The EMBO Journal vol.10 no.11 pp.3147–3155, 1991

Long range repression conferring boundaries of *Ultrabithorax* expression in the *Drosophila* embryo

Jürg Müller and Mariann Bienz

Zoological Institute, University of Zürich, Winterthurerstrasse 190, 8057 Zürich, Switzerland

Communicated by M.Bienz

In an attempt to reconstruct the embryonic expression pattern of the homeotic gene *Ultrabithorax* (*Ubx*) by stable integration of fusion constructs, we identified three key control regions called PBX, ABX and BXD. Each of these confers an expression pattern mimicking certain aspects of *Ubx* expression. The PBX and ABX patterns are limited to the *Ubx* domain with anterior boundaries at parasegments 6 and 5. In contrast, the BXD pattern extends from head to tail. PBX or ABX expression boundaries are imposed on the BXD pattern, if PBX or ABX is linked to BXD. These boundaries, although not the PBX and ABX expression limits themselves, are dependent on *Polycomb* function. We conclude that PBX and ABX are recognized by repressors which act across large distances to suppress BXD activity. Stable and heritable *Ubx* expression boundaries are thus mediated by this process of long range repression.

Key words: expression boundaries/homeotic gene/long range repression/*Polycomb*

Introduction

Homeotic genes in *Drosophila* assume a key role in the control of development (Lewis, 1963, 1978). They are activated in the early embryo in spatially restricted domains at unique positions along the anteroposterior axis (reviewed by Akam, 1987). The boundaries of these expression domains are maintained during subsequent development. Homeotic gene products are required throughout development (Morata and Garcia-Bellido, 1976) to control the formation of position-specific external and internal structures in the embryo, the larva and the adult (Lewis, 1963, 1978; Wakimoto and Kaufman, 1981; Hooper, 1986; Tremml and Bienz, 1989). They exert their function apparently by virtue of a protein domain highly conserved in many living organisms (McGinnis *et al.*, 1984), notably in yeast and prokaryotic gene regulators (Laughon and Scott, 1984; Shepherd *et al.*, 1984), which enables them to bind DNA (Beachy *et al.*, 1988) and to activate transcription (Thali *et al.*, 1988; Krasnow *et al.*, 1989; Samson *et al.*, 1989; Winslow *et al.*, 1989). Regulation of homeotic gene expression constitutes a link between early embryonic positional information and subsequent position-specific morphogenesis.

The initial activation of homeotic genes in the early embryo occurs in response to anteroposterior positional information which is thought to be provided by segmentation genes (Nüsslein-Volhard and Wieschaus, 1980; reviewed by Akam, 1987; Ingham, 1988), in particular by the gap genes (White and Lehmann, 1986; Harding and Levine, 1988; Irish *et al.*, 1989). The products of the segmentation genes, however, are only transiently available in the early embryo and, thus, the maintenance of homeotic gene activity throughout development has to rely on other factors. Genes of the *Polycomb* (*Pc*) group, generally acting as repressors of homeotic gene activity (Lewis, 1978), have an essential function in the maintenance of this activity (Struhl, 1981; Jürgens, 1985). In some cells, positive autoregulation may account for maintenance of homeotic gene activity (Bienz and Tremml, 1988; Kuziora and McGinnis, 1988).

Presence of a homeotic gene product in a metamer is in many cases sufficient to confer a certain developmental pathway (Lewis, 1978; Schneuwly *et al.*, 1985; Gonzales-Reyes *et al.*, 1990). Therefore, regulation of homeotic gene expression, particularly its restriction along the anteroposterior axis, is of paramount importance. In order to understand the regulatory processes which govern homeotic gene expression, we attempted to reconstruct the embryonic expression pattern of *Ultrabithorax* (*Ubx*), one of the homeotic genes. We set out to identify *cis*-acting control regions by linking fragments from the *Ubx* gene to β-galactosidase (βgal) coding sequences and by analysing the βgal expression patterns in transformed embryos. We found three comparatively small control regions, located in remote areas of the *Ubx* gene, which evidently play a crucial role in generating the embryonic *Ubx* expression pattern. They act together to generate a βgal pattern which mimics essential aspects of the endogenous *Ubx* expression pattern, notably its restriction along the anteroposterior axis. Linked to a *Ubx* cDNA, they confer *Ubx* function in the larval epidermis. The function of these control regions and their way of action provide insight into the logic by which the pattern of this homeotic gene is created.

Results

Three important Ubx control regions

The *Ubx* gene is one of three large genes constituting the bithorax complex (Sanchez-Herrero *et al.*, 1985). It comprises >105 kb of genomic sequence (Bender *et al.*, 1983) of which the *Ubx* transcription unit spans 75 kb (O'Connor *et al.*, 1988; Kornfeld *et al.*, 1989). There is a unique transcription start site (Saari and Bienz, 1987). The 30 kb of 5′ flanking sequence contain *Ubx* regulatory mutations (Bender *et al.*, 1983) as well as promoters for various upstream transcripts that are mostly non-coding (Lipshitz *et al.*, 1987).

We previously found that the region near the *Ubx* transcription start site contains *cis*-regulatory sequences conferring *Ubx*-like expression in the embryonic visceral mesoderm (Bienz *et al.*, 1988), including target sites for autoregulation (Müller *et al.*, 1989). Yet, neither this region nor the 3′ flanking region of the gene (3.9 kb from the

© Oxford University Press

translation stop codon; M.Bienz, unpublished) is sufficient to direct any *Ubx*-like expression in other germ layers of the embryo. However, a number of regulatory mutations mapping in remote areas of the gene, outside the *Ubx* coding regions, affect the *Ubx* expression pattern (White and Wilcox, 1985a; Beachy *et al.*, 1985). Most of these are caused by insertion of transposable elements or by rearrangement breakpoints, but some represent internal deletions (two *pbx* deletions at −30 kb, two *abx* deletions at +47 kb; Bender *et al.*, 1983; see Figure 1a). We surmised that the genomic fragments covered by the deletion overlaps may carry important *cis*-regulatory control elements. Therefore, we joined these fragments (called PBX and ABX) individually to the *Ubx* proximal promoter

