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The *bx* region enhancer, a distant *cis*-control element of the *Drosophila Ubx* gene and its regulation by *hunchback* and other segmentation genes

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The *Drosophila* homeotic gene *Ultrabithorax (Ubx)* is regulated by complex mechanisms that specify the spatial domain, the timing and the activity of the gene in individual tissues and in individual cells. In early embryonic development, *Ubx* expression is controlled by segmentation genes turned on earlier in the developmental hierarchy. Correct *Ubx* expression depends on multiple regulatory sequences located outside the basal promoter. Here we report that a 500 bp DNA fragment from the *bx* region of the *Ubx* unit, ~30 kb away from the promoter, contains one of the distant regulatory elements (*bx* region enhancer, BRE). During early embryogenesis, this enhancer element activates the *Ubx* promoter in parasegments (PS) 6, 8, 10 and 12 and represses it in the anterior half of the embryo. The repressor of the anterior *Ubx* expression is the gap gene *hunchback* (*hb*). We show that the *hb* protein binds to the BRE element and that such binding is essential for hb repression *in vivo*. *hb* protein also binds to DNA fragments from *abx* and *bxd*, two other regulatory regions of the *Ubx* gene. We conclude that *hb* represses *Ubx* expression directly by binding to BRE and probably other *Ubx* regulatory elements. In addition, the BRE pattern requires input from other segmentation genes, among them *tailless* and *fushi tarazu* but not *Krüppel* and *knirps*.

Key words: distant enhancer/gap gene/homeotic gene/parasegmental expression

Introduction

Drosophila early development depends on the sequential activation of a hierarchy of zygotic genes. The specific distribution of maternal determinants in the egg activates a set of early zygotic segmentation genes, the gap genes, in distinct but partially overlapping domains. Combinations of these gene products in turn provide spatial cues for the activation of the next tier of genes in specific patterns that lead to the subdivision of the embryo into metameric units (reviewed by Ingham, 1988). The identities of individual segments are subsequently assigned by the homeotic genes. One of the major homeotic genes is *Ultrabithorax (Ubx)*, a member of the bithorax complex (BX-C) that controls the posterior part of the body (Lewis, 1978; Sanchez-Herrero *et al.*, 1985). The role of the *Ubx* gene varies in different tissues: in the epidermis it specifies primarily parasegments (PS) 5 and 6. In the mesoderm it is required in most of the abdominal segments, in the region from PS6 to PS13 (Hooper, 1986) but the visceral mesoderm only requires *Ubx* in PS7 (Bienz and Tremml, 1988). A major role of *Ubx* is in the central nervous system where it is expressed in PS5-PS13 but, as in the epidermis, it is needed principally in PS5 and 6 (Teugels and Ghysen, 1985).

During embryogenesis, the *Ubx* expression pattern is initiated at the blastoderm stage as a band of weak transcription in the region of the egg between 25 and 50% egg length (EL), measured from the posterior tip. During germ band extension, expression becomes stronger and evolves into a transient pair-rule pattern which subsequently resolves into a complex parasegmental pattern spanning PS5-13, with the highest level of expression in PS6. After germ band retraction, expression of *Ubx* is found predominantly in the ventral nervous system (Akam and Martinez-Arias, 1985; White and Wilcox, 1985a; Beachy *et al.*, 1985; White and Lehmann, 1986). Genetic interactions indicate that a number of early-acting genes, such as the gap gene *hunchback (hb)*, the pair-rule gene *fushi tarazu (ftz)* and the terminal gene *tailless (tll)*, are involved in the correct activation of *Ubx* expression (White and Lehmann, 1986; Ingham and Martinez-Arias, 1986; Irish *et al.*, 1989; Reinitz and Levine, 1990). A *Ubx* promoter fragment containing only 3.1 kb upstream sequence (*Ubx* basal promoter) directs expression in a pattern very different from that of the endogenous *Ubx* gene (Bienz *et al.*, 1988). Therefore, the correct regulation of *Ubx* expression by early segmentation genes requires sequences located outside the basal promoter.

The sequences essential for proper *Ubx* expression are distributed over more than 100 kb and include regulatory regions that dictate *Ubx* function in specific parts of the embryo, specific tissues or groups of cells. *abx, bx, bxd* and *pbx* are four such regulatory regions identified by genetic analysis (Lewis, 1978). Mutations in each of these cause homeotic tranformations of discrete subsets of cells without affecting the identities of other parts of *Ubx*-controlled segments (reviewed by Duncan, 1987). *bxd* and *pbx* mutations have been mapped upstream of the *Ubx* promoter and transform PS6 towards PS5, while *abx* and *bx* mutations reside within a large intron 25-50 kb from the promoter and cause PS5 → PS4 tranformations in the adult cuticle (Figure 1). The specific defects caused by *abx, bx, bxd* and *pbx* mutations have been correlated with decreased amounts of the *Ubx* protein in corresponding groups of cells in embryos or imaginal discs (White and Wilcox, 1985b; Cabrera *et al.*, 1985). These observations suggest that each regulatory region might encode tissue-specific enhancers that specify *Ubx* expression in a subset of cells. The final *Ubx* expression pattern would then result from the cumulative and/or combinatorial effect of these enhancers. If the enhancer-like activity of such regulatory regions could be demonstrated, important questions to be explored are precisely what aspects of *Ubx* expression each one governs,

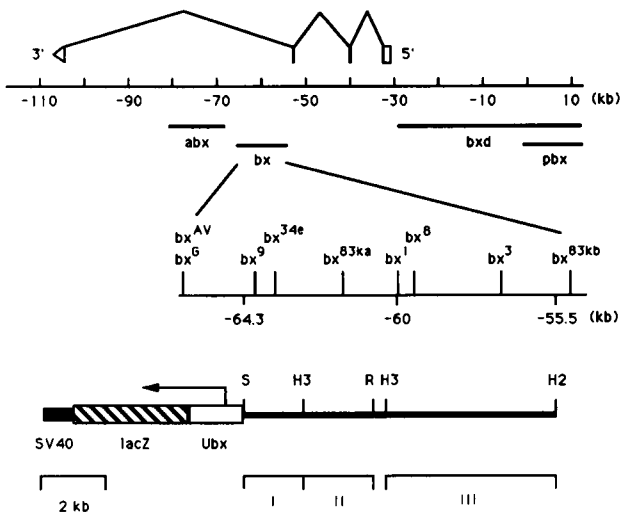

Fig.1. Localization of the *bx* region enhancer (BRE). The *Ubx* transcription unit is shown on top with bars indicating the positions of the *abx*, *bx* and part of the *bxd* and *pbx* regulatory regions. The scale, in kb, is numbered according to Bender *et al.* (1983). The *bx* region is shown enlarged below, with insertional *bx* mutations indicated. The *bx*–*Ubx*–*lacZ* fusion construct is at the bottom. The *bx* sequence is indicated by a solid bar, with some of the restriction sites used in the constructions (S:*Sal*I; H3: *Hin*dIII; R: *Eco*RI; H2: *Hin*cII). The open, hatched and shaded boxes represent the *Ubx* promoter, *lacZ* gene and SV40 T antigen polyadenylation region, respectively. The arrow indicates the direction of transcription. The *bx* DNA is an 8.8 kb *Sal*I–*Hin*cII fragment from −55.5 to −64.3 on the *Ubx* map, I, II, III are three *bx* sub-fragments individually tested. *bx*I gave an expression pattern identical to that of the entire 8.8 kb fragment while II and III produced either the basal pattern only (Figure 2f, Bienz *et al.*, 1988) or variable patterns attributable to position effects.

how they are able to influence the activity of the promoter at such great distances, how the regulation of each is achieved and how they interact with one another.

