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Molecular mechanisms of pattern formation by the BRE enhancer of the *Ubx* gene

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The core activity of the *Ubx* gene enhancer BRE (*bx* region enhancer) is encoded within a 500 bp module. *bx* DNA outside this active module increases the level of expression, expands the expression into ventro-lateral ectoderm and partially stabilizes the late expression pattern. The products of the gap genes *hb* and *tll* and of the pair-rule gene *ftz* bind to the 500 bp BRE module and control directly its initial pattern of expression. *ftz* enhances expression in even-numbered parasegments within the correct spatial domain whose boundaries are set by *hb* and *tll*. In addition, *en* and *twi* products activate the enhancer, probably directly. *en* broadens the parasegmental stripe while *twi* cooperates with *ftz* to enhance expression in the mesoderm. Binding sites for the five regulators are closely clustered, often overlapping extensively with one another. *In vitro*, *hb* blocks the binding of *ftz* and can also displace *ftz* protein pre-bound to an overlapping site, suggesting that competitive binding and/or interference by *hb* sets the initial boundaries of the domain of expression. Our results also suggest that this interaction is short-range and the long distance interactions among different enhancers may depend on each enhancer's ability to complex with the promoter.
Key words: competitive repressor/enhancer competition/ homeotic domain/maintenance of expression

Introduction

Homeotic genes encode nuclear factors, presumably transcriptional regulators, whose activity results in the assignment of specific identities to the metameric units constituting the body plan of the developing embryo (reviewed by Ingham, 1988; McGinnis and Krumlauf, 1992). The segmentally appropriate expression of homeotic genes is therefore essential for correct development and differentiation. In *Drosophila*, the positional cues for the proper activation of the homeotic genes are provided by the early, spatially patterned expression of the gap, pair-rule and segment polarity genes (Ingham and Martinez-Arias, 1986; White and Lehmann, 1986; Martinez-Arias and White, 1988; Irish *et al.*, 1989; Reinitz and Levine, 1990). After gastrulation, however, the activity of the segmentation genes subsides and the expression of homeotic genes enters a second phase in which the correct pattern is maintained by sustaining activity where they had been originally active and suppressing activity where they had been repressed. Genetic analysis has identified a number of genes involved in the maintenance of repression: the *Polycomb* (*Pc*) group, and a smaller number required to promote expression: the *trithorax* (*trx*) group (reviewed by Duncan, 1987 and McGinnis and Krumlauf, 1992). However, the molecular nature of the patterned activation or of maintenance of homeotic gene expression is not well understood.

The *Ultrabithorax* (*Ubx*) gene spans >100 kb, along which are scattered multiple *cis*-control regions (Peifer *et al.*, 1987). Several enhancer elements from these regions have been identified using *lacZ* reporter constructs. Three of them, ABX, BRE and PBX from the genetically defined *abx*, *bx*, *pbx* regulatory regions respectively, generate patterns of expression partially resembling that of the endogenous *Ubx* gene (Simon *et al.*, 1990; Müller and Bienz, 1991; Qian *et al.*, 1991). The enhancer activities of these three elements have been mapped to short, autonomously acting sequences 500-1000 bp in length. All three elements activate the *Ubx* promoter in a pair-rule pattern within embryonic parasegments (PS)5-13, the normal domain of *Ubx* expression. The ABX element is active in the odd-numbered parasegments PS5, 7, 9, 11 and 13, with the anterior border set at PS5. The BRE and PBX elements activate expression in the even-numbered parasegments PS6, 8, 10 and 12, with the anterior border set at PS6. In addition, several other enhancer elements have been identified in the *bxd* region (Müller and Bienz, 1991; V.Pirrotta and D.McCabe, unpublished), some expressing in the even-, some in the odd-numbered parasegments. The similarity between the patterns of activity of some of these elements, e.g. BRE and PBX, suggests a certain degree of redundancy although we do not know if they differ at the cellular level. It appears therefore that a number of basic pattern elements are encoded by different regulatory regions whose function must be coordinated and integrated if the expression of the gene as a whole is to assume the correct pattern and detail.