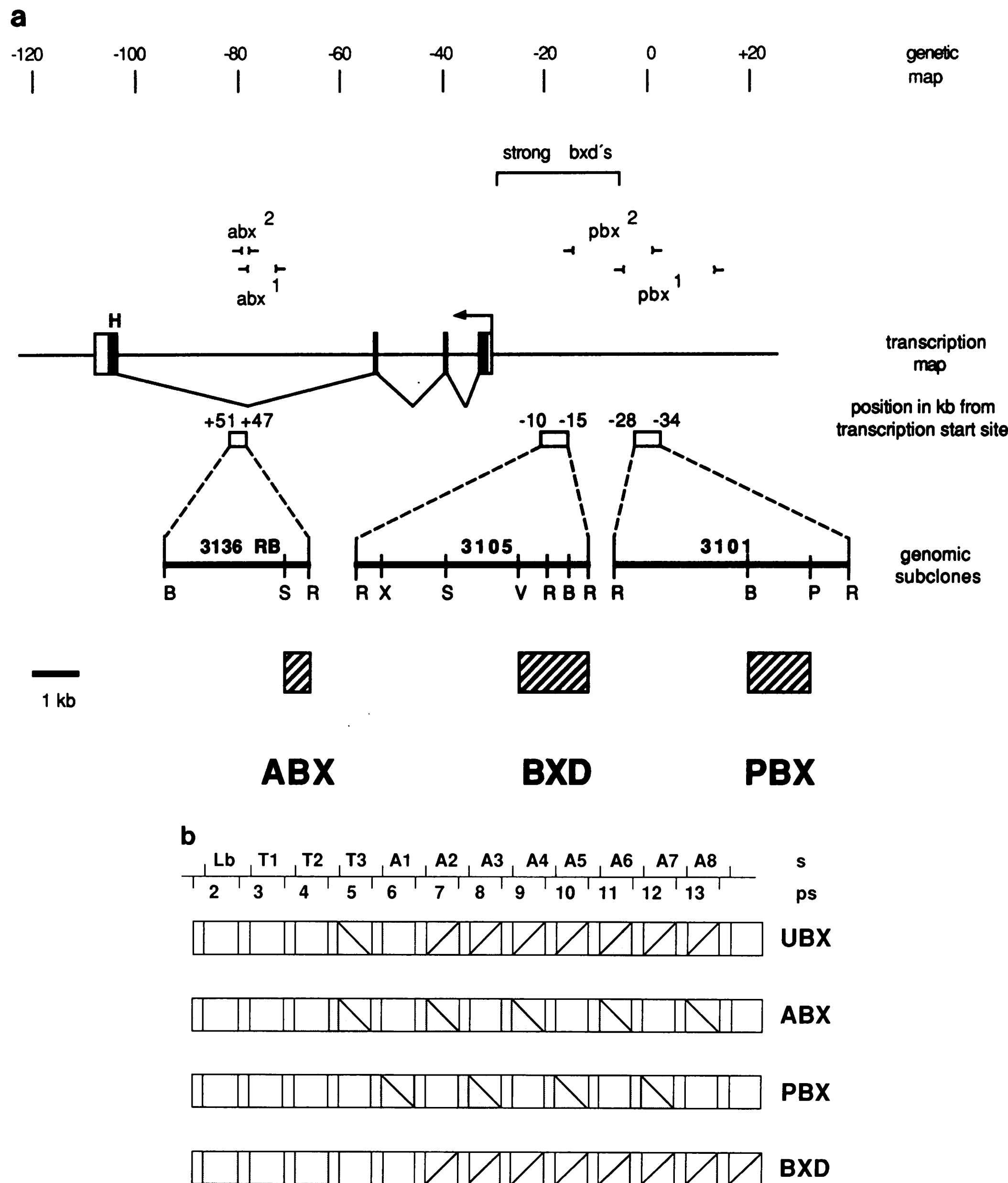

Fig. 1. Three important *Ubx* control regions. (**a**) Location of PBX, BXD and ABX control regions with respect to genetic and transcription map of *Ubx* gene (Bender *et al.*, 1983; O'Connor *et al.*, 1988; Kornfeld *et al.*, 1989). Below, maps of genomic subclones (3101, 3105, RB fragment of 3136; B, *Bam*HI, R, *Eco*RI, S, *Sal*I, all sites shown; P, *Pst*I, V, *Eco*RV, X, *Xho*I, not all sites shown; scale to the left) containing these control regions (minimal fragments indicated by hatched boxes) and their positions (in kilobases) with respect to transcription start site (arrow, direction of transcription; black boxes, protein exons with H, homeobox; open boxes, mRNA leader and trailer). Above, map positions of mutations (*pbx* and *abx* deletions, region of strong *bxd* breakpoints; Bender *et al.*, 1983) that led to identification of control regions. (**b**) Schematic drawings of *Ubx* expression pattern as previously described (White and Wilcox, 1985b; Beachy *et al.*, 1985; White and Lehmann, 1986; Irvine *et al.*, 1991) and βgal patterns conferred by each individual control region (minimal fragments) in thoracic (T1−T3) and abdominal (A1−A8) segments (s) or parasegments (ps) of the ectoderm. The following key features of these patterns are drawn: restriction along the anteroposterior axis (*Ubx* expression and ABX pattern in ps5−13, PBX pattern in ps6−12, BXD pattern in ps2−14); patterns within individual ps (slopes indicate graded expression within ps, observed in early germ band embryos; *engrailed* (*en*) expression domains anteriorly within ps marked by vertical bars); levels of expression (strong, average and weak expression indicated by stippling intensity). Note that the *Ubx* expression pattern in ps6 apparently corresponds to the superimposition of a thoracic ps5 (ABX) and an abdominal ps7−13 (BXD) pattern. βgal patterns conferred by the genomic subclones 3101, 3105 and 3136RB show the same key features, although there is additional βgal expression in other cells, due to different enhancers contained within adjacent regions of these subclones.

(−3.1/+1 kb) and the βgal gene (Bienz *et al.*, 1988) and analysed embryos transformed with these constructs for βgal staining. We also tested additional constructs containing adjacent fragments (see Materials and methods), as well as one (called BXD) derived from the region in which strong *bxd* breakpoint mutations map (Bender *et al.*, 1983; see Figure 1a).

We found that three of the fragments tested confer a βgal pattern in the embryo (PBX, ABX, BXD pattern; Figure 1b) that shows certain essential features of the endogenous *Ubx* expression pattern (White and Wilcox, 1985b; Beachy *et al.*, 1985; Irvine *et al.*, 1991). In the following paragraphs, we shall outline what we consider to be the most important aspects of these patterns. We tested each pattern in homozygous Ubx^- embryos and found that they were unaltered; thus none of them appears to be dependent on endogenous *Ubx* function.

The PBX and ABX patterns

The most striking aspect of the PBX and ABX patterns is that they are limited along the anteroposterior axis to a domain within which endogenous *Ubx* is expressed (Figure 1b). The PBX pattern is essentially restricted to even-numbered parasegments (ps) within ps6−12 in the ectoderm, the ABX pattern to odd-numbered parasegments within ps5−13 (Figure 2a and b). In either case, βgal staining first appears after gastrulation during stage 7 (stages according to Campos-Ortega and Hartenstein, 1985) in blocks whose anterior limits are sharp and coincide with parasegmental boundaries. These staining blocks develop into short individual gradients with highest expression levels anteriorly within parasegments. The βgal patterns resemble endogenous *Ubx* expression in ps5 (Figure 1b) where we observe a good correspondence between βgal and *Ubx* antibody staining (Figure 2b, bottom). The PBX and ABX patterns remain essentially unaltered throughout embryonic development, although they eventually fade out, suggesting that the two control regions are active exclusively in early embryos (late staining may reflect persisting βgal protein). We also see faint βgal staining in ps6−12 of the mesoderm in PBX transformants. The ABX pattern has been described in detail by Simon *et al.* (1990).

