gene; the larger sizes can be distinguished by Southern blots. In experiments with several different pairs of insertions, the recombinants represented approx. 20% of the progeny of the males treated with the Flp recombinase.

We used this recombination method to make duplications and deletions within the Ubx transcription unit. The HC71-1 and HC166D insertions are both in the largest *Ubx* intron, but nearly 41 kb apart. Their positions allowed us to delete sequences proximal to the *abx* region, which were unaffected by any mutant lesions yet mapped. In order to avoid potential influences of a P element, we exposed the initial deletion derivative to transposase for one generation, and recovered two clean deletions of the P element. PCR tests showed that less than 50 bp of the P element remained at the site of the deletion. Heterozygotes of the final 41 kb deletion show a dominant phenotype; a slight haltere enlargement with a few tiny black bristles, like that seen in *Ubx* mutant heterozygotes. Homozygotes usually die at the pharate adult stage; a rare escaper is shown in Fig. 6A. The animals show an additional notum in the third thoracic segment, as well as a strong transformation of anterior haltere to wing, of hypopleura to sternopleura, and of anterior third leg to second. We did not detect transformation of posterior haltere or loss of the first abdominal tergite, which indicates that the PS6 function of *Ubx* is not affected by the deletion. The largest deletion formerly available was the abx^{CAC4} mutation,which removes a 14 kb subset of the region between HC71-1 and HC166D; its phenotype was weaker and much more variable (Peifer and Bender, 1986).

We examined the *lacZ* pattern in embryos homozygous for the 41 kb deletion (retaining the 5 kb P element). β-galactosidase expression was almost completely ablated, with only faint stripes of expression in every other segment, beginning in posterior T2 (anterior PS5) (not shown). The same faint pattern was seen with rearrangement breakpoints proximal to the $bx^{Plac(-61)}$ enhancer trap. These results imply that the major PS5 enhancer(s) lies proximal to HC166D and $bx^{Plac(-61)}$ (Fig. 1), but that a weak pair-rule enhancer maps distal to the HC166D insertion site.

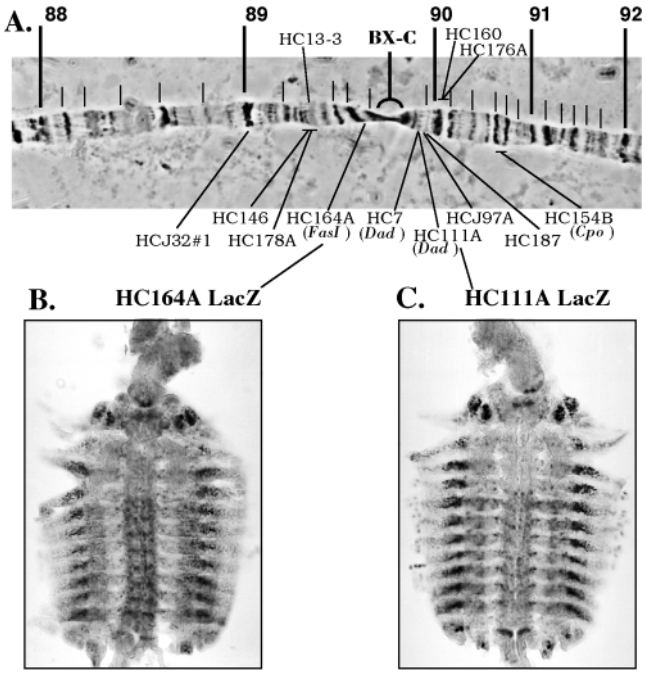

Fig. 4. 'Near miss' insertion sites near the BX-C. (A) A section of the right arm of the third chromosome centered on the 89E constriction, the site of the BX-C. The positions of twelve homing pigeon insertions are shown. The HC164 insertion lies in the promoter of the *Fasciclin 1* gene, the HC7 and HC111A insertions interrupt the transcription unit of *Daughters against dpp*, and HC154B is in the *Couch potato* promoter. (B) The embryonic β-galactosidase staining (*lacZ*) pattern of the HC164 line, the closest insertion to the BX-C on the proximal side. (C) The *lacZ* pattern of HC111A, a nearby insertion on the distal side. Note the partial restriction of β-gal staining to parasegments 6-12, most apparent in the central nervous system. Eight of the twelve near-miss insertions showed some such PS6 restriction; HCJ32#1, HC7, HCJ97A, and HC167A did not.