A major difference between the expression pattern directed by these enhancers and that of the endogenous *Ubx* gene is that the former fail to be correctly maintained as development proceeds. To varying degrees and at different developmental stages after initial activation, ectopic expression appears more anterior than the normal boundary of the *Ubx* expression domain, as well as in some cells within the normal domain that do not normally express. This resembles the ectopic expression characteristic of the *Ubx* gene in embryos lacking the function of one or more genes of the *Pc* group. In addition, the small individual enhancer elements fail to generate correct and reproducible expression in the larval imaginal discs, the primordia that give rise to adult body structures (Simon *et al.*, 1990; Qian *et al.*, 1991). This may be due to the same failure to maintain appropriate expression

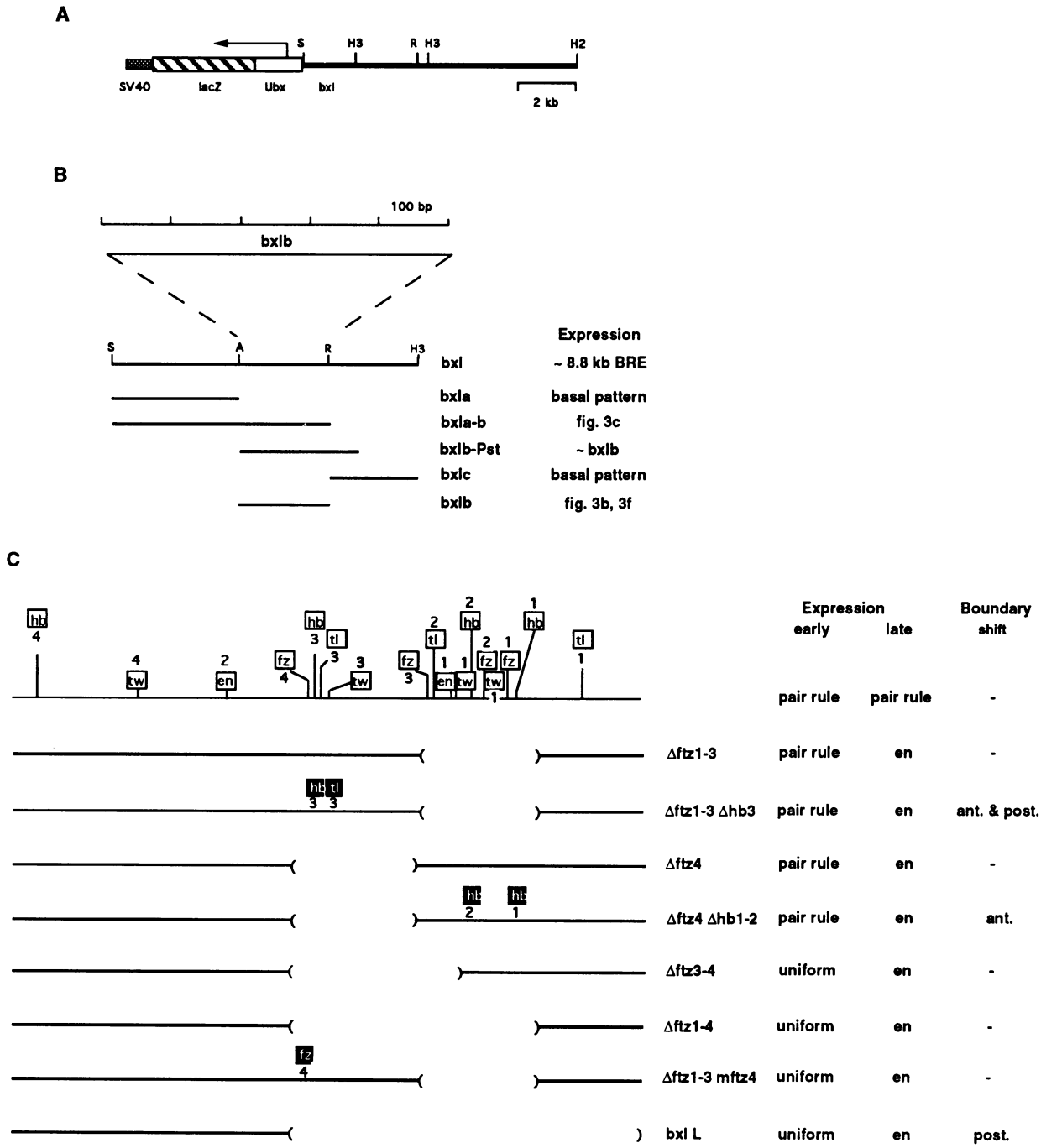

Fig. 1. Dissection of the BRE module. (**A**) The 8.8 kb BRE−*Ubx*−*lacZ* fusion construct (Qian *et al.*, 1991) of which *bxl* is the 1.7 kb *SalI*−*Hin*dIII fragment adjacent to the *Ubx* promoter. (**B**) The *bxl* fragment containing the active module and its deletion derivatives. The different deletion constructs are shown with solid lines indicating the DNA sequence present. Their names and expression patterns are listed on the right. At the top of the drawing, the 500 bp active module (*bxlb*) is shown at an expanded scale (marked in 100 bp intervals). Restriction sites: A, *Apa*I; H2, *Hin*dII; H3, *Hin*dIII; R, *Eco*RI; S, *Sal*I. (**C**) Deletions