The BXD pattern

Of all other fragments tested, only one from the *bxd* region conferred a βgal pattern (called BXD pattern; Figure 2c) that differed significantly from the weak background pattern in the ectoderm due to the proximal promoter (Bienz *et al.*, 1988). The BXD pattern first appears at stage 9 extending from head to tail (ps2−14). Initially, it consists of narrow stripes abutting posterior parasegment boundaries. Later, the βgal stripes widen and develop into staining blocks closely resembling endogenous *Ubx* expression in abdominal segments: staining is strongest posteriorly, but almost undetectable anteriorly within parasegments. The BXD pattern remains strong throughout development, notably in the central nervous system (CNS). We note that the individual staining blocks in anterior parasegments of the CNS are wider than those of ps7−14 (Figure 1b).

Linking of control regions

We asked whether the combination of individual control regions in the same construct would lead to additive or non-additive patterns. We constructed pairwise combinations of each of the three control regions joined to the proximal *Ubx* promoter (PB and BP, AB and BA, AP and PA; first letter indicates proximity to the promoter). We also made two constructs bearing all three control regions upstream of the promoter, in the same order (ABP) or at the same relative distance from the transcription start site (BPA) as they occur within the chromosome.

Transformants obtained with AP and PA constructs all show a very similar pattern which essentially consists of a PBX and an ABX pattern superimposed on each other (Figure 3a). The two patterns are thus additive. The βgal staining blocks in odd-numbered parasegments are stronger in AP than in PA transformants, indicating that relative proximity of an individual control element to the promoter favours the intensity of the pattern it confers.

In contrast, the patterns generated by the other double-combination constructs show a strong tendency to be highly non-additive (Figure 3b). In some of the BP and PB transformant lines, βgal expression outside of ps6−13 is essentially eliminated. Similarly, we obtained AB and BA lines in which βgal staining is restricted to ps5−13. In these cases, we observe a sharp boundary of βgal staining coinciding with the anterior margin of ps6 (BP and PB) or ps5 (AB and BA), with very little if any βgal staining anterior to these boundaries (ectopic βgal staining tends to be confined to the dorsal epidermis and to the head). The boundaries in these lines persist throughout embryonic development. In the majority of the double-combination transformant lines, the BXD pattern outside of ps6−12 or ps5−13 is still visible faintly and thus not completely suppressed; occasional lines show no suppression of the BXD pattern (Table I). Although the degree of suppression in individual lines is somewhat variable, probably due to interference by adjacent chromosomal sequences (see Discussion), suppression of the BXD pattern outside of ps6−12 or ps5−13 is strictly correlated with the presence of PBX or ABX, respectively, and never observed in the absence of these control regions (Table I). Relative proximity of the PBX or ABX control regions to the proximal *Ubx* promoter does not effect the efficiency of suppression, but PBX-mediated suppression tends to be generally more efficient than ABX-mediated suppression.

We made two further constructs (BβP and BβA) in which the PBX or ABX control regions are located ~7 kb downstream of the transcription start site (and thus ~10 kb from the BXD control region) to test whether PBX and ABX could suppress the BXD pattern from this remote position. The BβP and BβA transformants show the same range of βgal staining patterns as the BP and BA transformants. We observe virtually complete suppression of the BXD pattern outside of the *Ubx* domain in some transformant lines; other lines show incomplete suppression or no suppression at all (Table I). We conclude that the PBX and ABX control regions mediate repression independently of their position with respect to the gene and across considerable distances.

Both triple-combination constructs are capable of generating a βgal staining pattern which is restricted to ps5−13 (Figure 3b). We also obtained some lines in which the βgal pattern is suppressed only partially or not at all outside of ps5−13 (Table I). Long range suppression appears to be particularly efficient in the ABP construct in which the BXD control region is flanked by ABX and PBX.

The βgal patterns within individual parasegments of double- and triple-combination transformants are additive,

