

## Target sequences for *hunchback* in a control region conferring *Ultrabithorax* expression boundaries

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### Summary

Boundaries of *Ultrabithorax* expression are mediated by long-range repression acting through the PBX or ABX control region. We show here that either of these control regions confers an early band of  $\beta$ -galactosidase expression which is restricted along the anteroposterior axis of the blastoderm embryo. This band is succeeded by a stripe pattern with very similar anteroposterior limits. Dissection of the PBX control region demonstrates that the two patterns are conferred by distinct *cis*-regulatory sequences contained within separate PBX subfragments. We find several binding sites for *hunchback* protein within both PBX subfragments. Zygotic

*hunchback* function is required to prevent ectopic PBX expression. Moreover, the PBX pattern is completely suppressed in embryos containing uniformly distributed maternal *hunchback* protein. Our results strongly suggest that *hunchback* protein directly binds to the PBX control region and acts as a repressor to specify the boundary positions of the PBX pattern.

Key words: homeotic gene regulation, anteroposterior axis, expression boundaries, gap gene function, *hunchback*, *Ultrabithorax*.

### Introduction

Gap segmentation genes are expressed in broad and partly overlapping bands at different positions along the anteroposterior axis of the early *Drosophila* embryo (Knipple *et al.* 1985; Tautz *et al.* 1987; Nauber *et al.* 1988; Stanojevic *et al.* 1989; Pankratz *et al.* 1989). Their limits of expression are determined primarily by maternal genes (reviewed in Nüsslein-Volhard *et al.* 1987; see also Driever *et al.* 1989; Struhl *et al.* 1989). Gap gene products show homologies to transcription factors, mostly to zinc finger proteins (Rosenberg *et al.* 1986; Tautz *et al.* 1987; Nauber *et al.* 1988). They function regionally to control the activity of pair-rule segmentation genes (Carroll and Scott, 1986; Ingham *et al.* 1986; Frasch and Levine, 1987; Howard *et al.* 1988; Goto *et al.* 1989; Harding *et al.* 1989; Howard and Struhl, 1990), thereby ensuring the correct establishment of segment primordia within their functional domains (Nüsslein-Volhard and Wieschaus, 1980).

A second function that has been ascribed to the gap genes is the control of homeotic gene expression (reviewed by Akam, 1987; Ingham, 1988). This was first indicated by the phenotypes of gap gene mutants (Wieschaus *et al.* 1984; Lehmann and Nüsslein-Volhard, 1987; Bender *et al.* 1987). More direct

evidence was provided by the findings that the distribution of homeotic gene products is altered in gap mutants (White and Lehmann, 1986; Harding and Levine, 1988; Irish *et al.* 1989a; Reinitz and Levine, 1990). Irish *et al.* (1989a) found, by focusing on the very first consequences of gap mutations, that these affect homeotic gene expression as early as they affect pair-rule gene expression. Based on this, they suggested that the observed effects of gap mutations are likely to be due to direct interactions of gap gene products with regulatory regions of homeotic genes.

In an attempt to reconstruct the embryonic expression pattern of the homeotic gene *Ultrabithorax* (*Ubx*), we identified two separate control regions in this gene (called PBX and ABX) which confer  $\beta$ -galactosidase ( $\beta$ gal) patterns confined to the *Ubx* expression domain (Müller and Bienz, 1991). As these patterns appear early in the embryo, we asked whether the PBX and ABX control regions might be activated directly by products of the segmentation genes. In particular, we were interested whether the limits of the  $\beta$ gal patterns along the anteroposterior axis might be determined by gap proteins. Here, we analyse the RNA patterns that are conferred by these control regions at the beginning of embryogenesis. We dissect the PBX control region and search it for different *cis*-acting control elements as

well as for gap protein binding sites. We examine the PBX pattern in mutant embryos lacking individual gap proteins. We provide evidence that *hunchback* (*hb*) protein directly interacts with the PBX control region as a repressor to confer PBX expression boundaries.

## Materials and methods

### Fly strains and transformation

Embryos of a *cn;ry<sup>42</sup>* strain were injected with the various constructs, and transformants were isolated and made homozygous as described (Bienz *et al.* 1988). The following mutant alleles were used: *hb<sup>7M</sup>* (Lehmann and Nüsslein-Volhard, 1987), *Kr<sup>l</sup>* (Wieschaus *et al.* 1984), *kni<sup>FC13</sup>* and *osk<sup>346</sup>* (Tearle and Nüsslein-Volhard, 1987). Homozygous mutant embryos were obtained at a frequency of 1/4 in the case of gap mutations; all embryos obtained from homozygous *osk* mothers were mutant.

### Plasmids

Minimal PBX and ABX fragments (Fig. 1, top two lines), cloned into HZ50PL (Hiromi and Gehring, 1987), were described before (Müller and Bienz, 1991). Four PBX subfragments (Fig. 1) were inserted separately, *via* a subcloning step into bluescript, as *Xba*I–*Kpn*I fragments into HZ50PL.

### In situ hybridisations and antibody stainings

Embryonic sections were prepared and hybridised with uniformly <sup>35</sup>S-labelled RNA probes as described (Müller *et al.* 1989;  $\beta$ gal probe as in Müller *et al.* 1989; *Ubx* probe: *Stu*I–*Xho*I fragment from *Ubx* cDNA; Gonzales-Reyes *et al.* 1989). For antibody stainings, whole embryos were fixed and incubated with a polyclonal rabbit serum against  $\beta$ gal protein (Cappell) and/or a rabbit serum against *eve* protein (Frasch *et al.* 1987) as described (Lawrence *et al.* 1987; Tremml and Bienz, 1989).

### Footprinting analysis

Extracts containing gap proteins (overexpressed in *E. coli* with T7 RNA polymerase; Rosenberg *et al.* 1987) were prepared as described by Kadonaga *et al.* (1987). Various DNA fragments were end-labelled with Klenow enzyme and used for DNAase I footprinting according to Kadonaga *et al.* (1987). Both strands of the minimal PBX control region (*Pst*I/*Bam*HI fragment; Müller and Bienz, 1991) were screened for *hb*, *Kr* and *kni* footprints.