Some of these elements are able to control the *Ubx* promoter on a homologously paired chromosome by a transvection mechanism that requires the activity of the *zeste* gene product (Lewis, 1954; Kaufman *et al.*, 1973). Previous studies have identified a number of *zeste* binding sites within the regulatory regions of *Ubx* (Benson and Pirrotta, 1988), suggesting that regulation over a distance might be achieved by juxtaposing distant elements to the promoter through the action of the *zeste* protein. The presence of a strong *zeste* binding site in the *bx* region led us to study the enhancer activity of this regulatory element. In this paper, we show that the *bx* region contains an enhancer element (*bx* region enhancer or BRE) that directs promoter expression in a parasegmental pattern related to the early embryonic *Ubx* pattern. The BRE enhancer is regulated by the same set of segmentation genes that controls the expression of *Ubx*, suggesting that it is one of the distant enhancers that regulate the expression of the *Ubx* gene. In particular, we show that direct repression of this enhancer, and possibly of enhancers in the other *Ubx* regulatory regions, by the gap gene *hb* is responsible for establishing the correct anterior boundary of the *Ubx* expression domain.

Results

The bx region contains a strong embryonic enhancer

The *bx* region is defined by the sites of a number of *bx* mutations that exhibit a common phenotype with differing severity. Most of these mutations are caused by the insertion of transposable elements, most frequently the *gypsy* element, and have been mapped within a 10 kb interval between positions −55 and −65 in the 2Ubx map (Peifer and Bender, 1986). This region contains also a strong *zeste* binding site identified by immunoprecipitation (Benson and Pirrotta, 1988). To identify potential enhancers in the *bx* regulatory region, an 8.8 kb fragment including almost the entire *bx* region was placed in either orientation upstream of a *Ubx*–*lacZ* fusion gene driven by the *Ubx* basal promoter (Figure 1) and the constructs were introduced into the fly genome by P element-mediated germ line transformation (Rubin and Spradling, 1982). In transgenic embryos, the *bx* DNA directed a strong, dynamic expression from the *Ubx* basal promoter, independent of its orientation. The control construct, containing the *Ubx* promoter only, gave a very weak basal pattern similar to that described by Bienz *et al.* (1988). *Ubx* protein is not detectable in normal embryos until four hours after fertilization (White and Lehmann, 1986), probably due to the length of the *Ubx* gene, which requires more than one hour for complete transcription (Kornfeld *et al.*, 1989). The reporter protein β-galactosidase, in contrast, can be detected one hour earlier by antibody staining, probably because the *LacZ* gene is much smaller than the *Ubx* gene and is more rapidly transcribed.

Antibody staining reveals that β-galactosidase is first expressed in a region of the embryo spanning 25–50% EL at the cellular blastoderm stage. At the onset of gastrulation, this domain resolves into three stripes of stronger expression, with a fourth stripe, weaker and about half as wide as the other three, appearing during germ band extension. The four stripes are laid out in pair-rule fashion, corresponding with PS6, 8, 10 and 12. Double staining with anti-β-galactosidase and anti-*engrailed* (*en*) antibodies (DiNardo *et al.*, 1985) shows that the first three stripes are wider than the actual parasegments (Figure 2), consisting of the even parasegment plus the anterior part of the odd parasegment (defined by the *engrailed* stripe). Expression in the ectoderm is strongest in the anterior part of the parasegment but in the mesoderm it appears uniform across each stripe. During germ band extension, the anterior boundary of the pattern coincides with *engrailed* stripe 6 (beginning therefore with PS6). However, during germ band retraction, weak expression appears in the posterior part of PS5, corresponding to the anterior T3 segment which is typically affected by *bx* mutations (Figure 2e). After germ band shortening, the stain appears to be localized predominantly in the lateral ectoderm and is much weaker in the ventral neurogenic region or in the mesoderm.

To determine whether the expression of this enhancer depends on the function of the endogenous *Ubx* gene, we crossed the enhancer–*lacZ* transposon into flies carrying *Ubx* null mutations. Embryos lacking functional *Ubx* product [$Df(3)bxd^{100}$ or Ubx^{1} embryos, data not shown] produced a normal pattern of *lacZ* expression, in agreement with the observation of Bienz and Tremml (1988) that the expression of *Ubx* in the visceral mesoderm, but not that in the ectoderm, depends on autocatalytic activation by the endogenous *Ubx* protein. Overall, the expression directed by the *bx* DNA during embryogenesis may be viewed as a part of the total *Ubx* expression pattern. It is very similar to the initial *Ubx* pattern up to the germ band extension stage. Afterwards, however, it does not continue to resolve from the pair-rule to the parasegmental pattern and remains conspicuously absent from the nervous system, the major

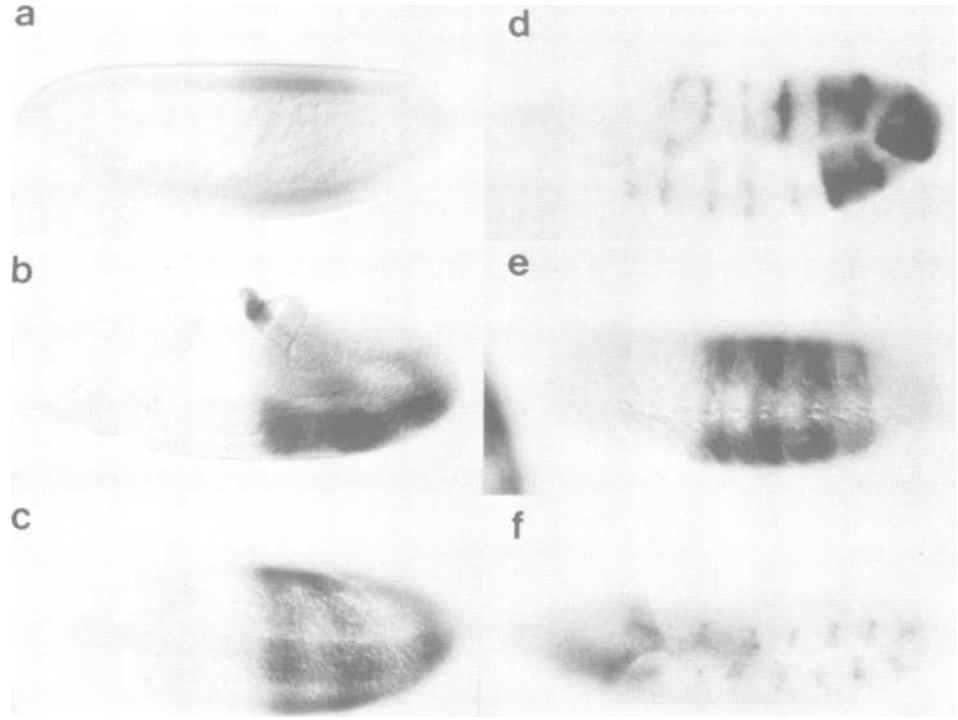

Fig. 2. Antibody detection of BRE-directed *Ubx−lacZ* gene expression during embryogenesis. Whole mount embryos were either stained with anti-β-galactosidase antibody (a, b, c, e, f), or double-stained with anti-β-galactosidase and anti-*en* antibodies (d). Optical sections of the stained embryos are shown with anterior end to the left and dorsal side up. (**a**) At the cellular blastoderm stage, the earliest stage, β-galactosidase is evenly distributed from 25 to 50% EL (0% is the posterior tip). (**b**) As gastrulation starts, the blastoderm domain is resolved into three stripes, with a fourth one appearing shortly afterwards. Staining persists in the dorsal region and later gives rise to a pattern of staining cells in the amino-serosa corresponding to *Ubx*-expressing cells in these tissues. (**c**) Ventral surface view of the stain pattern at early germ band elongation. Note that the ventral mesoderm displays the same staining pattern as the lateral ectoderm at this stage. (**d**) Typical pair-rule pattern at germ band elongation (stage 10, following Campos-Ortega and Hartenstein, 1985). The *en* stripes position the four β-galactosidase bands to PS6, 8, 10 and 12. (**e**) Stage 13 embryo: ventral lateral view after germ band retraction. The ventral neurogenic region stains more weakly than the lateral ectoderm. Note that weaker expression is now found in the posterior part of PS5. Later, at the time of head involution and dorsal closure, the pattern starts to break down and expression is found in all thoracic and head segments and in the posterior terminal region. (**f**) Basal pattern expressed by the *Ubx−lacZ* gene without *bx* enhancer at the end of the germ band elongation (stage 11). This weak basal pattern, very similar to that described by Bienz *et al.* (1988), is detected using more concentrated antibody and longer staining. Note that in the *bx*-directed pattern shown in (a)−(e), the ectopic anterior expression from the basal promoter is suppressed. The results shown in this figure were from *bx−Ubx−lacZ* transformant line 2; four independently transformed lines gave an identical pattern of expression.

tissue of *Ubx* expression in the late embryo. These results indicate that other elements must be responsible for *Ubx* expression in odd-numbered parasegments and in the ventral nerve cord. In the late embryo, the spatial restriction that initially confined expression to the region between PS6 and PS12 begins to break down and activity begins to be detected in more anterior parasegments and in the head region as well as in the posterior terminal region.