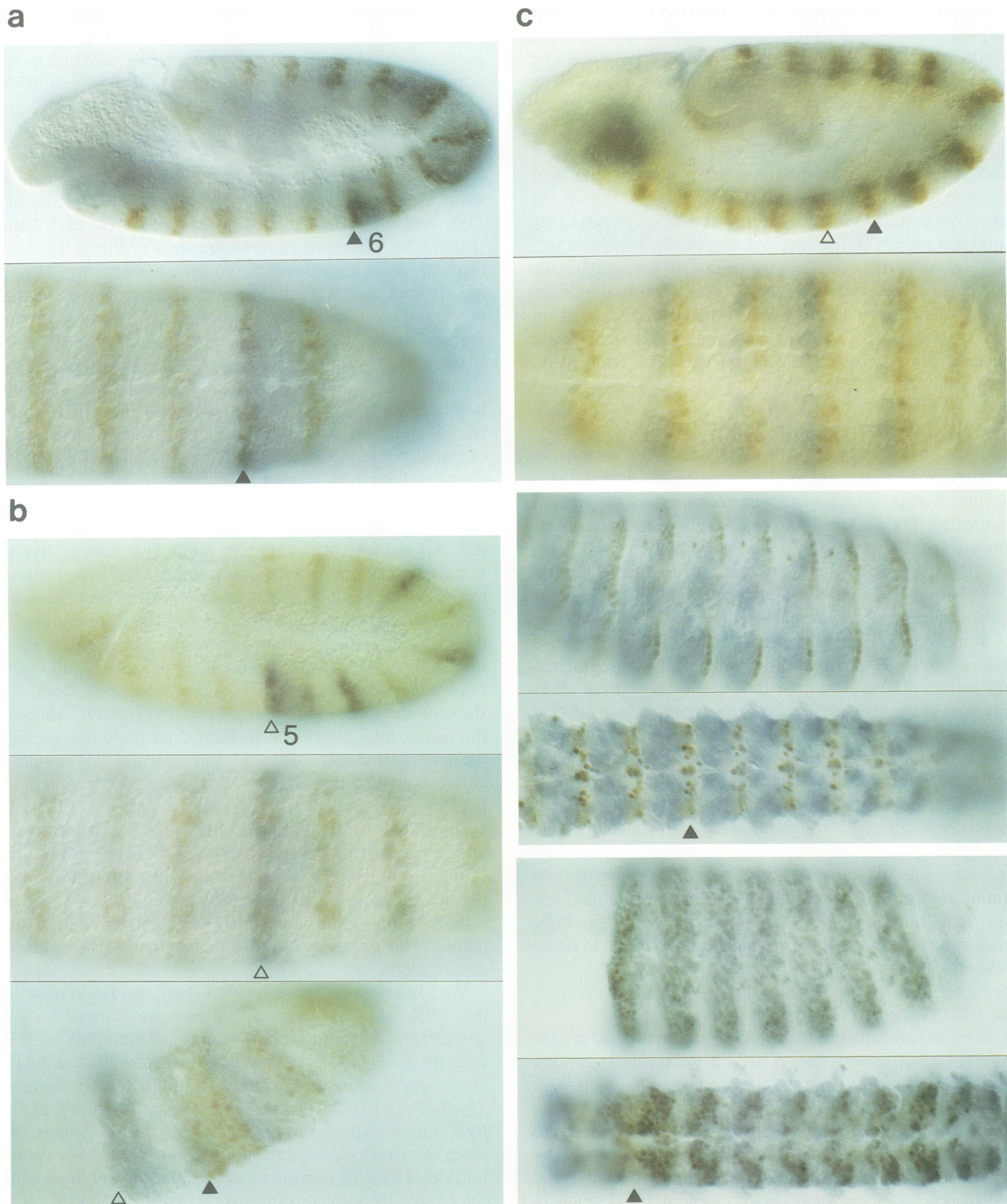

Fig. 2. Three *Ubx*-like βgal patterns. Stage 11 embryos stained with βgal antibody (grey) to show PBX (**a**), ABX (**b**) and BXD (**c**) pattern (top panel, lateral view; second panel, ventral view). Bottom two double-panels in (c) show BXD pattern in dissected lateral halves (top) or CNS (bottom) of stage 14 embryos. Most embryos are co-stained with anti-Engrailed antibody (brown) to show anterior ps margins (ps5 indicated by open, ps6 by black triangles). Some dissected embryos (b, bottom and c, bottom double-panel) are co-stained with *Ubx* antibody (brown) to show correspondence between βgal and *Ubx* pattern in ps5 (ABX) or in abdominal segments (BXD). Note that PBX- and ABX-mediated βgal staining is strong anteriorly within ps, closely resembling the βgal patterns obtained with *fushi tarazu* and *even-skipped* fusion genes (Lawrence *et al.*, 1987). In contrast, BXD-mediated βgal staining is strong posteriorly within the ps (initially, top panels in c, abutting *en* staining; later, middle double-panel in c, alternating with *en* staining). Constructs used for transformants: (a) pry3101BR (pattern identical to the one mediated by minimal PBX fragment, 3101BP); (b) pry3136 (pattern similar to the one mediated by minimal ABX fragment, although in the latter no βgal staining in ps6); (c) pry3105VR (containing minimal BXD fragment). Anterior of ps to the left.