and mutations of the 500 bp *bxlb* active module. The expression patterns of these various mutants were tested with the mutated active module incorporated in the 1.7 kb *bxl* fragment. The positions of the different footprint sites are indicated schematically and mutated footprint sites are shown by reverse contrast symbols. The pattern of expression at gastrulation is either pair-rule, when active *ftz* sites are present, or uniform, when these are absent or very weak. The pattern at late germ band extension is still pair-rule, when strong *ftz* sites are present, or *engrailed*-like when they are absent or weak. Mutations or deletions that relieve the *hb* and *tll* repression cause expansion of the pattern anteriorly and posteriorly, respectively.

in the late embryo or to the lack of some imaginal disc-specific control element. Clearly, correct maintenance of *Ubx* expression requires additional sequences which, in contrast to the pattern-forming elements, probably do not confer specific pattern information by themselves.

The initial patterns displayed by the *Ubx* enhancers imply that these elements are targets of the early segmentation genes. The availability of these enhancers, especially in manageable sizes in the 500−1000 bp range, greatly facilitates the identification of *trans*-acting factors that control the expression of this homeotic gene. We have already shown that the BRE contains a cluster of binding sites for the product of the gap gene *hunchback* (*hb*) and that their deletion or mutation abolished *hb*-mediated repression in the