Elongated *Ubx* transcript

The Flp-induced recombination experiment also generated duplications, which should dramatically elongate the *Ubx* transcription unit. We examined most carefully the duplication with an intact (19 kb) copy of the P element that gives a *Ubx* transcript length of 138 kb. Homozygotes of this duplication look wild type. However, rare individuals (approx. 2%) show a reduction of the notum and malformation of the wings. When the duplication is heterozygous with a Ubx^{-} chromosome (Ubx^{1}, $Ubx^{9.22}$, *Df bxd*^{100}, or *Df P2*), the notum reduction is much more penetrant (approx. 70%) and more severe (Fig. 6B). These animals also show an enlargement of the haltere, with anterior bristles, characteristic of a weak *bx* transformation. Neither the haltere nor the notum phenotype was seen in the ry^{-} duplication derivative, with only a 5 kb P element present at the duplication site (124 kb *Ubx* transcript). The notal transformation affects PS4, where *Ubx* is not normally

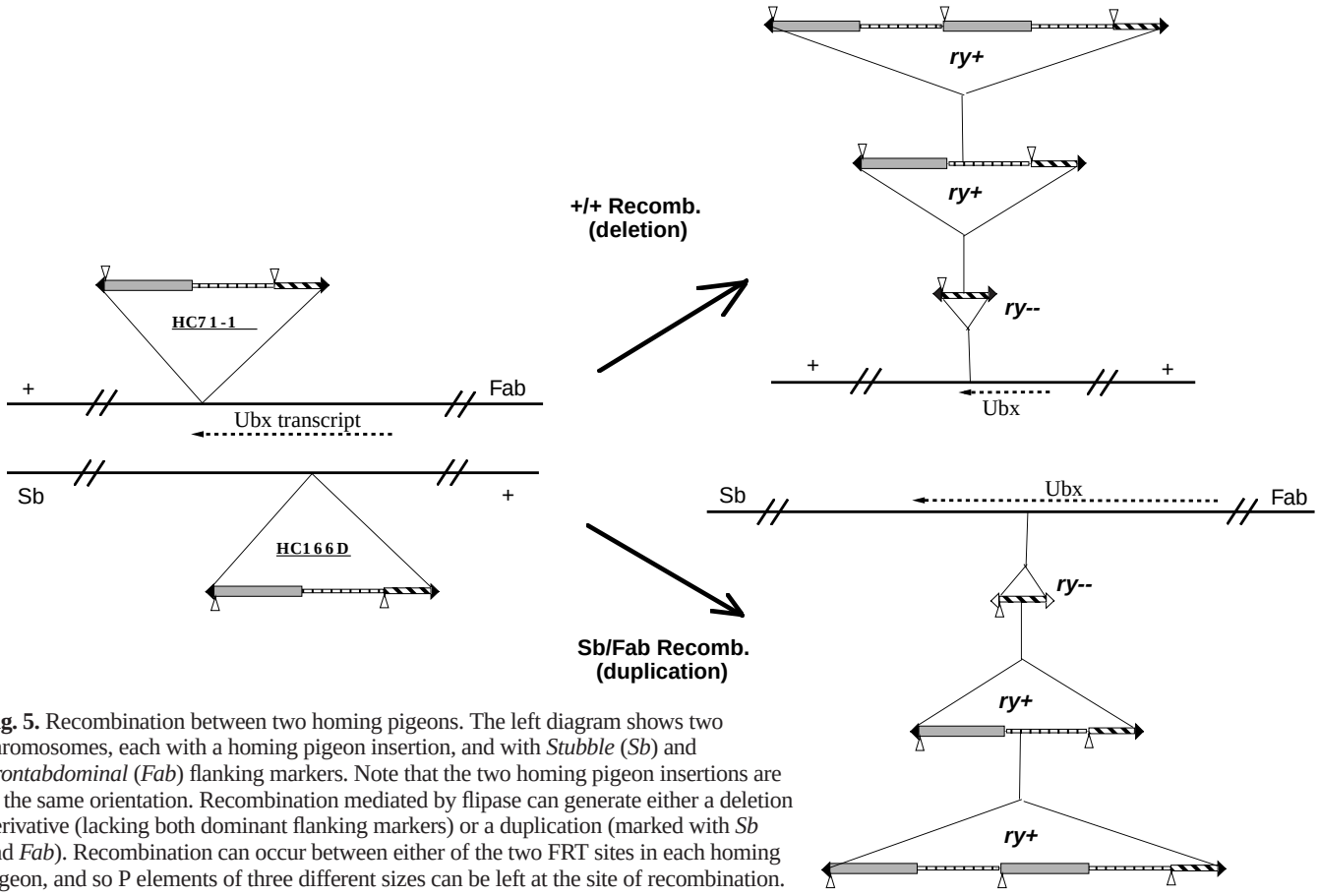

Fig. 5. Recombination between two homing pigeons. The left diagram shows two chromosomes, each with a homing pigeon insertion, and with *Stubble* (*Sb*) and *Frontabdominal* (*Fab*) flanking markers. Note that the two homing pigeon insertions are in the same orientation. Recombination mediated by flipase can generate either a deletion derivative (lacking both dominant flanking markers) or a duplication (marked with *Sb* and *Fab*). Recombination can occur between either of the two FRT sites in each homing pigeon, and so P elements of three different sizes can be left at the site of recombination.

expressed. We suspect that the P element in the middle of the duplication induces misexpression of *Ubx* in PS4. We were not able to detect misexpression of *Ubx* protein in the larval wing disc, but prolonged staining with X-Gal did reveal weak expression of β-galactosidase in the notal portion of these wing discs. It is unclear why misexpression might be more pronounced in duplication hemizygotes than in homozygotes.