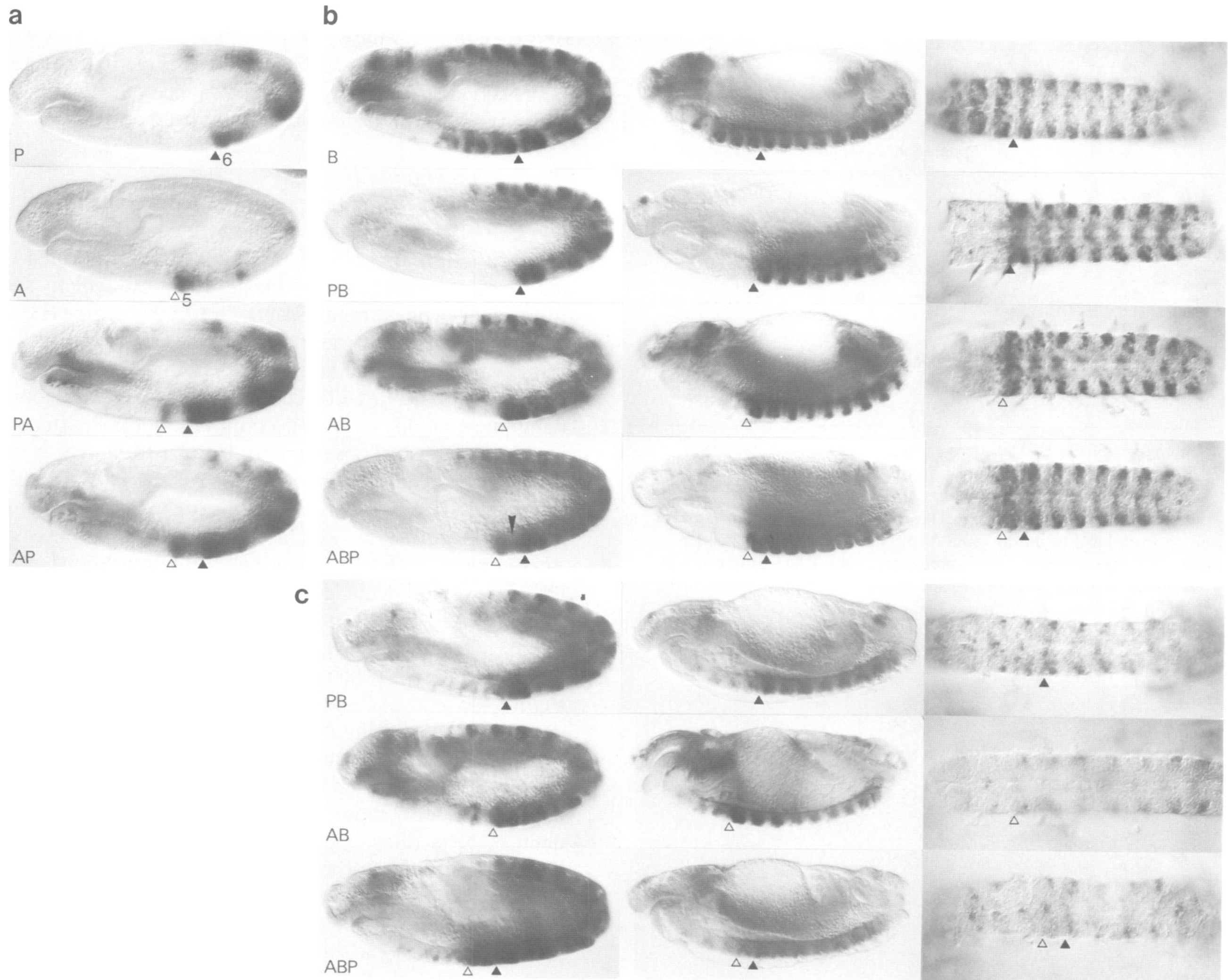

Fig. 3. Combination constructs: βgal patterns in wild-type and Pc^- embryos. (**a and b**) Wild-type embryos stained with βgal antibody, transformed with PBX (P), ABX (A), BXD (B) or combination constructs (PA, AP, PB, AB, ABP). Anterior limits of βgal expression at ps5 (open triangles) or ps6 (black triangles); the BXD pattern extends through ps2–14. Suppression of BXD pattern anteriorly to ps6 or ps5 and posteriorly to ps13 in PB, AB and ABP transformants: limits of βgal expression in these transformants due to long range repression. The BXD pattern in ps5 of ABP transformants (arrowhead) suppressed only very slightly. (**c**) Pc^- embryos. Ectopic βgal staining anteriorly to ps6 (PB) or ps5 (AB, ABP), corresponding to a derepressed BXD pattern (derepression posteriorly to ps13 not visible, due to focal plane). βgal staining is generally weaker, most likely due to repression by other bithorax complex genes (Struhl and White, 1985). βgal patterns in PBX, ABX, PA and AP transformants are unaltered in Pc^- embryos. Stage 11 (a; b and c, left panels), stage 13 (b and c, middle panels), dissected CNS of stage 14 (b and c, right panels; CNS of Pc^- embryos usually distorted and, thus, βgal staining in different ps not on same focal plane). Construct used for all transformants contain PBX, ABX and BXD minimal fragments (except ABP construct containing whole PBX subclone 3101). Anterior to the left.

although the relative intensities of the underlying PBX, ABX and BXD patterns depend on the embryonic stage (PBX and ABX patterns predominate early, the BXD pattern at later stages) as well as on the relative proximity of the control elements to the promoter (e.g. the BXD pattern is very strong in BPA transformants). Unexpectedly, this is also true for ps5 of BPA and ABP transformants in which we observe an ABX as well as a BXD pattern, despite the fact that the BXD pattern ought to be suppressible in this parasegment by the PBX control region. Indeed, careful inspection reveals that the BXD pattern is weakly suppressed in ps5 of ABP transformants (arrowhead in Figure 3b), suggesting residual PBX-mediated suppression in this case. Evidently, the simultaneous presence of the ABX control region in the ABP and BPA constructs prevents PBX-mediated suppression in ps5 which, in the absence of the ABX control region (BP and PB constructs), is very efficient in this parasegment.

Requirement for Polycomb

Genes of the *Pc* group function to preserve the boundaries of homeotic gene expression (Lewis, 1978; Struhl, 1981; Jürgens, 1985). To ask whether the βgal expression boundaries in our transformants are dependent on *Pc* function, we analysed the βgal patterns of individual lines in homozygous Pc^- embryos. Surprisingly, we found that neither the ABX nor the PBX pattern nor the pattern conferred by the AP and PA combination constructs are altered in *Pc* mutants: the anterior boundaries of all these patterns remain unchanged throughout embryonic development.

Table I. Long range repression in different transformant lines

Construct and total number of lines		Number of lines with		
		complete suppression	incomplete suppression	no suppression
B	6	0	0	6
AB	7	3	2	2
BA	8	1	5	2
BP	7	2	5	0
PB	2	1	0	1
ABP	8	3	3	2
BPA	7	1	3	3
BβA	16	4	1	11
BβP	6	2[a]	2	2

Suppression of the BXD pattern outside of the *Ubx* domain was classified as complete if there was virtually no ectopic βgal staining (see text), and as incomplete if the BXD pattern was faintly visible outside of ps6–12 or ps5–13. The BXD pattern was equally strong in all ps from head to tail in some of the lines bearing combination constructs (no suppression). Suppression of the BXD pattern outside of ps5–13 was never observed in any of the BXD transformant lines.
[a]In these two lines, the anterior boundary is at ps5 (to be discussed elsewhere).

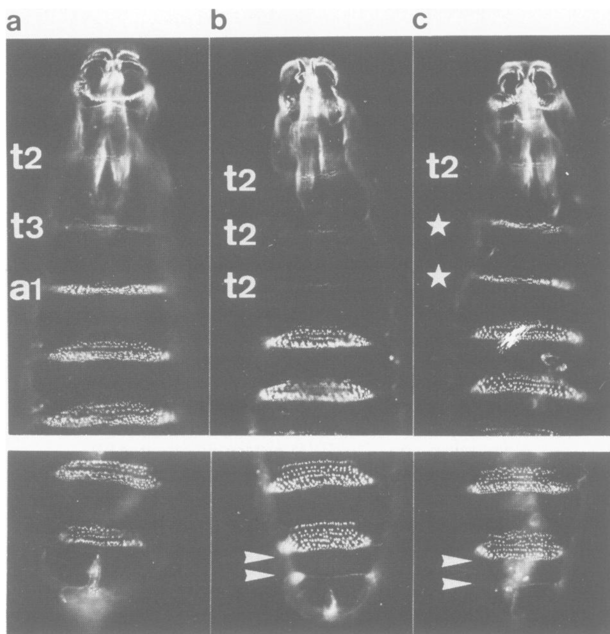

Fig. 4. Function of a *Ubx* minigene. Cuticle preparations of freshly hatched larvae with (c) or without (a, b) a *Ubx* minigene. (a) wild-type; (b and c) homozygous $Ubx^-Abd\text{-}B^-$ mutants, recognizable by their $Abd\text{-}B^-$ phenotype (bottom panels: naked region between arrowheads in b and c, not visible in a). Ventral denticle belts in (b) indicate transformation of third thoracic (t3) and first abdominal (a1) segments into t2, due to lack of *Ubx* function in ps5 and ps6. Denticle belts similar to those in the wild-type, visible in these two parasegments (asterisks in c) have formed due to the *Ubx* minigene.

In contrast, transformants from all other combination constructs show striking ectopic βgal expression anteriorly in homozygous Pc^- embryos (Figure 3c). Derepression is first visible at stage 11 and becomes more pronounced at later stages. The pattern of the ectopic βgal expression, especially the one in the CNS, suggests that this is mediated by the BXD control region. It is also reminiscent of ectopic *Ubx* expression in embryos mutant for *Pc* or *Pc*-like genes (most similar to the *Ubx* pattern in *extra sex combs*$^-$ embryos; Gould *et al.*, 1990). We conclude that the long-range suppression of the BXD pattern mediated by the PBX and ABX control regions requires *Pc* function.

Rescue experiments

We exchanged the βgal gene in the ABP construct with a *Ubx* cDNA to ask whether this *Ubx* minigene could provide *Ubx* function in Ubx^- embryos. We obtained 12 independent transformant lines, four of which show rudimentary wings, reminiscent of a *Cbx* phenotype (Lewis, 1978; *Cbx* mutations cause ectopic *Ubx* expression in wing imaginal discs; White and Akam, 1985). We ascribe this phenotype to the *Cbx*-like ectopic transposon expression in wing imaginal discs which is conferred by the PBX control region (unpublished observation); the *Cbx* phenotype is fully penetrant and thus observed in all ry^+ flies. We crossed flies from various *Ubx* minigene-bearing lines into a strain containing a *Ubx* as well as, for marking purposes, an *Abdominal-B* (*Abd-B*) mutation. In homozygous Ubx^- larvae, a ventral denticle belt typical for the first abdominal segment (A1, derived from ps6) cannot be formed (Lewis, 1978); hatched homozygous *Abd-B* larvae can be identified by their altered posterior end (Casanova *et al.*, 1987; Figure 4).