## Results

We previously described the  $\beta$ gal staining patterns conferred by the PBX and ABX control region in embryos at the extended germ band stage (Müller and Bienz, 1991; Fig. 1). These patterns are limited to parasegments (ps) 6–12 in the case of PBX and to ps5–13 in the case of ABX.  $\beta$ gal staining appears in even-numbered (PBX) or odd-numbered (ABX) parasegments in stripes with sharp anterior margins, coinciding with ps boundaries; the stripes fade towards posterior.

We determined the earliest time point in development at which these patterns become detectable. We

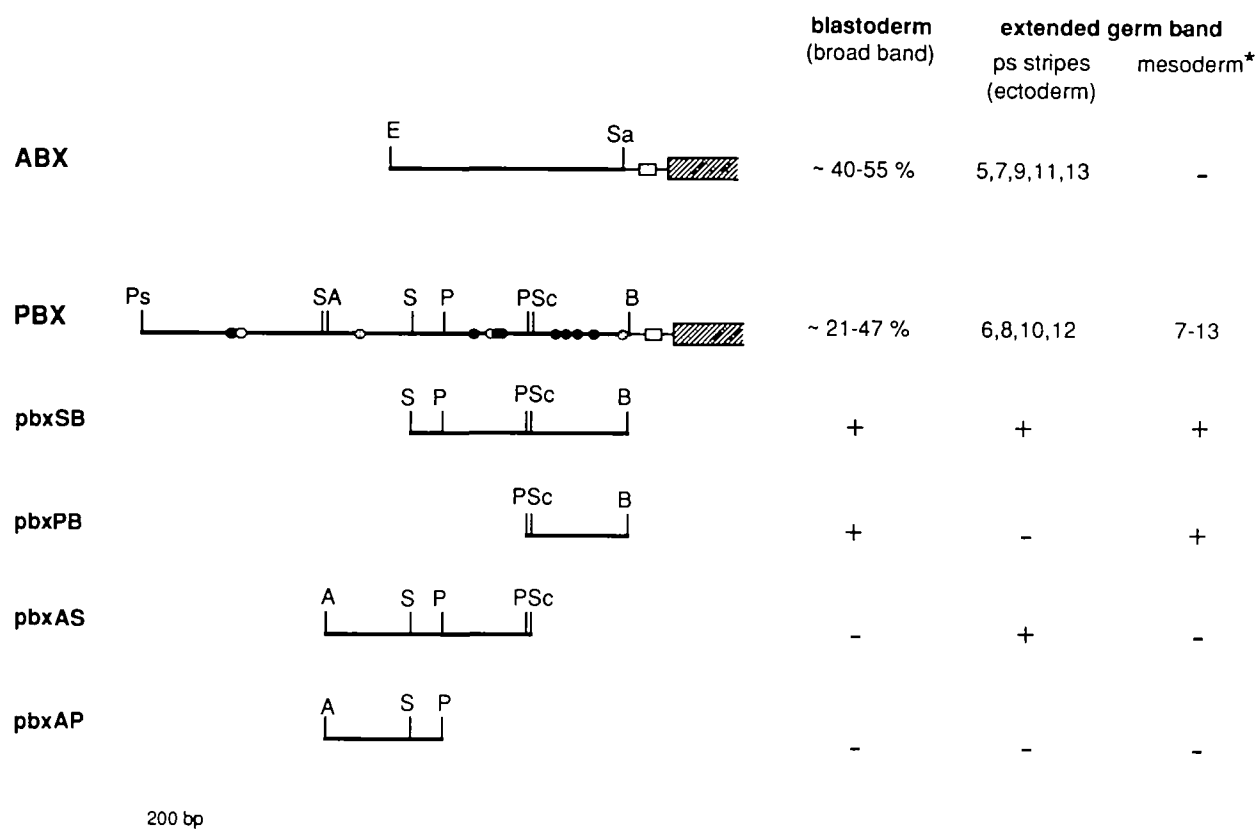
hybridised parallel sections of PBX and ABX transformants with radioactive RNA probes to detect  $\beta$ gal transcripts and to compare the distribution of these transcripts to that of endogenous *Ubx* transcripts. In both cases, we found broad bands of  $\beta$ gal RNA expression at the syncytial blastoderm stage (Fig. 2). The PBX RNA pattern is very strong and appears to precede *Ubx* expression; the ABX RNA pattern is weaker and becomes detectable at about the same time as *Ubx* RNA. The margins of the broad  $\beta$ gal bands are not sharp, and their positions are therefore difficult to determine. Also, the posterior limits appear to shift somewhat towards posterior with time, due to progressively increasing accumulation of transcripts. Nevertheless, from measurements of several different blastoderm embryos, we estimate that  $\beta$ gal RNA is detectable between ~21 and 47% egg length in the case of PBX, and between ~40 and 55% egg length in the case of ABX (in each case  $\pm 2\%$ ; the posterior pole corresponds to 0% egg length). Soon after, and before the onset of gastrulation, the broad bands of  $\beta$ gal RNA expression appear to resolve into stripes and thus undergo a pair-rule modulation. At the same time, the anterior margins of  $\beta$ gal expression begin to sharpen. The stripe patterns evidently correspond to the  $\beta$ gal staining patterns as described (Müller and Bienz, 1991). The PBX RNA pattern resembles the distribution of mature *Ubx* transcripts in the early embryo: the latter extends between ~20 and 50% egg length and shows a similar pair-rule modulation (Akam and Martinez-Arias, 1985).

We chose the PBX control region for further analysis because of its resemblance to *Ubx* RNA expression. Also, PBX-mediated expression is strong and can be visualised at the blastoderm stage by  $\beta$ gal staining, if constructs are used that contain an hsp70 RNA leader instead of the *Ubx* RNA leader (there appear to be sequences in the *Ubx* RNA leader that delay early translation; S.Hoppler and M.B., unpublished).

### Dissection of the PBX control region

We made four constructs containing different PBX subfragments linked to the hsp70 promoter and the  $\beta$ gal gene (Fig. 1) in order to locate and dissect *cis*-regulatory sequences required for the early PBX pattern. Several transformant lines were obtained in each case, and their patterns were analysed by  $\beta$ gal antibody staining.