Duplications and deletions were also generated between the HC71-1 and HC16-1 insertions, which are 4.5 kb apart near the proximal end of the *Ubx* transcription unit (Fig. 1). Deletion homozygotes (with only the 5 kb ry^- P element remaining) were without phenotype. Duplication chromosomes (with a 19 kb P element) over a Ubx^- chromosome show the mild *bx* phenotype, but not the notum reduction characteristic of the HC71-1/HC166D duplication lines.

HC184B and the homing fragment boundary

Nearly all homing pigeon insertions into the BX-C show segmentally limited *lacZ* patterns; the single exception is HC184B. This insertion was initially thought to lie outside the BX-C because its *lacZ* pattern showed no hint of segmental restriction (Fig. 7B), and the homozygotes looked wild type (Table 2). Its insertion site (mapped by inverse PCR) actually lies in the middle of the BX-C, just 15 bp proximal to the *Eco*RI site that marks the proximal edge of the genomic homing fragment. Thus, the *lacZ* reporter is flanked by inverted copies of the homing fragment.

We suspected that something in the homing fragment might block Polycomb-mediated repression from either side. This model could be tested by removing the homing fragments, one at a time. The HC184B element was first treated with flipase (as diagramed in Fig. 2), to remove the proximal P element homing fragment and the adjacent *rosy* gene (Fig. 7C). The resulting *lacZ* pattern is predominantly restricted to parasegments 6-12, and homozygotes showed a reduced tergite

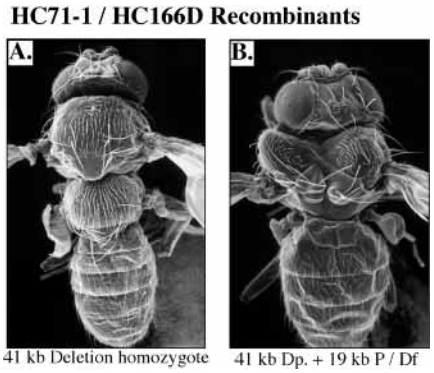

Fig. 6. Phenotypes of deletion and duplication derivatives, generated by recombination between the HC71-1 and HC166D insertions. (A) A rare adult homozygous for a deletion derivative with the P element removed. (B) A fly with the duplication derivative containing a full length (19 kb) P element at the site of recombination, heterozygous with a deficiency for the proximal half of the BX-C (*DfP2*). Both phenotypes are variable; the flies shown are among the most completely transformed.