Among the progeny from crosses with lines exhibiting a *Cbx* phenotype, we found two classes of homozygous $Abd\text{-}B^-$ larvae: those which lacked A1 denticle belts (the Ubx^- larvae), but also those with virtually normal looking A1 denticle belts (those carrying the transposon; Figure 4c). Denticle belts anterior to the second thoracic segment (T2, derived from ps4) are unaffected in the latter, however their ps5-derived denticle belts are broader than those normally seen in T3 and look somewhat A1-like, a phenotype also seen in many of the $Abd\text{-}B^+$ larvae (those carrying the transposon). These A1-like denticle belts derived from the posterior part of ps5 are probably due to the BXD pattern which is only poorly suppressed in ps5 of ABP transformants. The high frequencies of A1-like denticle belts among the progeny of a cross suggest that each Ubx^- larva containing the transposon shows rescue activity. We conclude that the ABP minigene provides *Ubx* function in ps5 and ps6 of the embryonic epidermis, thereby rescuing some of the most conspicuous aspects of the larval Ubx^- phenotype. We did not detect any rescue activity in those minigene transformant lines which lack the *Cbx* phenotype, presumably due to very low or absent transposon expression.

Discussion

We identified two types of functionally distinct control regions in the *Ubx* gene: those which act early in the embryo to confer spatially restricted expression along the anteroposterior axis, and one which acts later to confer an expression pattern extending through all parasegments from head to tail. The early control regions suppress the activity of the late one outside of the *Ubx* expression domain. We conclude that the early control regions, PBX and ABX, are recognized by repressors.

The three control regions, when combined, interact to generate a pattern resembling *Ubx* expression. They are capable of conferring *Ubx* function in those parasegments

which are most affected by *Ubx* mutation (ps5 and 6). It is therefore very likely, and we shall assume this to be the case for this discussion, that they function the same way in controlling the expression of the chromosomal *Ubx* gene.

Long range repression

The PBX and ABX control regions mediate repression over considerable distances (>7 kb; in the chromosomal gene perhaps over >40 kb) and independent of their position upstream or downstream of the transcription start site. Thus, they have properties opposite to those of an enhancer, but similar to those of a transcriptional silencer in yeast (Brand *et al.*, 1985). We do not know at present whether, like yeast silencers (Brand *et al.*, 1985), they are capable of repressing heterologous promoters. Alternatively, long range repression in the case of *Ubx* could be dependent on PBX or ABX repressors interacting with *Ubx*-specific proteins bound to the *Ubx* proximal promoter and/or the BXD control region.

Up till now, there are few examples of long range repression known in higher organisms (e.g. Doyle *et al.*, 1989), and it is unclear what the molecular basis of long range repression may be. For example, repression could be due to global structural features of a gene such as its association with compacted chromatin (Wolffe and Brown, 1988) or with the nuclear lamina (Diffley and Stillman, 1989). We favour a model in which the PBX and ABX repressors, similarly to enhancer-binding proteins (reviewed by Ptashne, 1988), interact with proteins bound to the proximal promoter, thereby looping out the intervening DNA. They may promote the assembly of a complex, like a stable transcription complex (reviewed by Brown, 1984; Wolffe and Brown, 1988), which however lacks transcriptional activation potential. Whether such a complex activates or blocks transcription might depend not only on its particular structure, but also on its protein composition. As in the case of globin gene silencing (Dillon and Grosveld, 1991), proteins bound to the proximal promoter (see Müller *et al.*, 1989) might play an important role in this decision between activation and repression function. A precedent for repression based on looping is found in the arabinose operon in *Escherichia coli* (Lobell and Schleif, 1990).

We found evidence that the formation of a long range repression complex may be interfered with by an activating transcription complex nearby which pre-exists or forms simultaneously (ABX-mediated activity interfering with PBX-mediated repression: ps5 pattern in ABP and BPA transformants). The variable efficiency of long range suppression in individual transformant lines could be due to a similar interference of active chromosomal enhancers fortuitously juxtaposed to a transposon. Interference of this kind evidently does not occur in the chromosomal *Ubx* gene: the *Ubx* expression pattern in ps5 (White and Wilcox, 1985b; Beachy *et al.*, 1985; Figure 1b) implies that BXD-mediated expression is suppressed in this parasegment, presumably by the chromosomal PBX control region. Interference between activating and repressing transcription complexes may be avoidable if the corresponding control regions are located far apart from each other or at opposite sides from the proximal promoter, as in the case of the chromosomal *Ubx* gene.

The role of Polycomb

Pc function is required for long range repression mediated by the PBX and ABX control regions. It is possible that *Pc* protein is a component of these long range repression complexes and that it bestows on them the stability which may be necessary for them to be heritable through cell divisions. Such a role of *Pc* would be consistent with the fact that *Pc* protein is associated *in vivo* with the *Ubx* locus (Zink and Paro, 1989) and with its function in the maintenance of *Ubx* expression boundaries required continuously through development (Struhl, 1981; Busturia and Morata, 1988).

The distribution of *Pc* transcripts is not graded nor restricted along the anteroposterior axis of the embryo (Paro and Hogness, 1991). Thus, *Pc* protein itself is hardly sufficient to specify the position of *Ubx* expression boundaries. As mentioned, the boundaries of the PBX and ABX patterns themselves are not dependent on *Pc* function. Indeed, early *Ubx* expression boundaries do not depend on the function of *extra sex combs*, an early acting member of the *Pc* group of genes (Akam and Struhl, 1985), but are due to blastoderm repressors such as *hunchback* (Irish *et al.*, 1989) and *tailless* (Reinitz and Levine, 1990) whose distribution in the blastoderm embryo is restricted along the anteroposterior axis (Tautz *et al.*, 1987; Pignoni *et al.*, 1990). Recent evidence strongly suggests that *hunchback* directly interacts as a repressor with the PBX control sequence to set the anterior PBX expression boundary (Zhang, *et al.*, 1991; see also Qian *et al.*, 1991). We imagine that *Pc* protein recognizes and interacts with the initial repressor complex set up by *hunchback*, thereby converting it into a stable and heritable repression complex. Whatever its structure, this complex would have to be capable of reforming after each round of replication in the absence of the blastoderm repressors which initially established it.