We found that transformants containing the subfragment pbxSB (0.6 kb) show a  $\beta$ gal expression pattern similar to the PBX pattern as described (Müller and Bienz, 1991). A characteristic feature of this pattern are the four stripes in ps6, 8, 10 and 12 which are recognisable in pbxSB transformant embryos at the extended germ band stage (Fig. 3B), apparently with some  $\beta$ gal staining extending into the anteriormost regions of adjacent odd-numbered parasegments (ps5, 7, 9, 11 and 13). We also observe a somewhat lower level of continuous  $\beta$ gal staining between the stripes in the epidermis as well as throughout ps6–13 in the mesoderm. We believe this continuous staining to be a



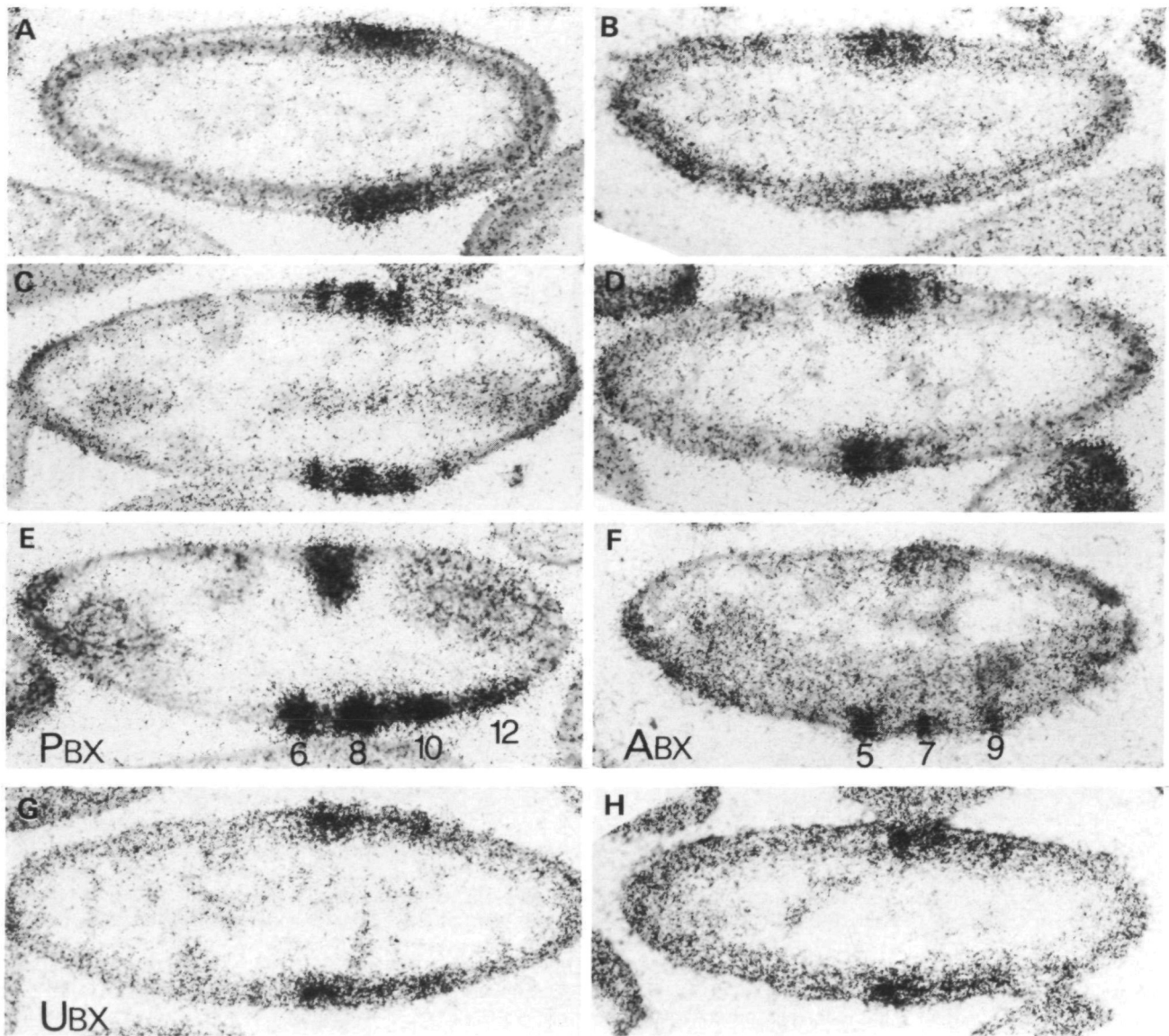
**Fig. 1.**  $\beta$ gal patterns conferred by the ABX and PBX control regions. Top two lines, maps of plasmids containing the minimal ABX or PBX control region (Müller and Bienz, 1991) linked to HZ50PL (Hiromi and Gehring, 1987; white square, hsp70 TATA-box; N-terminal end of  $\beta$ gal gene stippled); underneath, maps of 4 different PBX subfragments (A, *ApaL1*; B, *BamH1*; E, *EcoR1*; P, *Pvu2*; Ps, *Pst1*; S, *Ssp1*; Sa, *Sal1*; Sc, *Sac2*; all sites indicated, except for those within the HZ50PL cloning cassette). Short sequence stretches protected by *hb* (black dots), *Kr* (dotted circles) or *kni* protein (open circles) within the minimal PBX fragment are marked. At the right-hand side,  $\beta$ gal staining patterns conferred by the various constructs. Both control regions mediate a band of expression at the blastoderm stage with limits along the anteroposterior axis, as indicated in % egg length (% refer to RNA expression), and a similarly limited stripe pattern at the extended germ band stage, predominantly in odd-numbered ps (ABX) or even-numbered ps (PBX). Weak continuous  $\beta$ gal staining within ps7-13 of the mesoderm is observed in PBX transformants. \*, mesoderm expression always correlates with weak continuous  $\beta$ gal staining in the epidermis (probably reflecting early blastoderm expression) as well as some staining in odd-numbered ps (see text).

remnant of the early  $\beta$ gal expression (see below). The stripe pattern is preceded by a broad band of  $\beta$ gal expression, first visible at the mid-blastoderm stage (Fig. 3A), with somewhat fuzzy boundaries. The limits of this broad  $\beta$ gal band appear to be the same as the limits of early  $\beta$ gal PBX-mediated RNA expression (Fig. 2; the  $\beta$ gal protein band is widened posteriorly compared to the  $\beta$ gal RNA band, probably reflecting a higher sensitivity of protein compared to RNA detection). Double-labelling with  $\beta$ gal as well as *even-skipped* (*eve*) antibody (Frasch *et al.* 1987) confirms that the anterior boundary of the broad  $\beta$ gal band is somewhere within ps6 ( $\beta$ gal staining is clearly detectable in *eve* stripe 4, or ps7, but undetectable in *eve* stripe 3, or ps5; Fig. 5A). Posterior  $\beta$ gal staining is approximately co-extensive with *eve* staining in ps13.