We conclude from these results that the *bx* region contains a spatially regulated enhancer element (BRE). This enhancer may be the element affected by the classical *bx* mutations and might provide an explanation for their adult phenotypes (Peifer and Bender, 1986). The domain of expression directed by the BRE element in the early embryo only covers PS6−12, while *bx* phenotypes typically transform PS5 to PS4; however, by the beginning of germ band retraction, expression extends to the posterior part of PS5. Most *bx* mutations are due to the insertion of transposable elements that are known to alter the regulation of nearby genes (see, for example, Geyer *et al.*, 1990). Since the phenotypic effect of *bx* mutations is not detectable during embryonic stages (White and Wilcox, 1985b), it is possible that these mutations affect *bx* function principally in the late embryo and imaginal discs. In fact, the 8.8 kb *bx* DNA stimulates β-galactosidase expression in the imaginal discs while the *Ubx* basal promoter does not. However, the expression is not restricted to discs derived from PS5 (the haltere and third leg discs) but is found also in the first leg, second leg, wing and eye-antenna discs. The imaginal disc expression therefore does not follow the parasegmental pattern of expression established in the early embryo and, in particular, it extends to more anterior compartments than those affected by the *bx* mutations. Within the discs also, the expression pattern of the fusion gene does not obey the parasegmental restrictions and varies from line to line, presumably under the influence of the chromosomal insertion site. This suggests that the imaginal *bx* function requires the interaction of the *bx* region with other control element(s) to produce the correct pattern in the haltere and third leg discs and to repress the expression in discs more anterior than PS5.

To map the sequences responsible for the BRE enhancer activity, we subdivided the 8.8 kb *bx* fragment into three intervals and tested them individually. The entire pattern of expression could be obtained with a 1.7 kb *SalI−Hin*dIII fragment (*bx*I) from −64.3 to −62.6 on the *Ubx* map (Figure 1). The embryonic pattern was consistent in all the lines obtained with this fragment and was essentially identical to that produced by the entire 8.8 kb DNA. Fragment *bx*II,

which contains the *zeste* binding site, and *bx*III did not produce specific patterns in the embryo. When the *bx*I fragment was further divided into three, a 500 bp *Apa*I−*Eco*RI fragment in the middle (see below and Figure 5c) was found to retain all the pattern information although the level of expression was reduced. The other two fragments

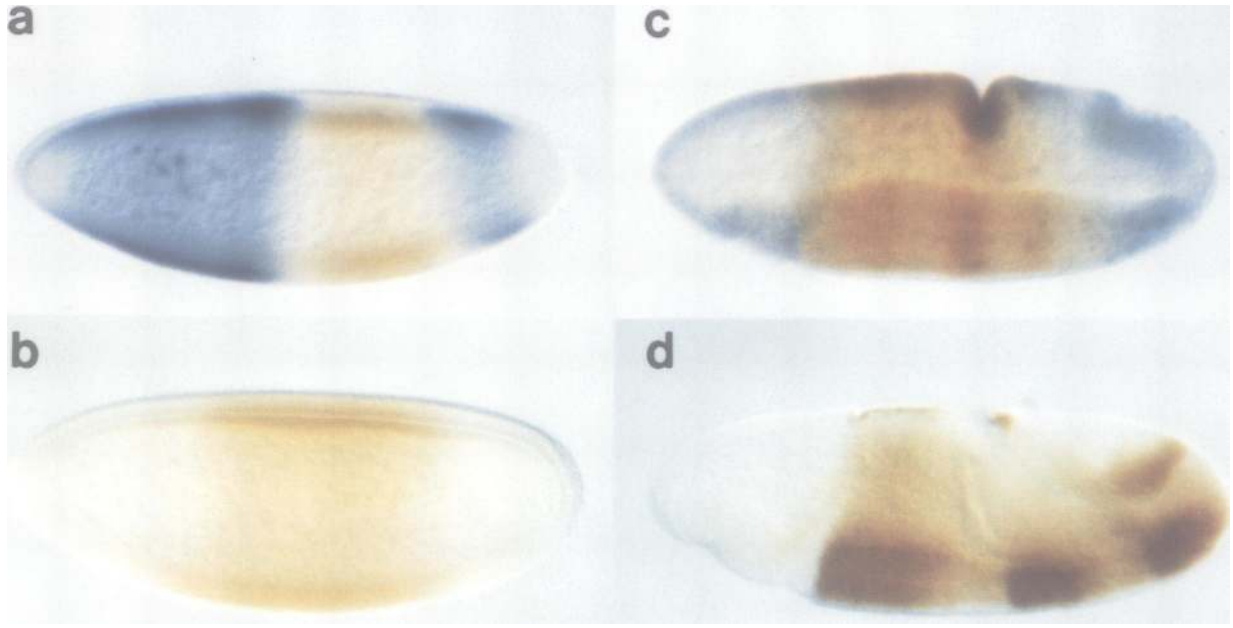

Fig. 3. *hb* suppresses BRE activity in the anterior half of the embryo. In wild-type embryos carrying *bx*−*Ubx*−*lacZ* (**a**), double staining with anti-*hb* and anti-β-galactosidase antibodies reveals that the domain of *hb* (blue) and the domain of BRE-directed expression (brown) are spatially complementary. In homozygous amorphic *hb* mutant (hb^{14F}) embryos (**b**, cellular blastoderm; **c**, early gastrulation, **d**, germ band extension), the anterior border of the BRE expression domain shifts from 50 to 75% EL, confirming the role of *hb* in setting the anterior boundary. hb^{14F} is phenotypically null but still produces a reduced level of *hb* antigen (Tautz, 1988) which is presumably inactive. Panel c shows a reduced level of *hb* staining from 75% EL to the anterior tip and an apparently normal or slightly weaker posterior *hb* stripe in hb^{14F} embryos. The BRE pattern in another *hb* null allele hb^{7M} which expresses no zygotic *hb* protein (Tautz, 1988) is identical to that in hb^{14F} (not shown). The posterior BRE border is set by *tailless* in addition to *hb* (Figure 4a).