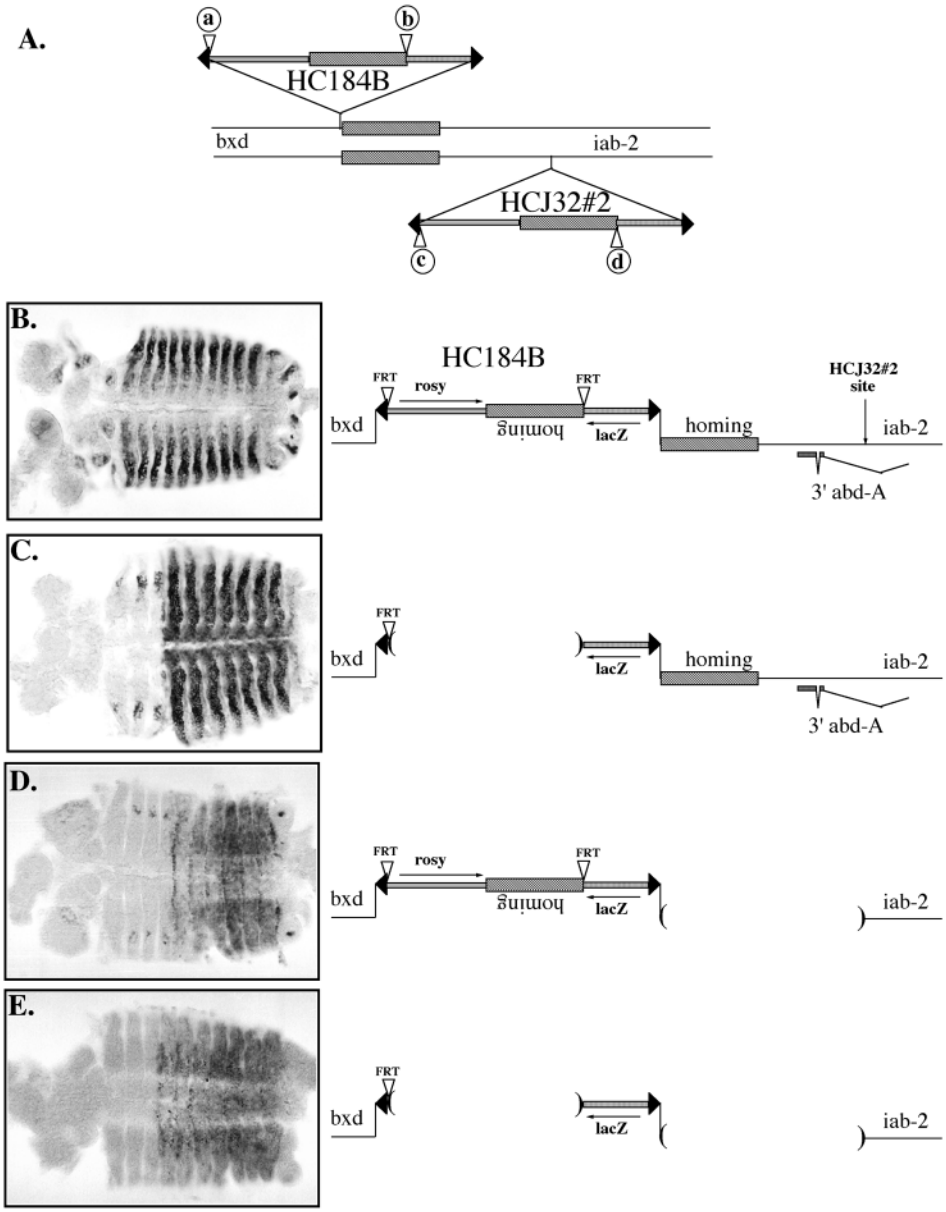

Fig. 7. The HC184B insertion and its derivatives. (A) A diagram of the HC184B and HCJ32#2 homing pigeon insertions used for Flp-mediated recombination. The four FRT sites that can be used are labelled a through d. (B) The embryonic *lacZ* pattern of the full length HC184B, showing β-gal expression in all parasegments. The map shows the full length HC184B insertion in its BX-C genomic site. The P element (shown on the raised part of the diagram) is inserted immediately next to the genomic copy of the homing fragment. Thus, the *lacZ* reporter is flanked by inverted copies of the homing fragment. (C) The flipped-out derivative of HC184B (recombination between FRT sites a and b), with the homing fragment only on the distal side of *lacZ*. β-gal expression is restricted to PS6-12, although there is weak lateral expression in PS3-5. (D) The *rosy*$^{+}$ recombinant between HC184B and HCJ32#2 (using FRT sites a and c, or b and d), with the homing fragment only on the proximal side of *lacZ*. β-gal expression is now restricted to PS7-12, again with weak lateral expression in more anterior parasegments. (E) The *rosy*$^{-}$ recombinant between HC184B and HCJ32#2, which is deleted for both copies of the homing fragment (using FRT sites a and d). β-gal expression is in PS6-12, but it is now completely repressed in more anterior parasegments. In D and E, the copy of *lacZ* is derived from the HCJ32#2 insertion; it is drawn in vertical alignment with the HC184B copy of *lacZ* to emphasize the symmetry of the homing fragment copies.

on the first abdominal segment, a weak *bxd* phenotype. Alternatively, we induced Flp-mediated recombination between HC184B and a homing pigeon insertion 14 kb to the distal side, HCJ32#2 (as in Fig. 5). The *rosy*$^{+}$ deletion recombinant retained one complete copy of the homing pigeon, but removed the distal genomic homing fragment plus a 7 kb genomic segment beyond it (Fig. 7D). The *lacZ* pattern is predominantly restricted to PS7-12. This deletion is homozygous lethal, due to the loss of the 3′ exons of *abd-A*. Both derivatives retaining one copy of the homing fragment show some weak *lacZ* expression in thoracic segments (Fig. 7C,D), suggesting that the Polycomb-mediated repression from either side was incomplete. However, when both homing fragments are deleted (in the *rosy*$^{-}$ recombinant between HC184B and HCJ32#2), *lacZ* is completely repressed in the anterior parasegments (Fig. 7E). These observations all support the notion that the homing fragment contains a blocking or boundary element.

DISCUSSION

Mechanism of homing