The shorter fragment pbxPB (0.3 kb) confers  $\beta$ gal expression in a broad band similar to the one in pbxSB transformants, although  $\beta$ gal staining is strong only at the ventral side (the presumptive mesoderm), but

weaker at the dorsal side (the presumptive epidermis; Fig. 3C). Accordingly, we observe weak continuous  $\beta$ gal staining in the epidermis at later stages, but quite strong continuous staining in the mesoderm (Fig. 3D). The margins of the continuous  $\beta$ gal staining have not sharpened; they are within ps6 and within ps13 in the epidermis. Mesodermal staining extends through ps7-13. Based on the anteroposterior extent of the continuous  $\beta$ gal staining and its relative intensities in the two germ layers as well as within their primordia, we believe that this staining reflects  $\beta$ gal expression from the preceding blastoderm stage. As in pbxSB transformants, we observe some  $\beta$ gal staining in odd-numbered ps of pbxPB transformants; however, the strong  $\beta$ gal staining blocks in even-numbered ps are completely missing.

In contrast, transformants of the near-complementary fragment pbxAS (0.6 kb) show exclusively  $\beta$ gal stripes in ps 6, 8, 10 and 12 in the epidermis of extended germ band embryos (Fig. 3F). This pattern is reminis-



**Fig. 2.** Early  $\beta$ gal transcript patterns in ABX and PBX transformants. Sections of embryos at blastoderm stage (A, B) or after onset of gastrulation (C, D; G, H, parallel sections of C, D) were hybridised with radioactively labelled  $\beta$ gal (A–F) or *Ubx* probes (G, H). Both PBX (A, C, E) and ABX transformants (B, D, F) show a broad band of  $\beta$ gal expression (A, B), resolving later into a stripe pattern (C–F; numbers indicate ps). Expression boundaries sharpen after onset of gastrulation, but remain similarly positioned throughout these stages. Anterior to the left.

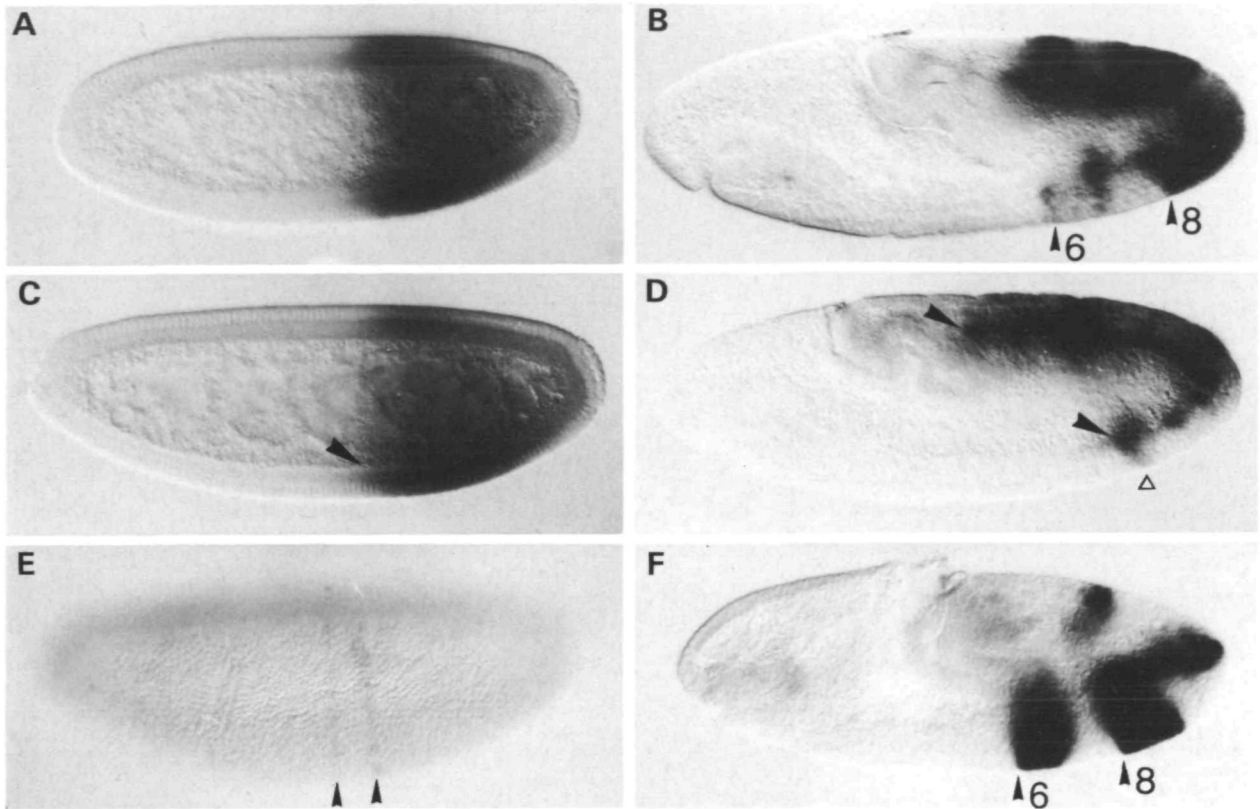
cent of the pattern generated by *fushi tarazu* (*ftz*)– $\beta$ gal fusion genes (Hiromi *et al.* 1985; Lawrence *et al.* 1987), suggesting that it may be a consequence of *ftz* activity. There is no other  $\beta$ gal staining in the ectoderm or in the mesoderm of pbxAS transformants. The *ftz*-like stripe pattern is first detectable after gastrulation (Fig. 3E); there is no  $\beta$ gal expression prior to this stage.