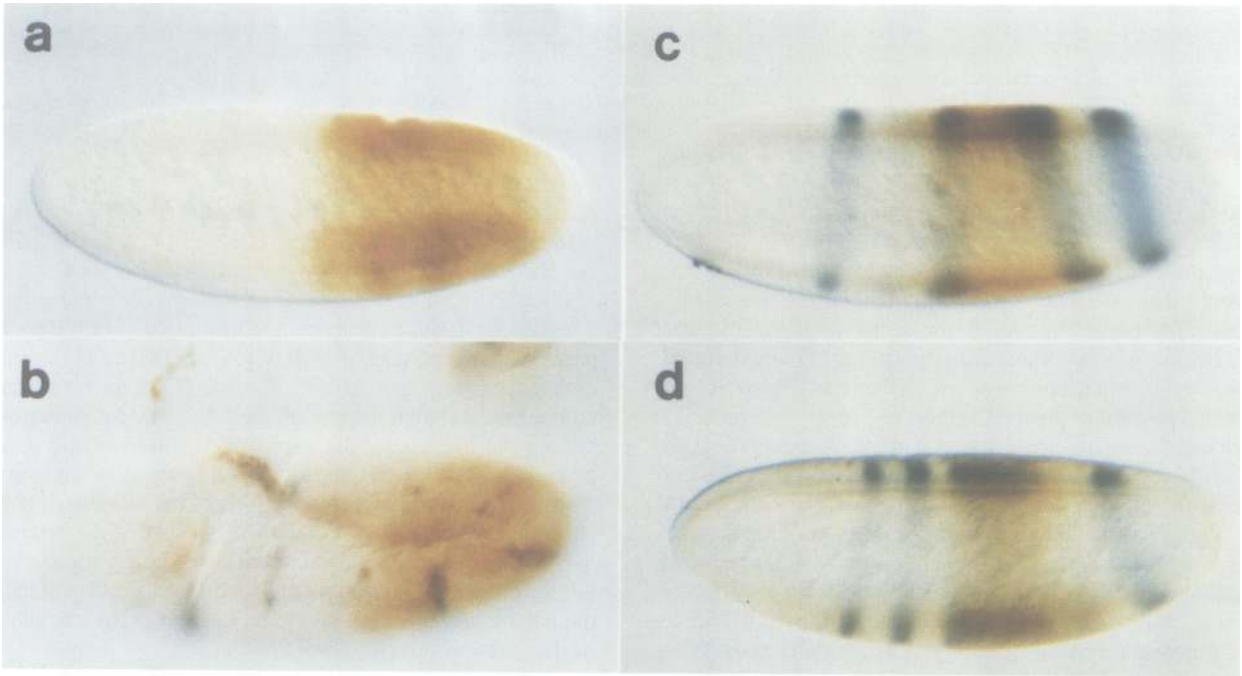

Fig. 4. Effects of mutations in *tll*, *ftz*, *Kr* and *kni* on BRE expression. (**a**) tll^{8} embryo at the cellular blastoderm stage. The posterior border of BRE expression is shifted almost to the posterior tip (compare with Figure 2a). Note that in the hb^{14F} embryo, there is no posterior shift (Figure 3b). (**b**) ftz^{bH34} embryo at the germ band elongation stage (compare with Figures 2d or 7d). The normally strong BRE expression in even numbered parasegments never develops at any stage and staining remains at the low level normally seen in the odd parasegments. Partial *engrailed* bands are visible only at PS1, 3, 7 and 9 in this focal plane. (**c**) and (**d**) Kr^{1} and kn^{IIE72} embryos at the cellular glastoderm stage double stained with anti-β-galactosidase (brown) and anti-*ftz* antibodies (blue). The BRE pattern (brown) is essentially normal in both mutant embryos from the beginning of cycle 14, when both BRE and *ftz* expression first became visible. The altered *ftz* patterns served to identify the mutant embryos (Carroll and Scott, 1986).

do not display any pattern by themselves but apparently contribute to give the normal level of activity.

The hunchback gene limits the anterior expression of the BRE

A striking feature of the BRE pattern is the sharp anterior border at 50% EL, similar to the anterior boundary of expression of the endogenous *Ubx* gene at the onset of transcription (Akam and Martinez-Arias, 1985). In contrast, the basal *Ubx* promoter alone gives a pattern of weak expression that extends into the head region. In the presence of the BRE, even the basal pattern is suppressed in the thorax and head, suggesting that the BRE contains a silencer for the anterior half of the embryo. The best candidate for a *trans*-acting repressor to set the anterior border of *Ubx* is the product of gap gene *hunchback (hb)* (Lehmann and Nüsslein-Volhard, 1987). *hb*, one of the earliest acting zygotic gap genes, is required for the development of head and thorax and is expressed strongly in the anterior half of the embryo, fading off at around 50% EL (Tautz *et al.*, 1987; Tautz, 1988). White and Lehmann (1986) observed that in *hb* mutant embryos, *Ubx* expression is shifted anteriorly, implying that *hb* could be the principal regulator directly or indirectly limiting the anterior extent of the *Ubx* domain of expression. *hb* encodes a zinc finger DNA-binding protein (Tautz *et al.*, 1987) and is known to regulate transcription of at least one pair-rule gene, *even-skipped (eve)* (Stanojevic *et al.*, 1989). Figure 3 shows that, in wild-type embryos, the domain of BRE-directed expression is complementary to that of *hb* expression. Furthermore, in *hb* mutant embryos (hb^{14F} in Figure 3; also hb^{7M}, not shown), the expression of the BRE−*Ubx*−*lacZ* fusion transposon, like that of the endogenous *Ubx* gene, is shifted markedly forward from 50% EL to about 75% EL. Note, that although no zygotic *hb* is expressed, these embryos still contain maternal *hb* product in their anterior region which may contribute to setting an anterior boundary. These results indicate that the BRE element is regulated by *hb*, either directly or indirectly. As Figure 3 shows, at the blastoderm stage, *hb* expression occurs also in a posterior domain (10−20% EL). However, the domain of BRE-directed expression does not expand posteriorly in an *hb* mutant, suggesting that additional repressive interactions may be involved.

The hb binding sites in the BRE mediate anterior suppression

To determine whether the *hb* suppression of the BRE results from a direct or an indirect interaction, we tested for the ability of bacterially expressed *hb* protein (Stanojevic *et al.*, 1989) to bind to BRE DNA. The immunoprecipitation experiment shown in Figure 5a demonstrates that *hb* protein binds specifically to the *bx*I fragment. Further experiments localized strong binding to the 500 bp *Apa*I−*Eco*RI fragment in the middle of *bx*I that encodes the pattern information, and a weak binding site to a 220 bp *Hin*cII−*Hin*dIII fragment at the right end of *bx*I (Figure 5c). Whether the strong and weak binding sites interact *in vivo* remains to be determined. When analyzed by DNase I footprinting, the middle *Apa*I−*Eco*RI fragment was found to contain three strong *hb* binding sites each about 25 bp in length (Figure 5 b and c). *hb* has recently been shown to recognize a 10 bp A-rich consensus sequence in the promoters of the *eve* gene and of the *hb* gene itself (Stanojevic *et al.*, 1989; Treisman and Desplan, 1989). Two of the *bx*I footprinting sites contain one copy of this core sequence while the third site contains two copies (Figure 5c). Beyond this A-rich consensus sequence, these three *hb* footprinting sites differ.

The co-localization of the strong *hb* binding sites and the enhancer pattern element within the same 500 bp DNA fragment argues strongly that *hb* suppresses the enhancer directly by binding to these sites. To confirm this, we deleted the *hb* recognition consensus sequences in all three strong *hb* binding sites of *bx*I by site-directed *in vitro* mutagenesis (Figure 5c and Materials and methods) and examined the interaction of the mutated enhancer with *hb* by the DNA binding assay *in vitro* and by the expression pattern *in vivo*. Figure 6 shows that the *hb* protein binds to the mutated