Models for P element homing generally invoke a homotypic interaction between proteins bound to the homing fragment and identical proteins bound to the genomic copy of that DNA fragment. Such binding would tether the P element donor plasmid before P transposition. Most DNA binding proteins are bound to hundreds of places throughout the genome, and so homotypic interactions of such proteins would target a P element to so many places that insertions might appear random. P elements containing Polycomb response elements may be homing to many endogenous Polycomb binding sites in this manner (Fauvarque and Dura, 1993; Chiang et al., 1995). Homing to a single chromosomal location, as we report here, would require that the DNA binding proteins mediating the homing be present at only one or a few sites in the genome.

A DNA element unique to the homeotic complexes is perhaps

the boundary element separating segmental domains. Such boundaries have been best defined by the *Fab* and *Mcp* deletions (Mihaly et al., 1998). The homing fragment appears to contain a boundary element; the analysis in Fig. 7 shows that it can block Polycomb repression from either side. From its position in the BX-C, the normal function of this boundary must be to separate the *bxd* and *iab-2* domains. There is independent evidence for a transition between the *bxd* and *iab-2* regulatory regions within the homing fragment, from mapping of the split in the homeotic complex found in *Drosophila virilis* (M. McLaughlin and W. B., unpublished results).

Some properties of the putative boundary in the homing fragment are difficult to explain, especially in ignorance of a biochemical mechanism. It is not clear why, in most homing pigeon insertions, the putative boundary doesn't block the influence of PS6 pattern elements residing on the homing fragment (as in Fig. 2), but it does block PS6 maintenance in the HC184B insertion (Fig. 7B) and PS6 expression in the derivative of Fig. 7D. Perhaps boundaries work in pairs, in which case the *lacZ* pattern would be influenced by the nature and proximity of endogenous boundary elements near the site of the P insertion.

It is unclear if other BX-C boundary elements share the homing property. Several groups have worked with P elements containing the *Mcp* or *Fab* boundaries, but most used the cell-autonomous *white* gene as a transformation marker, and they would have missed insertions into the BX-C. In a few cases where *rosy* was the transformation marker (Busturia and Bienz, 1993; Busturia et al., 1997), no P element insertions into the BX-C were reported. We have found two examples of P elements containing the Mcp region inserted into the BX-C (McCall et al., 1994, and M. O'Connor and A. H., unpublished results), but we have not studied Mcp-containing elements systematically. We also note that the 'bluetail' P insertion into the BX-C (Galloni et al., 1993) was one of 23 ry^+ transformants of a P element carrying a 1.1 kb fragment from the *Fab-8* boundary, recently identified by Barges et al. (2000).