Finally, transformants of the subfragment pbxAP (0.3 kb) do not show any  $\beta$ gal expression, except for some staining of the midline at later embryonic stages (not shown). We conclude that we can separate *cis*-regulatory elements conferring the early band of  $\beta$ gal expression and the subsequent *ftz*-like stripe pattern. Although these two patterns are completely different,

their limits along the anteroposterior axis are nearly the same.

#### *The PBX pattern in mutant embryos*

We previously found that the PBX and ABX control regions contain target sites for repressors mediating *Ubx* expression boundaries at advanced embryonic stages (Müller and Bienz, 1991). We wondered whether the limits of the PBX pattern are due to repressors already at the onset of expression. The *hb* gene product, required for the anterior *Ubx* expression boundary (White and Lehmann, 1986; Irish *et al.* 1989a), is a good candidate for such a repressor. The PBX expression pattern is flanked by two zygotic *hb*



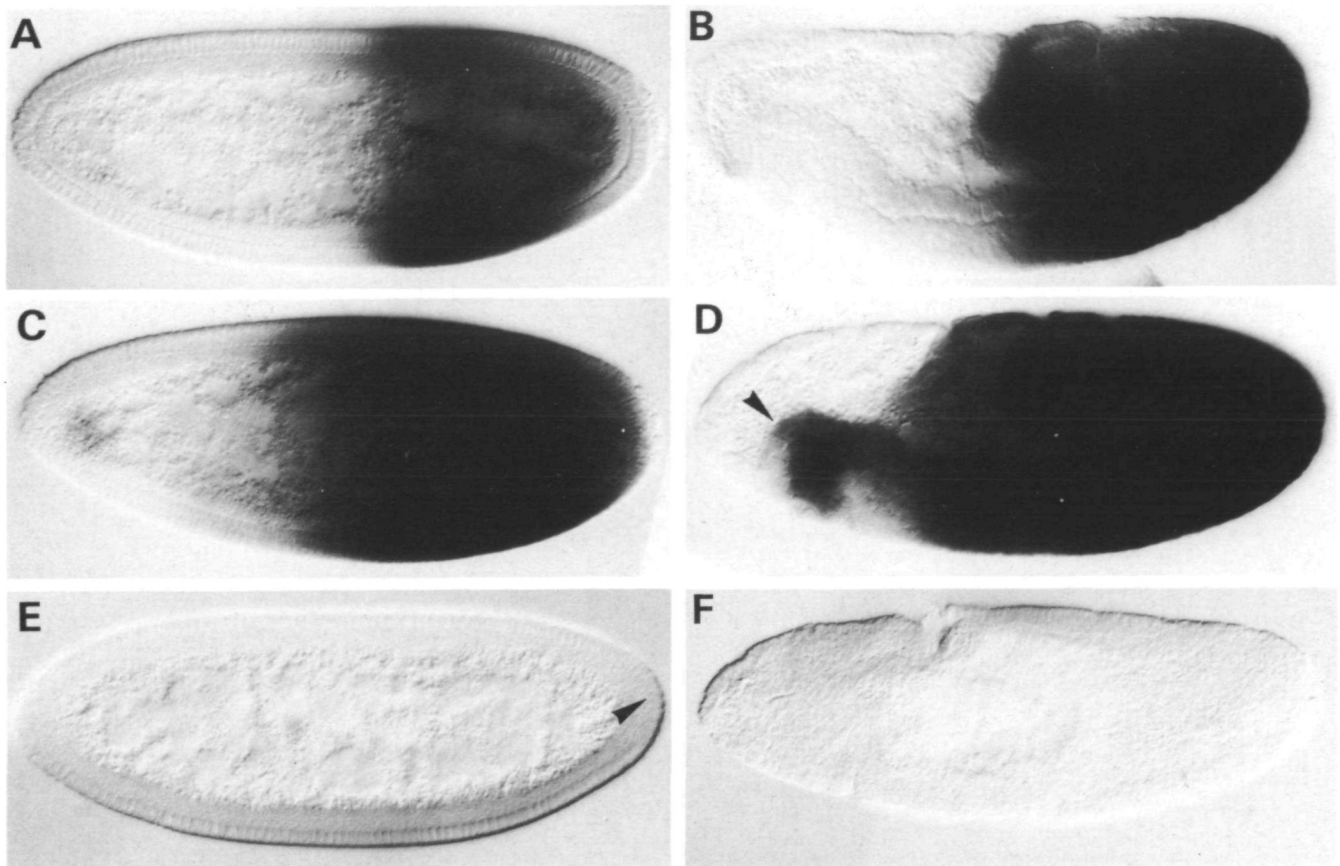
**Fig. 3.**  $\beta$ gal patterns conferred by different PBX subfragments. Embryos from a pbxSB (A, B), a pbxPB (C, D) or a pbxAS (E, F) transformant line, stained with  $\beta$ gal antibody. The early band of  $\beta$ gal expression is visible at the mid-blastula stage of pbxSB (A) and pbxPB (C), but not in pbxAS transformants;  $\beta$ gal staining in the latter starts to be detectable after gastrulation (E; arrowheads indicating appearing ps6 and ps8 stripe). The later *ftz*-like stripe pattern (anterior limits of ps6 and ps8 indicated by arrowhead) is visible in embryos of the extended germ band stage, most clearly in pbxAS transformants (F), but also in pbxSB transformants (B; continuous  $\beta$ gal staining in the epidermis partly obscures the stripe pattern; also, the stripe in ps6 is always very weak in transformants of this construct). The  $\beta$ gal stripes in even-numbered ps are missing in pbxPB germ band embryos (D).  $\beta$ gal staining in these is strongest in the mesoderm and its primordium (arrowheads in D and C, respectively). PBX transformants with mesoderm expression (pbxSB and pbxPB) also show some striping in odd-numbered ps (marked by triangle in D). Anterior to the left.

expression domains (Tautz *et al.* 1987; Tautz, 1988): *hb* protein forms an anteroposterior gradient occupying the anterior half of the embryo, but is also found in a stripe near the posterior pole (located between ~10 and 20 % egg length). We therefore asked whether the PBX pattern is altered in mutant *hb* embryos.