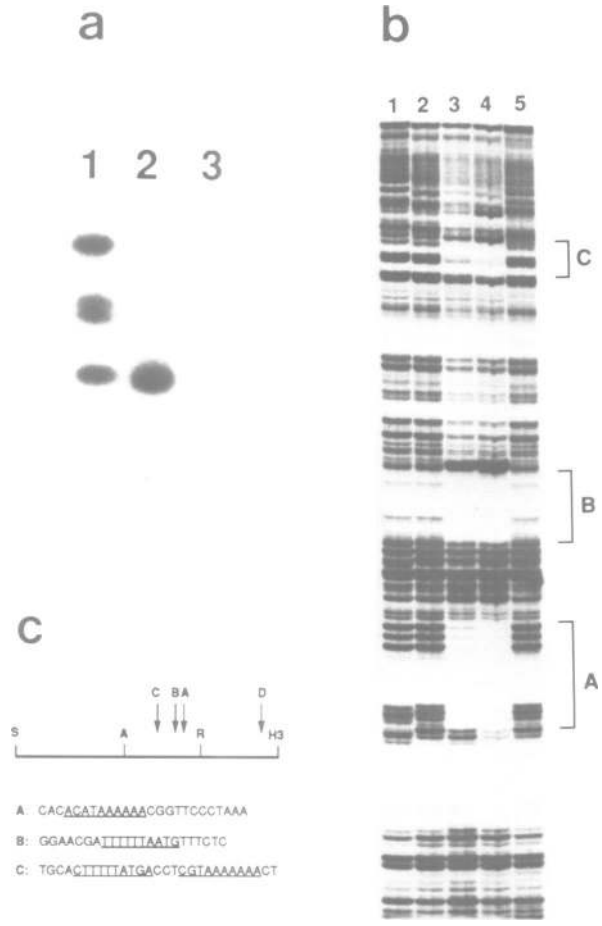

Fig. 5. Binding of *hb* protein to the BRE. (**a**) Immunoprecipitation of the BRE DNA with *hb* protein. Input DNA is the *bx*-pUC18 plasmid containing the 8.8 kb *bx* fragment, digested with *Sal*I−*Hin*dIII (Figure 1), and end-labeled. Lane 1, input DNA before immunoprecipitation; lane 2, 50 μg *hb* extract; lane 3, 50 μg *Kr* extract. Of the entire 8.8 kb *bx* DNA, only the 1.7 kb *bx* I enhancer element is co-precipitated with *hb* protein. Extract from bacteria expressing the *Krüppel* (*Kr*) protein failed to bind any of the input DNA fragments. Further experiments localized the strong binding region to a 500 bp *Apa*I−*Eco*RI fragment in the middle of the 1.7 kb *bx*I. (**b**) DNase I footprinting of the *Apa*I−*Eco*RI fragment. Lane 1, no protein; lane 2, 50 μg of protein from host bacteria without *hb* expression plasmid; lanes 3 and 4, 25 μg and 50 μg of *hb* extract; lane 5, 50 μg of *Kr* extract. (**c**) map of *hb* binding sites in the 1.7 kb *bx*I element. Sites A, B and C shown in (b) are indicated by arrows and their sequences are listed below the map with the A-rich consensus sequence underlined. A weaker binding site D is also indicated. Restriction sites: S: *Sal*I; A: *Apa*I; R: *Eco*RI; H3: *Hin*dIII.

enhancer much more weakly than to the wild-type enhancer under the same assay conditions. The residual binding is probably due to the remaining site D (Figure 5c). When placed in the *Ubx−lacZ* construct and expressed in wild-type embryos, the mutated enhancer lacked the *hb*-dependent anterior restriction and displayed the same anterior shift exhibited by the normal enhancer in *hb* mutant embryos. The new anterior border is now positioned right behind the cephalic furrow at PS2 instead of PS6 but, instead of simply expanding the four normal stripes, the mutated enhancer expands anteriorly by adding two new stripes in PS2 and PS4 (Figure 7). The shift of the anterior border resulting from the elimination of the *hb* binding consensus sequences proves that *hb* directly represses the BRE. Interestingly, in addition to shifting the anterior border, the mutation in the enhancer also relaxes the posterior border of the pattern. The fourth stripe, which is normally narrower than the others and covers only the anterior part of the PS12, is enlarged to about the same width as the first three stripes and an extra stripe appears at PS14 (Figure 7d). Together with the two new stripes generated by the anterior shift, we have a novel seven stripe pattern. The posterior shift may indicate that the posterior domain of *hb* expression (a band between 10 and 20% EL) also plays a role in defining the *Ubx* expression domain. We think it more likely that the deletion mutations disrupted the interaction of the enhancer not only with *hb* but also with the terminal gene *tll* (Strecker *et al.*, 1988) since, as will be shown below (Figure 4a), the posterior border of the BRE domain was shifted in *tll* but not in *hb* null embryos (Figure 3). The *tll* gene has recently been cloned and was shown to also encode a putative zinc finger DNA-binding protein (Pignoni *et al.*, 1990). Its binding sites in the BRE element may therefore overlap those of the *hb* protein.

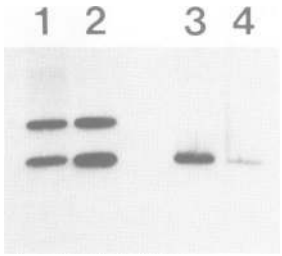

Fig. 6. Deletion of the *hb* binding consensus sequences in the BRE reduces the *hb* binding in the immunoprecipitation assay. Wild-type and deletion mutant *bx*I-Bluescript plasmids were digested with *SalI−Hin*dIII end-labeled, and equal amounts of the labeled DNAs were immunoprecipitated by hb protein extract. Lanes 1 and 2: wild-type and mutant *bx*I input DNA before immunoprecipitation. Lanes 3 and 4: wild-type and mutant *bx*I fragment after immunoprecipitation. The mutant *bx*I fragment was precipitated at about one-fourth the efficiency of the wild-type fragment. The upper band in the input DNA lanes is the vector.

In *hb* mutant embryos, the four stripes expressed by the normal enhancer, especially the first stripe, are expanded (Figure 3) but no new stripes are added to the pattern. This is clearly different from the seven stripe pattern of the mutant enhancer in wild-type embryos (Figure 7), although both expand anteriorly to the same extent. This difference can be explained in terms of the effect of *hb* on the pair-rule genes that create the metameric pattern, in particular those that activate *Ubx* expression. A major activator of *Ubx*, and most likely of BRE, is the pair-rule gene *ftz* which is expressed at blastoderm in a series of seven stripes roughly coinciding with the even parasegments. Genetic studies have indicated that *ftz* activity is necessary for the activation of *Ubx* expression in the even-numbered parasegments (Ingham and Martinez-Arias, 1986). We find that, in *ftz* mutant embryos, the strong BRE expression in the even-numbered parasegments is reduced to the level found in odd-numbered parasegements, resulting in a homogeneously low level of expression (see below and Figure 4b). In wild-type embryos, the relief of *hb* repression in the anterior of the embryo allows *ftz* to activate the mutated enhancer in PS2 and PS4; likewise, relief of *tll* repression in the posterior terminal region allows *ftz* to activate the mutated BRE in PS12 and PS14, resulting in a seven stripe pattern. In *hb* null mutant

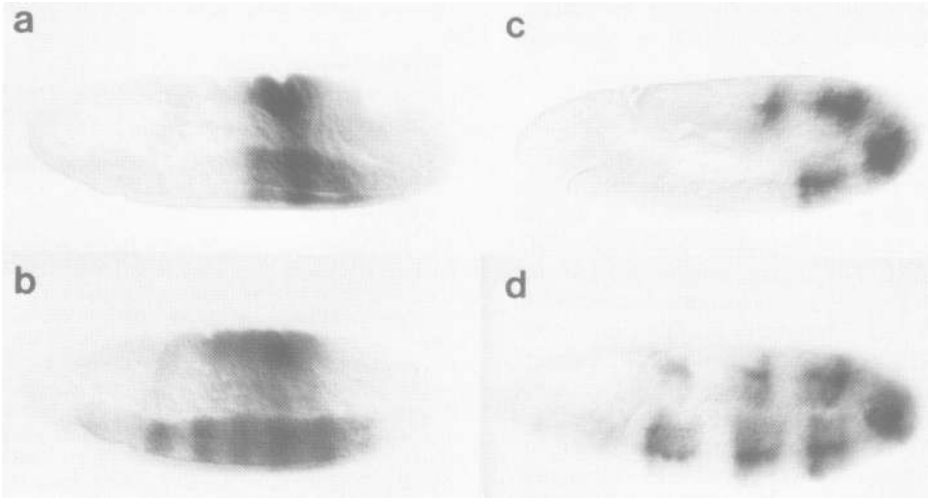

Fig. 7. Altered expression pattern of the mutant BRE element in which the *hb* binding sites have been deleted. (**a**) Normal BRE pattern at early gastrulation. (**b**) Early gastrulation embryo carrying the mutated BRE shows the anterior shift of the expression pattern. The anterior border now lies right behind the cephalic furrow. At this stage, the posterior shift is not apparent. (**c**) Normal BRE pattern at the extended germ band stage. (**d**) Novel seven stripe pattern at the germ band extension stage. As the result of the BRE mutation, three new stripes appear in PS2, 4, 14, and the normally narrow PS12 stripe is now enlarged to same width as the other stripes.