Concluding remarks

Functional significance has been ascribed to the large and apparently complex regulatory regions of the *Ubx* gene (e.g. Irvine *et al.*, 1991) and to their respective order along the chromosome (reviewed by Peifer *et al.*, 1987). Our results show that important *Ubx* control regions are comparatively short, though powerful as they apparently act across large, yet not unprecedented (Grosveld *et al.*, 1987) distances in the chromosomal gene. More importantly, they function autonomously, detached from their native chromosomal context, and essentially independently of their position with respect to each other and to the gene. Also, our rescue experiments suggest that there is no absolute requirement for a *Ubx* gene to be intact or located within the bithorax complex in order to function, in agreement with a conclusion previously reached (Struhl, 1984).

The discovery of long range repression in the *Ubx* gene emphasizes the fact that the gene is principally active and that an elaborate process, dependent on at least one, but probably a whole group of control genes (Jürgens, 1985), is required to suppress this activity in inappropriate parasegments. Thus, it appears that certain key control regions mediate shutting down (Gaunt and Singh, 1990) rather than 'opening up' (Peifer *et al.*, 1987) of a homeotic gene in a parasegment-specific way in the developing embryo. Interestingly, the boundaries of *Hox1.1* gene expression, a putative homeotic gene of the mouse, are also dependent on long range repression (Püschel *et al.*, 1991). This suggests that the principle of controlling the expression of homeotic genes along the anteroposterior axis by long range repression may be general and widespread as it is

employed in rather unrelated organisms such as mice and flies.

Materials and methods

Plasmids

Genomic subclones (3101, 3105 and 3136BR; provided by M.Akam) or minimal fragments (3101BP, 3105VR and 3136SR; see Figure 1a) were inserted as *Xba*I–*Kpn*I fragments (i.e. via a Bluescript subcloning step) into a modified -3.1*Ubx*–βgal fusion construct (plasmids called 'pry'; fusion construct as in Bienz *et al.*, 1988, but modified to contain a unique *Kpn*I site downstream of the existing unique *Xba*I site). The minimal fragments were also cloned into HZ50PL (Hiromi and Gehring, 1987; plasmids called 'hz'). For combination constructs, minimal PBX, ABX and BXD fragments were joined in various orders to the modified -3.1*Ubx*–βgal fusion gene. In the BβP and BβA constructs, the minimal PBX or ABX fragment was inserted between the *Sal*I and the *Bam*HI site (positions −109 and −110; O'Connor *et al.*, 1988) within the *Ubx* mRNA trailer which was used in these constructs instead of the hsp70 mRNA trailer (*Ubx* trailer and βgal gene were fused at their translation stop codons). The ABP construct contains the whole 3101 genomic subclone instead of the minimal PBX fragment, the BPA construct the *Ubx* mRNA trailer (to *Bam*HI site at −110) instead of the hsp70 trailer. For the *Ubx* minigene, the βgal gene in the ABP construct was substituted by a *Ubx* cDNA (Gonzales-Reyes *et al.*, 1990; provided by G.Struhl) including the complete *Ubx* trailer. In all constructs, the orientation of fragments was kept the same as in the chromosome. Detailed maps of plasmids are available on request (see also Zhang *et al.*, 1991.

Drosophila strains and transformation

Embryos of a *cn;ry*^{42} strain were injected with the various constructs; three to ten individual transformant lines per construct were isolated and made homozygous as described (Bienz *et al.*, 1988). Individual transformant lines with a transposon inserted in the second chromosome were analysed in *Ubx*^{1}*/triple* (Casanova *et al.*, 1987) or in homozygous *Pc*^{3} embryos. The function of the *Ubx* minigene was analysed in homozygous *Ubx*$^{9.22}$ *Abd-B*^{M5} larvae (Casanova *et al.*, 1987). Crossing of transposon-bearing and mutant strains was done as described (Bienz and Tremml, 1988).

Analysis of transformed embryos and larvae

Embryos were fixed and stained with a polyclonal rabbit serum against βgal protein (Cappell) and/or with a monoclonal antibody against Ubx (White and Wilcox, 1984) or engrailed (Patel *et al.*, 1989; provided by M.Wilcox) as previously described (Lawrence *et al.*, 1987; Tremml and Bienz, 1989). Cuticle preparations of freshly hatched larvae were done according to Gonzales-Reyes *et al.* (1990).

We observed few consistent differences between βgal pattern conferred by pry- and hz-based constructs: hz-derived patterns tend to be stronger than pry-derived patterns and there is generally more pry-derived 'background' staining, typically in the head and in the dorsal epidermis, due to proximal promoter elements (e.g. 'basal pattern'; Bienz *et al.*, 1988). 3101 and 3136BR subclones in hz constructs do not produce a PBX or ABX pattern (although the minimal fragment in each case does), probably due to the large distances between the PBX or ABX minimal fragment and the hsp70 TATA box within these constructs. Fragments from the following genomic subclones were also used for transformants as pry constructs, but did not show any βgal patterns other than the one due to the proximal promoter: 3104, 3106, 3108 and 3134.

Acknowledgements

We thank Michael Akam for providing genomic subclones of the *Ubx* gene, Bea Coton for help with the flies, Rob White, Mark Biggin and Gines Morata for discussion. Thanks also to Maya. This work was supported by the Swiss National Science Foundation (grant no. 31–26198.89 to M.B.).

References

Akam,M. (1987) *Development*, **101**, 1–22.

Akam,M. and Struhl,G. (1985) *EMBO J.*, **4**, 3259–3264.

Beachy,P.A., Helfand,S.L. and Hogness,D.S. (1985) *Nature*, **313**, 545–551.

Beachy,P.A., Krasnow,M.A., Gavis,E.R. and Hogness,D.S. (1988) *Cell*, **55**, 1069–1081.

Bender,W., Akam,M., Karch,F., Beachy,P.A., Peifer,M., Spierer,P., Lewis,E.B. and Hogness,D.S. (1983) *Science*, **221**, 23–29.

Bienz,M. and Tremml,G. (1988) *Nature*, **333**, 576–578.

Bienz,M., Saari,G., Tremml,G., Müller,J., Züst,B. and Lawrence,P.A. (1988) *Cell*, **53**, 567–576.

Brand,A., Breeden,L., Abraham,J., Sternglanz,R. and Nasmyth,K. (1985) *Cell*, **41**, 41–48.

Brown,D.D. (1984) *Cell*, **37**, 359–365.

Busturia,A. and Morata,G. (1988) *Development*, **104**, 713–720.

Campos-Ortega,J. and Hartenstein,V. (1985) *The Embryonic Development of Drosophila melanogaster*. Springer Verlag, Berlin.

Casanova,J., Sanchez-Herrero,E., Busturia,A. and Morata,G. (1987) *EMBO J.*, **6**, 3103–3109.

Diffley,J.F.X. and Stillman,B. (1989) *Nature*, **342**, 24.

Dillon,N. and Grosveld,F. (1991) *Nature*, **350**, 252–254.

Doyle,H.J., Kraut,R. and Levine,M. (1989) *Genes Dev.*, **3**, 1518–1533.