We crossed a pbxSB transformant line into a strain carrying a loss-of-function *hb* mutation (Lehmann and Nüsslein-Volhard, 1987) and found that a quarter of the offspring embryos (the putative *hb*-homozygotes) show an early  $\beta$ gal pattern with strikingly blurred anterior expression boundaries (Fig. 4). Measurements of 11 and 9 embryos, presumed to be wild type or *hb*<sup>-</sup>, show that the anterior  $\beta$ gal expression limit is located on average between 45 and 48 % egg length (wild type) or between 54 and 58 % egg length (*hb*<sup>-</sup>). The most anterior position at which  $\beta$ gal staining becomes maximal is also different in the two classes of embryos (39 % versus 45 % egg length). The posterior expression limit, harder to determine because of the shape of the embryo, is located approximately at 11 % (wild type) or 6 % (*hb*<sup>-</sup>) egg length; in the same sample of embryos,

maximal  $\beta$ gal staining is observed at ~23 % (wild type) or at ~14 % (*hb*<sup>-</sup>) from the posterior pole. By the beginning of germ band extension, we observe extensive ectopic  $\beta$ gal staining in the putative *hb*<sup>-</sup> embryos outside of the normal PBX expression domain, most noticeable in the mesoderm where staining extends into the head region (Fig. 4D). Double-labelling with  $\beta$ gal and *eve* antibody confirms that the embryos with widened  $\beta$ gal bands are the ones lacking zygotic *hb* function (Fig. 5C). We conclude that both limits of the PBX pattern, as early as this pattern becomes detectable, are determined by the activity of a repressor, and that this repressor is identical with or dependent on the *hb* protein.

A prediction from this is that the PBX-mediated expression might be suppressible by moderately high levels of uniformly distributed *hb* protein in the early blastoderm embryo. This situation is created in fertilised eggs laid by homozygous mutant *osk* mothers (Tautz, 1988). We crossed male pbxSB transformants to homozygous *osk* females and stained the offspring embryos with  $\beta$ gal antibody. As expected, every



**Fig. 4.**  $\beta$ gal staining patterns in  $hb^-$  and  $osk^-$  embryos. pbxSB transformants were crossed into a  $hb^{7M}$  strain or mated to homozygous  $osk^{346}$  females, and offspring embryos were stained with  $\beta$ gal antibody. The band of  $\beta$ gal expression at the mid-blastoderm stage is widened in  $hb^-$  (C), compared to wild-type embryos (A). Ectopic  $\beta$ gal expression is very prominent in  $hb^-$  embryos after onset of germ band extension (D compared to B, wild type), extending into the head in the mesoderm (arrowhead in D).  $osk^-$  embryos, recognisable by their lack of pole cells (arrowhead in E), do not show any  $\beta$ gal expression neither at early (E) nor at advanced stages in (F). Anterior to the left.

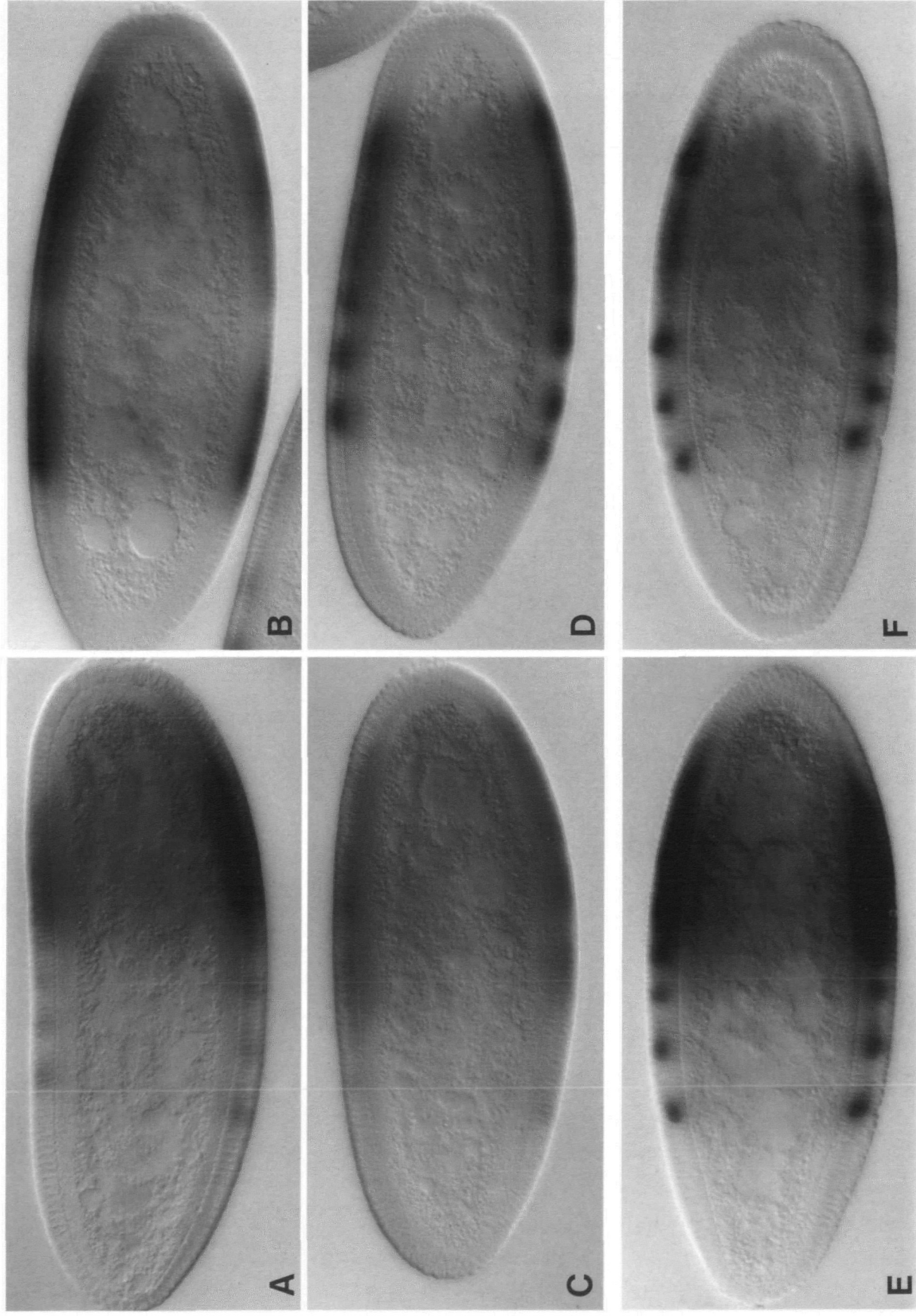
embryo from this cross was mutant, as judged by the lack of pole cells (Lehmann and Nüsslein-Volhard, 1986). We could not detect any  $\beta$ gal protein whatsoever in these embryos, not even at advanced stages (Fig. 4E and F). Thus, the PBX pattern is completely suppressed in embryos in which  $hb$  protein is uniformly distributed throughout. While this lack of the PBX pattern could reflect a failure of PBX-mediated activation (due to absence of an activator, e.g. *knirps* product, as a consequence of the *osk* mutation; Struhl, 1989; Hülkamp *et al.* 1989; Irish *et al.* 1989b), we think it more likely that it reflects  $hb$  protein acting as a repressor on the PBX control region (see below).