embryos, the first three anterior and the last two posterior *ftz* stripes are fused into broad anterior and posterior bands, the remaining two middle *ftz* stripes do not fuse but also become broader than normal (Carroll and Scott, 1986). Accordingly, in the absence of *hb* product, the four BRE stripes are enlarged and expanded anteriorly.

hb protein also binds to DNA fragments of the abx and bxd regulatory regions

Since the pattern displayed by the BRE element constitutes only a part of the endogenous *Ubx* expression pattern, there must be other regulatory elements located elsewhere in the *Ubx* gene. Besides the *bx* region, *abx* and *bxd/pbx* are two other important regulatory regions of the *Ubx* gene defined by genetic analyses. Since *Ubx* expression as a whole is subject to the *hb*-dependent anterior restriction, any enhancers contained in these regions would be expected to obey the same restriction. Using the immunoprecipitation assay, we scanned across 110 kb of DNA, including the *Ubx* transcription unit and 30 kb of upstream regulatory region, for the presence of additional *hb* binding sites. The results are summarized in Figure 8. There are only five significant *hb* binding fragments in the entire interval. Of the three stronger binding sites, one is located in the *bx* region and two in the *bxd* region. Of the two weaker binding sites, one is found in the *bxd* region, the other in the *abx* region. It is possible that some or all of these *hb* binding fragments are associated with enhancer elements. The relative strength of these binding sites might be significant. The *hb* concentration in the embryo does not drop sharply at 50% EL but rather is graded down progressively (Tautz, 1988). Sites with low binding affinity for *hb* would be occupied only at more anterior positions, where the *hb* concentration is higher, while sites with high affinity would continue to be occupied in more posterior parts of the embryo. This type of mechanisms has been shown to control the *bicoid*-dependent activation of *hb* in the anterior part of the embryo (Driever *et al.*, 1989; Struhl *et al.*, 1989). In our case, *hb* binds to the *abx* region more weakly than to the *bx* and *bxd* regions, suggesting that the enhancer in the *abx* region is able to function more anteriorly than enhancers in the *bx* and *bxd* regions. This is consistent with the genetic results impying that *abx* functions more anteriorly than *bx* and *bxd*.

BRE expression in tll, ftz, Kr and kni mutant embryos

hb is obviously only one of many factors involved in the activation of the BRE element. We have examined BRE expression in mutant backgrounds lacking other potential regulators. Of the four genes discussed in this section, *tll* and *ftz* alter BRE expression while *Kr* and *kni* has no effect, at least on the early pattern of BRE activation. In *tll* mutant embryos, the posterior border is relaxed and BRE expression extends nearly to posterior tip (Figure 4a). This effect could be indirect, mediated, for sample, by *hb*, whose posterior domain of expression is activated by *tll* (Schröder *et al.*, 1988). However, since the posterior border remains normal in *hb* mutant embryos (Figure 3), we conclude that *tll* is the major factor limiting posterior BRE expression. Figure 4b shows the effect of a *ftz* mutation on the expression of BRE: the strong four stripe BRE pattern never develops and expression remains at a low level in a homogeneous domain, indicating that *ftz* is required for the enhanced expression in the even-numbered parasegments. Based on this result and on the altered *ftz* expression in *hb* mutant embryos, we have suggested above that the difference between the BRE pattern in *hb* mutant embryos (Figure 3) and the BRE deletion mutant pattern in normal embryos (Figure 7) could be explained by the dependence of BRE expression on *ftz* activity. The shift of the posterior BRE border in *tll* mutants and the decrease of BRE expression in even-numbered parasegments in *ftz* mutants parallel the behavior of the *Ubx* gene in these mutants (Reinitz and Levine, 1990; Ingham and Martinez-Arias, 1986). The similarities indicate that, like *hb*, *tll* and *ftz* also regulate *Ubx* expression through distant enhancers, probably in a direct fashion.

The expression of the endogenous *Ubx* gene in PS6 is much stronger and different in detail for the rest of the metameric pattern. To account for this local enhancement, Ingham and Martinez-Arias (1986) proposed that the *ftz* and *Krüppel (Kr)* products act synergistically to achieve higher levels of *Ubx* expression. The BRE pattern does not show this preference for PS6. Initially, the expression is fairly uniform within the domain and, after the stripe pattern arises, it is somewhat stronger in PS8 than in PS6. *Krüppel (Kr)* and *knirps (kni)* are two major early-acting gap genes that are required for the abdominal segmentation of the embryo (Nüsslein-Volhard and Wieschaus, 1980). The expression domain of these two genes overlaps that of the BRE element (Gaul *et al.*, 1987; Pankratz *et al.*, 1989), raising the possibility that *Kr* and *kni* might be the early activators of the BRE. Irish *et al.* (1989) found that in *Kr* mutant embryos, strong early activation of the endogenous *Ubx* gene is not observed in the region corresponding to PS6 although expression in a broad posterior domain appears by early gastrulation. In contrast, in embryos lacking *Kr* or *kni*, the initial activation of the BRE element appeared at the normal time and was normal in spatial distribution and intensity from the earliest stage. Figure 4 c and d show the normal extent of expression at the cellular blastoderm stage, although the later stripe pattern is, of course, affected. Consistent with

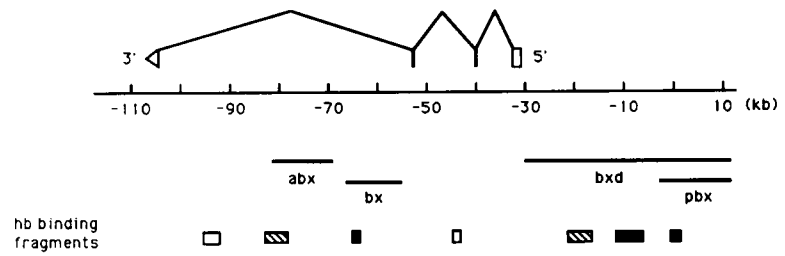

Fig. 8. *hb* binding sites in the *Ubx* locus. The *Ubx* transcription unit and the *abx*, *bx*, *bxd* and *pbx* regions are depicted as in Figure 1. The *hb* binding fragments identified by immunoprecipitation are indicated by boxes below. Filled boxes represent strong binding sites; hatched boxes, weak sites; open boxes, very weak sites. The *bx* region has a strong binding site, the *bxd* region has two strong and one weak site and the *abx* region has one weak binding site.

these results, we could not detect specific binding of the Kr protein to the BRE element (Figure 5a and b).

Discussion

The bx region enhancer, BRE

Several lines of evidence make it likely that the BRE contributes to *Ubx* expression during embryogenesis and, although imperfectly, corresponds to the genetically identified *bx* control element. The BRE expression pattern closely resembles the early *Ubx* pattern. It is regulated by the same set of segmentation genes that regulate the *Ubx* pattern: it is repressed by *hb* and by *tll*, which together set its boundaries, and it is positively regulated by *ftz*.

Physically, the sites of most *bx* mutations fall within the 8.8 kb BRE fragment. Within this region we have found no other enhancer activity that functions in the embryo or imaginal discs. All *bx* alleles but one are caused by transposon insertions, most frequently involving the *gypsy* element, and may have wider effects, perhaps influencing, for example, the *abx* region as well as *bx*. The exception is $bx^{34e\text{-}prv}$, a partial revertant of bx^{34e} in which the *gypsy* element excised leaving a deletion of ~9.5 kb that completely overlaps the 8.8 kb interval represented in our largest BRE construct (Peifer and Bender, 1986). Genetic evidence indicates that the downstream elements *abx* and *bx* make a substantial contribution to the expression of the native *Ubx* gene. Removal of most of the upstream *bxd* region reduces but does not eliminate *Ubx* expression in the embryo (Bienz *et al.*, 1988; Irvine *et al.*, 1990). However, strong mutations in either *abx* or *bx* fail to alter appreciably the distribution of *Ubx* expression at early embryonic stages (White and Wilcox, 1985b; Irvine *et al.*, 1990). We suppose that the domains of expression controlled by the upstream elements overlap substantially the pattern produced by the downstream elements, particularly at the early stages. Functional overlap is also suggested by the observation that *bx bxd* double mutants have a lethal phenotype much stronger than the viable phenotype displayed by either class separately (Lewis, 1951).