Boundary elements are likely to occur also in the *Antennapedia* complex, although none have yet been defined by small deletions. We saved third chromosome insertions of the homing pigeon mapping about 10 cM from the BX-C, to see if they fell into the ANT-C. Five were mapped by sequencing of inverse PCR products. Three of these matched sequences in 84DE, slightly distal to the ANT-C (on BACs AC008312, AC0088092, and AC008093). The clustering is intriguing, but it is not clear whether the proximity to the ANT-C is significant.

It was surprising that we failed to see significant homing when the P element was mobilized from the second chromosome by an endogenous source of transposase (jumping). The high frequency homing reported for the *engrailed* promoter sequences (near 50%; Hama et al., 1990) was seen using transformants produced by injection of bacterial plasmids into fly embryos. A subsequent study (Kassis et al., 1992) utilized a slightly smaller fragment from the *engrailed* promoter and generated insertions by jumping from sites on the same chromosome as the *engrailed* locus. In these experiments, less than 5% of the inserts landed in or near *engrailed*. Bacterial plasmids persist in the injected animal for days (Martin et al., 1986), which may allow time for the plasmid to be brought to the target locus. In contrast, the P element transposition intermediate may be too short-lived to find the target, unless it is very close by, as in the case of jumping from the BX-C on one homolog into the BX-C on the other (Fig. 3). The homing to the *linotte* promoter (Taillebourg and Dura, 1999) was based primarily on jumps from the X chromosome to the second. The 20% homing frequency in that study shows that homing is not incompatible with jumping, although the tightly localized homing to the *linotte* promoter may be qualitatively different from the homing seen with *engrailed* or the BX-C.

Hot spots

Within the BX-C, the P insertions containing the homing fragment are clearly not random. The most obvious hot spot is the *Ubx* promoter, which is marked by five independent insertions in the 200 bp upstream of the RNA start site (Fig. 1; Table 1; McCall et al., 1994). McCall et al. (1994) reported another P insertion (lacking the homing fragment) in the same small region, and P elements are seen to target the promoters of many genes (Spradling et al., 1995). Curiously, the promoters for *abd-A* and *Abd-B* are much less 'hot'.

There are three other sites of coincident insertions, in the *bx*, *bxd* and *iab-2* regions. The HC109Aβ and the HCJE24 insertions lie 509 bp apart within the major Polycomb response element of the *bxd* region (Chan et al., 1994). The HC7JA1 and HC7JA1J32#2 elements lie approx. 200 bp apart in the *iab-2* region, near a cluster of consensus binding sites for the *pleiohomeotic* protein; these sites are implicated in pairing-dependent repression (Shimell et al., 2000). In the *bx* region, three insertion sites lie within 300 bp; various reports have suggested that a PRE lies near this site (Qian et al., 1993, Chiang et al., 1995, Strutt et al., 1997), although the location and properties of this PRE have not been well defined. This same *bx* site was marked by the $bx^{Plac(-61)}$ insertion (McCall et al., 1994) which did not carry any BX-C DNA internal to the element. Thus, the preference for this site within the BX-C, as for the *Ubx* promoter, likely has nothing to do with the homing fragment. These sites may be targeted because of greater DNA accessibility in germ line nuclei. This would suggest that PREs in the BX-C have an distinctive structure prior to the specification of segments.

Segmental domains

It is important to map the segmental domains of the BX-C with precision, in order to investigate the roles of parasegment enhancers, Polycomb response elements, and boundary elements in the creation of these domains. The enhancer traps reported here redefine several segmental domains. The most proximal group (HC71-1, HC179, and HC16-1, see Fig. 1) show that the PS5 domain extends at least 20 kb more proximal than the proximal-most *bx* mutant lesion (Peifer and Bender, 1986), almost to the final exon of the *Ubx* transcription unit. Likewise, the HC184B line extends the limit of the *bxd* region, mapping 15 kb distal to the most distal *bxd* mutation so far reported (Bender et al., 1985).