We also monitored the PBX pattern in embryos homozygous for the gap mutations *Krüppel* (*Kr*) or *knirps* (*kni*). Double-labelling with  $\beta$ gal and *eve* antibody served to identify the homozygous mutants as well as to stage the embryos (Frasch *et al.* 1987; Frasch and Levine, 1987). Among the blastoderm embryos which show the first unambiguous signs of an altered *eve* stripe pattern, due to lack of *Kr* or *kni* function, we found several with a normal  $\beta$ gal staining band (Fig. 5B, D). In both types of mutants, this band of  $\beta$ gal

staining remains normal throughout the early stages. Alterations of the  $\beta$ gal pattern do not become apparent until after the onset of germ band extension (not shown); however, these are restricted to those regions of the embryo in which parasegment primordia are disrupted by *Kr* or *kni* mutation (Carroll and Scott, 1986; Frasch and Levine, 1987). These alterations are likely to reflect indirect consequences of these mutations. Therefore, there is no evidence that *Kr* or *kni* function is required directly for the PBX pattern.

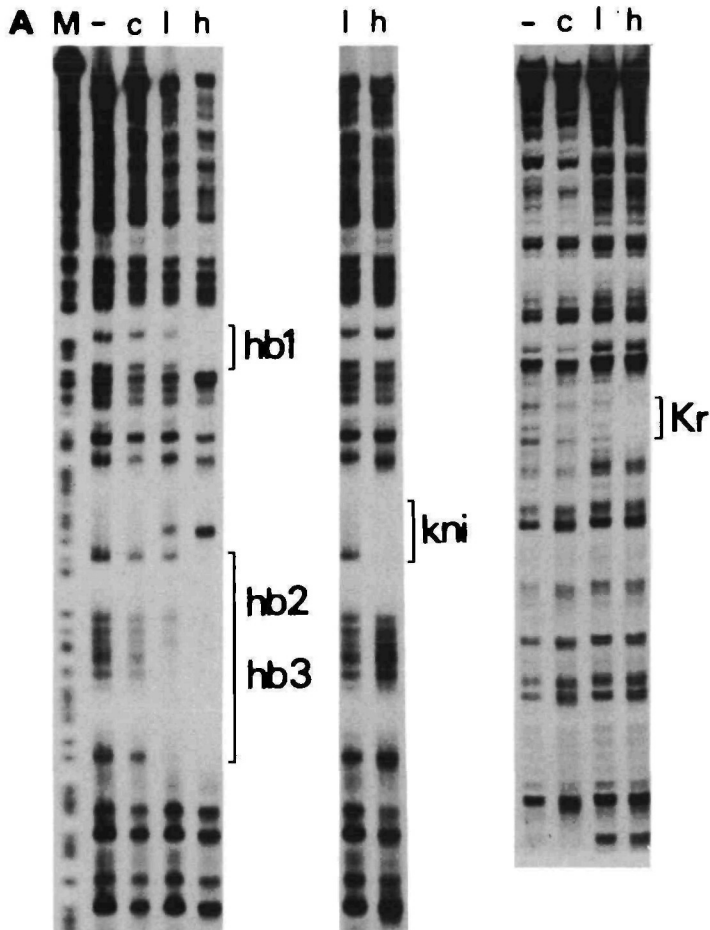
#### *Gap protein footprints in the PBX control region*

We asked whether any of the gap proteins might bind directly to sequences within the PBX control region. We tested this by footprint analysis, using protein overproduced in *E. coli*. We screened both DNA strands of the PBX fragment (Fig. 1) for putative gap protein binding sites. We found 8 short sequence stretches protected by  $hb$  protein as well as 2 sequence stretches each protected by *Kr* or *kni* protein (Fig. 6; the *Kr* footprint shown in Fig. 6A is weak compared to the one found in the pbxAS subfragment; see Fig. 1). The  $hb$  and *Kr* footprint regions each contain a



**Fig. 5.**  $\beta$ gal and *eve* patterns in wild-type and mutant embryos. Wild-type (A, E) and mutant embryos (B, *Kr*<sup>-</sup>; C, *hb*<sup>-</sup>; D, *F*, *kni*<sup>-</sup>) of the mid-blastoderm stage (A–D) or after the onset of gastrulation (E, F) were double-stained with  $\beta$ gal (brown) and *eve* (grey) antibody. The anterior boundary of  $\beta$ gal expression is located within *ps6* in the wild type (between *eve* stripe 3 and 4; note the sharpening of the boundary after onset of gastrulation: E compared to A), but blurred in *hb*<sup>-</sup> homozygotes (C; *eve* stripes were only weakly developed in

order not to obscure the extended  $\beta$ gal staining band). A normal  $\beta$ gal staining band is visible in *Kr*<sup>-</sup> (B) and *kni*<sup>-</sup> homozygotes (D). Embryos were chosen as young as possible to detect early consequences of *Kr* and *kni* mutations (cf. Irish *et al.* 1989a), hence the relatively weak  $\beta$ gal staining. Note that, in both cases, the *eve* stripes in the  $\beta$ gal staining domain are severely affected by the mutations (e.g. anterior boundary of  $\beta$ gal staining in F appears normal with respect to *eve* stripe 3, although *eve* stripe 4 is missing in *kni*<sup>-</sup>). Anterior to the left.