Gaunt,S.J. and Singh,P.B. (1990) *Trends Genet.*, **6**, 208–212.

Gonzales-Reyes,A., Urquia,N., Gehring,W.J., Struhl,G. and Morata,G. (1990) *Nature*, **344**, 78–80.

Gould,A.P., Lai,R.Y.K., Green,M.J. and White,R.A.H. (1990) *Development*, **110**, 1319–1325.

Grosveld,F., van Assendelft,G.B., Greaves,D.R. and Kollias,G. (1987) *Cell*, **51**, 975–985.

Harding,K. and Levine,M. (1988) *EMBO J.*, **7**, 205–214.

Hiromi,Y. and Gehring,W.J. (1987) *Cell*, **50**, 963–974.

Hooper,J. (1986) *EMBO J.*, **5**, 2321–2329.

Ingham,P.W. (1988) *Nature*, **335**, 25–34.

Irish,V.F., Martinez-Arias,A. and Akam,M. (1989) *EMBO J.*, **8**, 1527–1538.

Irvine,K.D., Helfand,S.L. and Hogness,D.S. (1991) *Development*, **111**, 407–424.

Jürgens,G. (1985) *Nature*, **316**, 153–155.

Kornfeld,K., Saint,R.B., Beachy,P.A., Harte,P.J., Peattie,D.A. and Hogness,D.S. (1989) *Genes Dev.*, **3**, 243–258.

Krasnow,M.A., Saffman,E.E., Kornfeld,K. and Hogness,D.S. (1989) *Cell*, **57**, 1031–1043.

Kuziora,M.A. and McGinnis,W. (1988) *Cell*, **55**, 477–485.

Laughon,A. and Scott,M.P. (1984) *Nature*, **310**, 25–31.

Lawrence,P.A., Johnson,P., Macdonald,P. and Struhl,G. (1987) *Nature*, **313**, 639–642.

Lewis,E.B. (1963) *Am. Zool.*, **3**, 33–56.

Lewis,E.B. (1978) *Nature*, **276**, 565–570.

Lipshitz,H.D., Peattie,D.A. and Hogness,D.S. (1987) *Genes Dev.*, **1**, 307–322.

Lobell,R.B. and Schleif,R.F. (1990) *Science*, **250**, 528–532.

McGinnis,W., Garber,R.L., Wirz,J., Kuroiwa,A. and Gehring,W.J. (1984) *Cell*, **37**, 403–408.

Morata,G. and Garcia-Bellido,A. (1976) *Wilhelm Roux Arch. Dev. Biol.*, 179, 125–143.

Müller,J., Thüringer,F., Biggin,M., Züst,B. and Bienz,M. (1989) *EMBO J.*, **8**, 4143–4151.

Nüsslein-Volhard,C. and Wieschaus,E. (1980) *Nature*, **287**, 795–801.

O'Connor,M.B., Binari,R., Perkins,L.A. and Bender,W. (1988) *EMBO J.*, **7**, 435–455.

Paro,R. and Hogness,D.S. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 263–267.

Patel,N., Martin-Blanco,E., Coleman,K.G., Poole,S.J., Ellis,M.C., Kornberg,T.B. and Goodman,C.S. (1989) *Cell*, **58**, 955–968.

Peifer,M., Karch,F. and Bender,W. (1987) *Genes Dev.*, 1, 891–898.

Pignoni,F., Baldarelli,R.M., Steingrimsson,E., Diaz,R.J., Patapoutian,A., Merriam,J.R. and Lengyel,J.A. (1990) *Cell*, **62**, 151–163.

Ptashne,M. (1988) *Nature*, **344**, 309–313.

Püschel,A.W., Balling,R. and Gruss,P. (1991) *Development*, **112**, 279–287.

Qian,S., Capovilla,M. and Pirrotta,V. (1991) *EMBO J.*, **10**, 1415–1425.

Reinitz,J. and Levine,M. (1990) *Dev. Biol.*, 140, 57–72.

Saari,G. and Bienz,M. (1987) *EMBO J.*, **6**, 1775–1779.

Samson,M.-L., Jackson-Grusby,L. and Brent,R. (1989) *Cell*, **57**, 1045–1052.

Sanchez-Herrero,E., Vernos,I., Marco,R. and Morata,G. (1985) *Nature*, **313**, 108–113.

Schneuwly,S., Klemenz,R. and Gehring,W.J. (1987) *Nature*, **325**, 816–818.

Shepherd,J.C.W., McGinnis,W., Carrasco,A.E., DeRobertis,E.M. and Gehring,W.J. (1984) *Nature*, **310**, 70–71.

Simon,J., Peifer,M., Bender,W. and O'Connor,M. (1990) *EMBO J.*, 9, 3945–3956.

Struhl,G. (1981) *Nature*, **293**, 36–41.

Struhl,G. (1984) *Nature*, **308**, 454–457.

Struhl,G. and White,R.A.H. (1985) *Cell*, **43**, 507–519.

Tautz,D., Lehmann,R., Schnürch,H., Schuh,R., Seifert,E., Kienlin,A.,

Jones,K. and Jäckle,H. (1987) *Nature*, **327**, 383–389.
Thali,M., Müller,M.M., DeLorenzi,M., Matthias,P. and Bienz,M. (1988) *Nature*, **336**, 598–601.
Tremml,G. and Bienz,M. (1989) *EMBO J.*, **8**, 2677–2685.
Wakimoto,B.T. and Kaufman,T.C. (1981) *Dev. Biol.*, 81, 51–64.
White,R.A.H. and Akam,M. (1985) *Nature*, **318**, 567–569.
White,R.A.H. and Lehmann,R. (1986) *Cell*, **47**, 311–321.
White,R.A.H. and Wilcox,M. (1984) *Cell*, **39**, 163–171.
White,R.A.H. and Wilcox,M. (1985a) *Nature*, **318**, 563–567.
White,R.A.H. and Wilcox,M. (1985b) *EMBO J.*, 4, 2035–2043.
Winslow,G.M., Hayashi,S., Krasnow,M., Hogness,D.S. and Scott,M.P. (1989) *Cell*, **57**, 1017–1030.
Wolffe,A.P. and Brown,D.D. (1988) *Science*, **241**, 1626–1632.
Zhang,C.-C., Müller,J., Hoch,M., Jäckle,H. and Bienz,M. (1991) *Development*, in press.
Zink,B. and Paro,R. (1989) *Nature*, **337**, 468–471.

Received on July 4, 1991; revised on July 26, 1991

Note added in proof

According to our recent results, the *Ubx* minigene *U12* is capable of supporting development of Ubx^1 homozygotes until late pupal stages (Castelli-Gair,J., Müller,J. and Bienz,M., in preparation). This rescue of the larval *Ubx* lethality confirms that the minigene contains most or all essential control regions necessary for embryonic and larval *Ubx* function.