The most surprising observation is that both the HCJ192B and HCJ200 insertions give *lacZ* patterns with the anterior edge at PS8, thus extending the *iab-3* domain. Two rearrangement breaks proximal to the HCJ200 site had been called *iab-4* lesions in our prior analysis ($iab\text{-}4^{66}$ and $iab\text{-}4^{45}$; Karch et al., 1985). These should now be called *iab-3* alleles. There is no contradiction implied by the redefinition, because breaks in the *iab-3* domain also lack *iab-4* function – they cut the *iab-4* domain away from the *abd-A* gene it is meant to regulate. The *iab-4* domain may

begin just distal to the HCJ200 site; another P element insertion less than 2 kb from HCJ200 shows *lacZ* expression with a PS9 anterior boundary (D. Fitzgerald and W. B., unpublished).

Ubx transcript length

Long transcription units in *Drosophila* may control the timing of appearance of their protein products (Thummel, 1992). For *Ubx*, the 78 kb transcription unit may coordinate protein production in early cell cycle times (Shermoen and O'Farrell, 1991) or may facilitate the cross regulation of the various homeotic genes (Kornfeld et al., 1989). In the latter scenario, *abd-A* (with a 22.4 kb transcription unit; Martin et al., 1995) may be better able to repress *Ubx* in segments where both are expressed, because of the delay in *Ubx* mRNA production. Likewise, *Ubx* may have a head start in repressing *Antennapedia*, whose maximum transcript length is 103 kb (Garber et al., 1983; Scott et al., 1983).

We saw surprisingly little effect of altered *Ubx* transcript lengths. Homozygotes with the shortened form of *Ubx* (37 kb) had normal abdominal segments (Fig. 6A). Heterozygotes with *abd-A* alleles (D24 and MX1; Karch et al., 1985) showed no loss of *abd-A* function relative to *abd-A*$^{-}$/+ animals; the second abdominal tergite was normal in size and pigmentation. Thus, we see no interference with *abd-A* function, although our test length for *Ubx* was still longer than *abd-A*. Likewise, homozygotes with the elongated *Ubx* transcription unit (138 kb) look normal; there was no indication of anterior transformation in PS5 or PS6, where *Ubx* expression overlaps with that of *Antennapedia*. Heterozygotes over *Ubx*$^{-}$ alleles did show a mild *bx* phenotype (Fig. 6B); this phenotype was also seen with the *ry*$^{+}$ HC71-1/HC16-1 duplication line (approx. 101 kb), but not with the *ry*$^{-}$ HC71-1/HC166D duplication line (124 kb). Thus, the mild *bx* phenotype is not a strict function of transcript length, but it may reflect a reduced level of transcription or splicing across the homing pigeon P element.

Alterations in the BX-C

The FRT sites in the homing pigeon element permit the formation of duplications or deletions between any pair of insertions, as long as the insertions are in the same orientation. The recombination products retain a P element with a *lacZ* reporter, which is useful for assaying nearby enhancers and Polycomb response elements. Of course, the P promoter might interfere with the adjacent regulatory elements, to give misleading homeotic phenotypes. In the case of a deletion recombinant, the P element can be removed by treatment with transposase. Removal of the P element from a duplication derivative might be more difficult, because P excision intermediates might be repaired by recombination between the tandem repeats.

The collection of P insertions can also be used in more conventional ways to make mutations. Small deletions can be made by imprecise excision (Salz et al., 1987), or generated in concert with P-mediated male recombination (Preston et al., 1996). Targeted gene conversion can be used to alter sequences within a few hundred bases of a P insertion site (Nassif et al., 1994), or to insert a large DNA fragments with reporter genes (Dray and Gloor, 1997).

The homing fragment might be useful for bringing other sorts of P elements into the BX-C. We hope to introduce probes for chromatin structure, such as promoters for foreign polymerases (McCall and Bender, 1996). The yeast GAL4 transcriptional activator might be substituted for *lacZ* in the homing pigeon; this would allow expression of transgenes under Gal4-UAS control (Brand and Perrimon, 1993), now restricted to particular segments. Insertions at the *Ubx* promoter, which express GAL4 in the *Ubx* pattern, might be used to test the function of altered forms of the *Ubx* transcription factor.

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