While this article was being written, two other works on the *Ubx* regulatory region appeared (Simon *et al.*, 1990; Irvine *et al.*, 1990). These papers, together with the present work, show that the *Ubx* regulatory region contains elements which produce patterns of expression in alternating parasegments. A control element in the *abx* region gives expression in odd parasegments beginning with PS5. At least two distinct elements are present in the *bxd* region, one expressing in even- and one in odd-numbered parasegments. The BRE is superficially similar to one of the *bxd* elements and produces a pattern in even-numbered parasegments beginning with PS6. These control elements program patterns of expression related to those expected for the genetically identified *abx*, *bx* and *bxd* control regions but the correspondence is not perfect, suggesting that additional controls or combinatorial interactions are required to reproduce the full anterior–posterior pattern. In addition to their differences in parasegmental pattern, the different genetic control elements appear to have different spectra of tissue specificities. The *abx* and *bxd* mutations clearly affect *Ubx* expression in the embryonic CNS as well as the epidermis, while *bx* mutants, including $bx^{34e\text{-}prv}$, have no detectable effects on the CNS (Little *et al.*, 1990; White and Wilcox, 1985b). The *bxd* and *bx* elements and, to a lesser extent, *abx* play a role in the mesoderm (Hooper, 1986). Consistent with these tissue specificities, the *abx* enhancer of Simon *et al.* (1990) is active in the epidermis and in the CNS while the BRE does not program expression in the CNS but is strongly expressed in the mesoderm as well as in the epidermis. The *bxd* enhancers have been analyzed in lesser detail but seem to affect all three tissues (Irvine *et al.*, 1990). Although there is overlap in the domains of expression of *bx* and *bxd* elements, there are important differences in detail. In the mesoderm, Hooper (1986) found that *bx* mutations cause substantial transformations of the larval abdominal musculature in segments A1 to A5, similar to the defects caused by *bxd* mutations. However, while *bxd* affects the more medial muscles, *bx* mutations transform the more lateral muscles, consistent with the intense expression of BRE in the lateral mesoderm and the weak expression in the ventral region of the later embryo (see Figure 2e).

A major discrepancy remains between the BRE pattern and the classical *bx* phenotypes. The *bx* mutations typically transform anterior T3 towards anterior T2 in the imaginal discs and the adult cuticle, that is a PS5 to PS4 transformation. This presupposes a normal *bx*-dependent expression in the posterior part of PS5 in the third leg and haltere imaginal discs. In the early embryo we do not detect BRE expression in PS5 until the end of term band extension when weaker expression becomes visible in this parasegment. However, direct comparison of BRE activity and imaginal or adult *bx* phenotypes is not possible because of the breakdown of parasegmental control. The failure to preserve the parasegmental specificity beyond the extended germ band stage was observed in different degrees with all the enhancer constructs (Simon *et al.*, 1990) except the largest one tested by Irvine *et al.* (1900). Most of these enhancer constructs also produced inappropriate expression in the imaginal discs, indicating that some additional layer of control is required in later development to determine correct parasegmental regulation. We conclude that the BRE program of expression corresponds at least in part to the genetically defined *bx* function but we cannot exclude the possibility that the *bx* region contains additional control elements that might correct or modify the pattern. It is possible also that combinatorial effects between enhancers might produce additional pattern elements not predictable from their separate behavior. In fact, the *abx20* construct tested by Simon *et al.* (1990) probably contained both the *abx* enhancer and the BRE described here. However the pattern of expression described by this construct can be accounted for by the simple addition of the BRE and *abx* pattern elements.

Regulation by segmentation genes

The parasegmental pattern of *Ubx* and of BRE expression implies that the segmentation genes have a major input in the activation of the *Ubx* gene. Mutations in gap, pair-rule and segment polarity genes all affect the correct establishment of *Ubx* expression (Ingham and Martinez-Artias, 1986; Ingham *et al.*, 1986; Ingham, 1988; Martinez-Arias and White, 1988; Irish *et al.*, 1989). Our results show that neither *Kr* nor *kni*, the two gap genes expressed in the *Ubx* domain, are required for the initial activation of BRE. The initial activation of the *Ubx* gene as a whole occurs also in a broad abdominal region but becomes sharply enhanced in

a narrow stripe at the position of PS6 and then in stripes at PS8, 10 and 12 (Akam and Martinez-Arias, 1985). This strong expression of *Ubx* is affected by *Kr* and *kni* mutations (Ingham *et al.*, 1986; Irish *et al.*, 1989) while the lower level initial BRE expression is not. It is possible that the initial activation of BRE is affected by ubiquitous factors and controlled primarily by negative regulators.

Our results reveal that at least one gap gene, *hb*, acts directly upon the BRE and probably on the other distant regulatory elements of *Ubx* to repress more anterior expression and determine the anterior boundary of the *Ubx* domain. Failure to suppress anterior expression of *Ubx* causes homeotic transformations (Gonzalez-Reyes *et al.*, 1990), demonstrating the importance of this repressive effect. *hb* also has a posterior domain of expression, which is activated at blastoderm and may contribute to setting the posterior border but our results indicate that, for BRE as for the endogenous *Ubx* gene, lack of *hb* is not sufficient to remove the posterior boundary of the early expression pattern. Instead, the major factor determining this boundary is the terminal gene *tll* (Figure 4b; Reinitz and Levine, 1990). The site-specific mutagenesis experiments show that the repressive effect of *hb* on BRE is mediated directly by one or more of the three *hb* binding sites in the BRE DNA but do not clarify the mechanisms of repression. The *hb* binding consensus sequences might overlap with the binding sites of an activator, permitting *hb* to block or compete with the binding of the activator. Alternatively, *hb* protein might have a direct negative effect on the promoter or it might interfere with the communication between enhancer and promoter. The deletion of the three *hb* binding sites does not inactivate the enhancer appreciably, indicating that possible activator binding sites do not overlap extensively with the *hb* binding sites. It is interesting to note that the deletions also relieve the negative effect of *tll*. Since this effect is not mediated through *hb*, it is likely that the *tll* product or a protein immediately controlled by *tll* also binds to one or more of the deleted sequences, suggesting that both *tll* and *hb* affect the action of the enhancer by the same mechanism.

After the cellular blastoderm stage, stronger expressing stripes composed of the even parasegments plus the anterior part of the odd parasegments are superimposed on the uniform initial domain of BRE. Our result shows that *ftz* is necessary for the strong expression of the BRE normally seen in the even parasegments. DNA binding experiments suggest that this is a direct effect since *ftz* protein binds to the same 500 bp fragment for the BRE that contains the *hb* binding sites (S.Qian, M.Capovilla and V.Pirrotta, unpublished results). However, the BRE stripes are wider than the stripes of *ftz* expression, strongly suggesting that some other pair-rule gene that interacts with *ftz* also enhances BRE expression.

Later expression

Input from the segmentation genes is essential for the establishment of the *Ubx* pattern and, as we have shown, to set the boundaries of the expression domain. *In vivo*, the *Ubx* gene continues to be expressed only in the appropriate segmental derivatives throughout the embryonic development and in the larval imaginal discs. What maintains this pattern after the cellular blastoderm, when the expression of the segmentation genes fades away in their segmental domains? Autoregulation, which has been shown to stabilize a pattern of expression in some cases (Kuziora and McGinnis, 1988; Bienz and Tremml, 1988), is not involved in the case of the BRE but it is possible that mutual interactions between homeotic genes play some role (Struhl and White, 1985). Two classes of genes are important in maintaining the *Ubx* pattern of expression through later development. One is the *Polycomb* group, which appears to act by keeping *Ubx* repressed in parts of the embryo in which it is not initially activated (Duncan, 1982; Jürgens, 1985; Struhl and Akama, 1985). The other is the trithorax class of genes, whose role is to sustain expression in the appropriate segments (Ingham, 1985). The BRE pattern of expression in late embryonic development and in the imaginal discs suggests that both of these mechanisms fail to act upon our constructs. The parasegmental restriction of BRE expression begins to be transgressed shortly after germ band retraction when staining appears in thoracic segments T1 and T2 as well as in the head region and in posterior terminal structures. In the imaginal discs, the expression of BRE is variable in different transformed lines and it appears in inappropriate discs or compartments.