**Fig. 6.** Gap protein footprints in the PBX control region. (A) The three *hb* footprints in the central Pvu2 fragment (Fig. 1) as well as the *Kr* and the *kni* footprint in the pbxSB fragment (-, no protein; c, control extract; l, h, low and high concentration of protein extract); protected residues are bracketed. (B) Sequence stretches protected by the gap proteins *hb*, *Kr* or *kni* (boxed) are indicated in the pbxSB sequence. (C) Sequences protected by *kni* protein in the pbxSB fragment (asterisk) and elsewhere in the PBX control region (downstream adjacent to the pbxSB fragment). A consensus sequence is derived (bottom line).

consensus sequence as previously determined (Stanojevic *et al.* 1989; Treisman and Desplan, 1989; Pankratz *et al.* 1989). The regions protected by *kni* protein show sequence similarities with 6 additional *kni* footprint regions found elsewhere in the PBX control region (downstream of the minimal PBX fragment; Fig. 6C). From these, we deduce a *kni* binding consensus sequence T/A AATGG A/G A/C C.

### Discussion

The early band of PBX-mediated expression is positioned between the two *hb* protein expression domains (Tautz *et al.* 1987; Tautz, 1988). Its widening anteriorly as well as posteriorly due to absence of zygotic *hb* function led to our main conclusion that *hb* protein acts as a repressor to position the PBX expression boundaries. The widened PBX pattern is still limited along the anteroposterior axis; however, the altered boundaries are most probably due to the maternal *hb* protein in the anterior region (Tautz, 1988; see also Struhl, 1989; Hülkamp *et al.* 1989; Irish *et al.* 1989b) and perhaps to *tailless* protein in the posterior region of the embryo (Reinitz and Levine, 1990). Our conclusion is reinforced by the observation that complete suppression of the PBX pattern is observed in a situation where *hb* protein is distributed uniformly throughout the embryo. A similar boundary-determining activity of *hb* protein with respect to the expression domains of other gap genes has been demonstrated previously (Hülkamp *et al.* 1990).

We found a cluster of 7 *hb* binding sites within a small fragment of the PBX control region that confers a PBX-like expression pattern in the blastoderm embryo. These binding sites are evenly distributed between two hardly overlapping subfragments (3 on the pbxAS, 4 on the pbxPB subfragment) either of which confers a  $\beta$ gal expression pattern with essentially the same boundaries, although the patterns themselves are completely different from each other. This, together with the fact that the boundaries in both cases depend on *hb* function, suggests strongly that *hb* protein exerts its boundary-determining function by binding as a repressor directly to the PBX control region. Recently, an expression pattern conferred by an intronic *Ubx* control region was described which resembles the PBX pattern and whose anterior boundary is determined directly by *hb* protein (Qian *et al.* 1991).

The PBX pattern resembles *Ubx* expression in early

embryos with respect to its expression boundaries and its pair-rule modulation (Akam and Martinez-Arias, 1985). As in the case of PBX-mediated expression, *Ubx* expression limits are dependent on zygotic *hb* function (White and Lehmann, 1986; Irish *et al.* 1989a), and *Ubx* expression is prevented in embryos containing uniformly distributed maternal *hb* protein (Irish *et al.* 1989a). Because of these parallels between PBX-mediated expression and early *Ubx* expression, it is very likely that the same molecules and mechanisms are responsible for both expression patterns.

Early PBX-mediated expression is not affected by the absence of *Kr* function, nor is the pattern reported by Qian *et al.* (1991), although initial *Ubx* expression at the blastoderm stage becomes undetectable under these conditions (Irish *et al.* 1989a). As expected from our result, the one strong *Kr* binding site that we found in the PBX control region is located within the pbxAS subfragment which is not capable of mediating early blastoderm expression. Early PBX-mediated expression must therefore be conferred by an activator other than *Kr*, perhaps by a general activator which is uniformly distributed in the embryo. In any case, initial activation at the blastoderm stage appears irrelevant for the generation of expression boundaries: the subfragment pbxAS is capable of conferring PBX expression boundaries without mediating expression prior to gastrulation (Fig. 1). Similarly, boundaries of *Ubx* expression can be generated in *Kr* mutant embryos although they lack initial *Ubx* expression (Irish *et al.* 1989a). Clearly, the two processes are separable and independent of each other. Whereas boundaries of *Ubx* expression are essential for the developing embryo (Gonzales-Reyes *et al.* 1990; Mann and Hogness, 1990), it is unclear whether the initial *Ubx* expression at the blastoderm stage is functionally significant.

We previously found that a mechanism of long-range repression mediated by the PBX control region acts to confer PBX expression boundaries at advanced stages, and that this long-range repression is dependent on *Polycomb* function (Müller and Bienz, 1991). We now find that the PBX expression boundaries themselves are generated by a repression mechanism. This however, does not require *Polycomb* function (Müller and Bienz, 1991) and may not act at a distance as we have not been able to separate PBX-mediated repression from PBX-mediated activation. It thus appears that PBX expression boundaries, and probably also *Ubx* expression boundaries, are generated in a two-step process involving two types of repression mechanisms. All current evidence suggests that *hb* protein is the primary repressor responsible for the first step of repression. Interestingly, this initial repression can be maintained throughout embryogenesis, as suggested by our result with *osk* mutant embryos, far beyond the developmental stage at which *hb* protein becomes undetectable (Tautz, 1988). It is possible that the second step of repression (Müller and Bienz, 1991) is dependent on the first one and thus on the same primary repressor. *hb* protein may therefore assume a pivotal role in the generation of stable and heritable *Ubx* expression boundaries.

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