Long-distance regulation

The BRE element, like the other genetically defined regulatory regions of *Ubx*, is located at a large distance from the *Ubx* promoter. *In vivo*, it is presumably able to control its activity across more than 30 kb. One possible mechanism that could account for the ability of regulatory elements to act over distances of this order was suggested by the phenomenon of transvection, first observed by Lewis (1954) and prominently exhibited by *bx* mutations. Transvection is the ability of regulatory elements such as *bx* to control the promoter of a second copy of the gene on a homologously paired chromosome. The phenomenon requires the activity of the *zeste* gene and could be explained by a model according to which *zeste* binding sites near the distant regulatory element and *zeste* binding sites near the promoter mediate the looping that brings the distant element in proximity to the promoter (Benson and Pirrotta, 1988). The presence of a *zeste* binding site little more than one kb distant from the BRE lends support to this model. However, *zeste* function is not essential for correct *Ubx* expression since normal development is possible in flies bearing total deletions of the gene (Goldberg *et al.*, 1989; V.Pirrotta, unpublished results). It is likely therefore that the mechanisms are more complex and that additional functions exist that can substitute for any role that *zeste* may have in gene regulation.

Materials and methods

Fly stocks and mutant embryo collection

Recipient flies for germ line transformation were $Df(1)w^{67c23(2)}$ in which the *white* gene is partially deleted causing complete lack of eye pigmentation (Pirrotta *et al.*, 1983).The mutant stocks used for analysis of genetic interactions are: *hb* null alles hb^{14F}; hb^{7M} (Tautz, 1988); *Ubx* null alles Ubx^{1}; $Dfbxd^{100}$ (Bienz and Tremml, 1988). ftz^{9H34} (Ingham and Martinez-Arias, 1986); tll^{8} (Strecker *et al.*, 1988); Kr^{1} (Preiss *et al.*, 1985). kni^{1IE72} (Tearle and Nüsslein-Volhard, 1987). To obtain embryos homozygous for various mutations and carrying the BRE–*Ubx*–*lacZ* transposon, the mutant strain was first crossed with transgenic flies carrying the transposon on a different chromosome and their non-balancer progeny were mated together to produce embryos for antibody staining.

Tranposon construction and germ line transformation

The *Ubx* basal promoter is a 1.65 kb *Stu*I fragment including 680 bp of 5′ upstream sequence and the entire 968 bp untranslated leader plus the

first 7 codons (Saari and Bienz, 1987). This fragment was adapted with a *Bgl*II linker and cloned into the *Bam*HI-digested Cahsneo−lacZ, a Carnegie 4-derived plasmid (V.Pirrotta, unpublished), to give *Ubx−lacZ* gene assembled in a P-transposon using the *hs−neo* gene as selective marker (Steller and Pirrotta, 1985). Translation of the *Ubx−lacZ* gene begins with the first 7 codons of *Ubx* fused in-frame to the *lacZ* coding sequence. The 8.8 kb *bx* DNA was generated by merging a *Sal*I−*Kpn*I fragment from lambda clone 2269 and a *Kpn*I−*Hinc*II fragment from clone 2261 of the *Ubx* chromosomal walk (Bender *et al.*, 1983) in a pUC18 plasmid. From this *bx*-pUC clone, the 8.8 kb *bx* DNA was excised out as a *Sal*I fragment and inserted into the *Sal*I site of the *Ubx−lacZ*−hsneo P-transposon, upstream of the *Ubx* promoter. Constructs with *bx* arranged in both orientations relative to the *Ubx* promoter were selected. Transposon injection and G418 selection were done as described by Steller and Pirrotta (1985). For *bx* sub-fragment, the *bx−Ubx−lacZ* fusion constructs were assembled in CaSpeR4, a derivative of the CaSpeR vector (Pirrotta, 1988) containing a more extensive polylinker. Transformant flies were identified by eye pigmentation (details of constructions available upon request).

Antibody staining

Embryos were fixed, stained and mounted by the methods of Lawrence and Johnston (1989). The rabbit anti-β-galactosidase antibody (Cappel) was preabsorbed against 0−14 h embryos overnight at 1:500 dilution and used at a further dilution of 1:10 for early stage embryos or 1:20 for later stage embryos. The anti-β-galactosidase antibody was detected as brown color using the Vectastain ABC-HRP kit (Vector Labs), as instructed. For double staining, the embryos were washed after anti-β-galactosidase stain and incubated overnight with secondary antibody, then reacted with the Vectastain ABC-AP kit reagents to produce a blue color. The rat anti-*hb* antiserum was preabsorbed at 1:20 dilution, used at a final dilution of 1:5000; the rabbit anti-*ftz* antiserum was preabsorbed at 1:100 dilutions, used at a final dilution of 1:1000; the mouse anti-*en* antibody was used at 1:1000 dilution.

Immunoprecipitation and DNase I footprinting

hb and *Kr* proteins were expressed from the T7 promoter in *E.coli* host strain BL2(DE3) (Stanojevic *et al.*, 1989). 100 ml cultures at OD_{600} = 1 were induced for 3 h with 0.4 mM IPTG. Cells were pelleted and resuspended in 0.5 ml of buffer Z (Hoey and Levine, 1988). After adding 0.25 mg lysozyme and sonicating, the insoluble fraction was pelleted and then solubilized in 1.5 ml buffer Z containing 4 M urea. The protein extracts were stored at −80°C and used directly for immunoprecipitation (Benson and Pirrotta, 1987) and DNase I protection assays.

DNase I footprinting was done according to Stanojevic *et al.* (1989) with slight modifications: 2−4 ng ^{32}P-labeled DNA, 0.5 μg poly(dI·dC), 0.25 μg sonicated calf thymus DNA and appropriate amounts of protein extract were incubated on ice for 30 min in 50 μl binding buffer (100 mM KCl, 35 mM HEPES pH 7.9, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM $ZnCl_2$, 12% glycerol and 0.06% NP-40). After incubation, 50 μl of 10 mM $MgCl_2$, 5 mM $CaCl_2$ was added and 1 μl of 0.04 μg/μl DNase I was mixed in to give a final concentration of 0.4 μg/ml. After 2−5 min digestion on ice, the reaction was stopped by adding 90 μl stop solution (1% SDS, 20 mM EDTA, 200 mM NaCl). The DNA samples were extracted with phenol−chloroform (1:1), then with chloroform, ethanol precipitated and analysed on a 6% acrylamide sequencing gel.

Site-specific deletions

To minimize the damage to the *bx* enhancer, only the core *hb* binding consensus sequences (shown by parentheses) in the footprinting site A, B and C (Figure 3c) were deleted: A: CACA(CATAAAAAA)CGGTTCC-CTAAA; B: GGAACGA(TTTTTT)AATGTTTCTC; C: TGCACTT-(TTTATGACCTCGTAAAAA)AACT. Site D was left intact.

The *hb* consensus sequences were deleted using a strategy based on the site-directed mutagenesis method of Kunkel (1985). The *bx* I fragment was cloned into the Bluescript phagemid vector and single-stranded template was prepared from host cells BW313. The template DNA prepared from the BW313 host contains uracil residues in place of thymine. It can serve as template for *in vitro* DNA synthesis but is unable to replicate in wild-type *E.coli* cells, thus simplifying the selection of deletion products. A synthetic oligonucleotide complementary to two sides of the target sequence was annealed to the single-stranded template to prime *in vitro* DNA synthesis. The double-stranded DNA product was then transformed into DH5α cells and deletion mutants were selected by DNA sequencing. The correct mutant clone then served as new template and the process repeated until all three consensus sequences were deleted. The oligonucleotide primers were made with 14 bp match in each side of the deletion target sequences. Sites A and B were deleted in two single cycles while the site C was deleted in two steps.

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