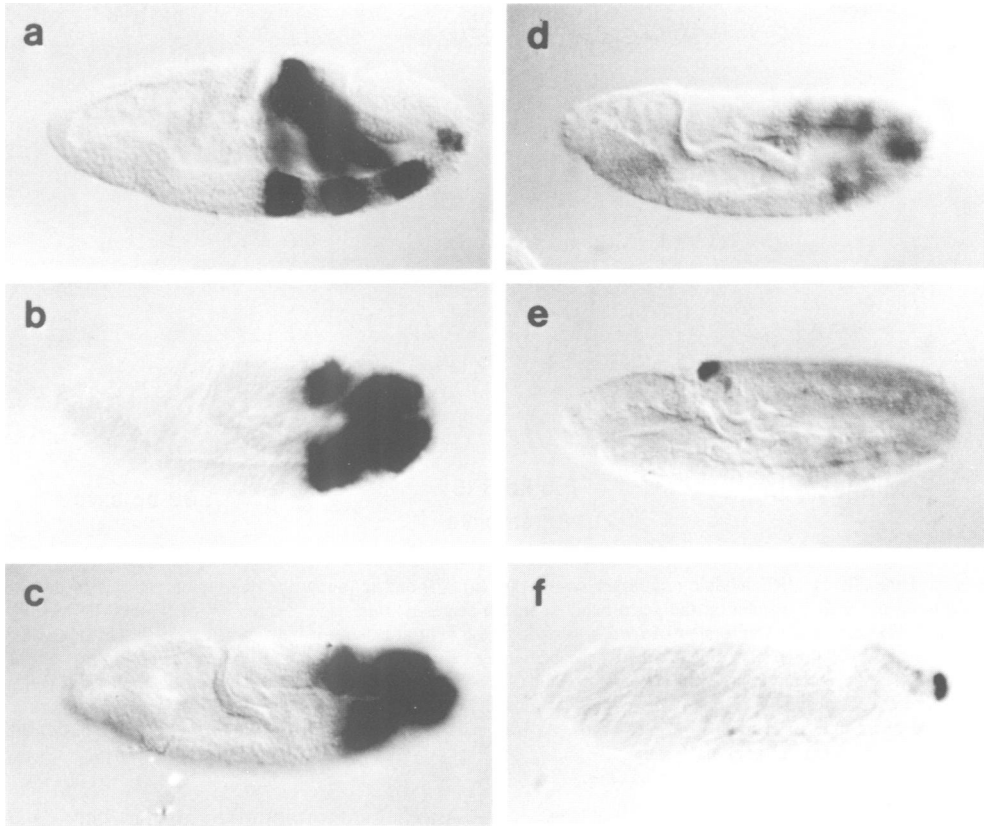

Fig. 2. RNA expression pattern of the *lacZ* reporter gene directed by the 8.8 kb BRE. Note the change of the stripe width from *ftz*-like (**a**) to wider than a parasegment (*ftz* plus *en*) (**b** and **c**) to *engrailed*-like (**d**). The stepwise change suggests that the enhancer is activated first by *ftz*, then by both *ftz* and *en*, and finally by *en* alone (see text). The *ftz*-dependent expression starts to fade as the germ band nears full extension (c and d). As the germ band retracts, the *en*-dependent expression also fades, leaving a weak, rather featureless expression in the normal expression domain PS6–12 (**e**). After germ band retraction (**f**), there is still a low level of expression in the lateral ectoderm, more noticeable in some cells of the peripheral nervous system. This expression (f) is still within the correct domain of expression (see also Figure 2e, Qian *et al.*, 1991) and might contribute to the endogenous *Ubx* expression in those tissues. Later, this low-level expression extends ectopically more anteriorly and eventually to the entire embryo, as shown by the antibody staining. The staining of the anal pad in (e) and (f) is specific for this transformant line and probably results from position effects.

anterior half of the embryo. This proved that *hb* acts as a repressor directly setting the anterior boundary during the initiation stage of *Ubx* expression (Qian *et al.*, 1991). Here, we report a detailed analysis of the structure of the BRE in terms of the binding sites of the segmentation genes *hb*, *fushi tarazu* (*ftz*), *tailless* (*tll*) and *engrailed* (*en*) and the dorso-ventral gene *twist* (*twi*). We show that the binding of these gene products and their interplay are responsible for generating the expression pattern directed by the BRE and we present data concerning the maintenance of the initiated expression pattern.

Results

The BRE active module

We have shown that an 8.8 kb interval spanning most of the *bx* region of the *Ubx* gene contains a single enhancer element (BRE) able to activate the *Ubx* promoter–*lacZ* reporter gene in the embryo (Qian *et al.*, 1991). The activating element in this region is localized within a 500 bp *Apa*I–*Eco*RI fragment (Figure 1) lying in the third intron of the *Ubx* gene, ~33 kb downstream from the transcription initiation site. Although sequences outside the 500 bp element contribute significantly to the BRE expression pattern, no other part of the 8.8 kb BRE fragment can activate the reporter gene by itself. The 500 bp fragment is therefore the only autonomous pattern-forming element. We will therefore refer to the entire 8.8 kb element as the BRE and to the 500 bp core element as the BRE module.

To characterize the BRE expression pattern with better resolution, we examined the distribution of the reporter RNA by *in situ* hybridization. Figure 2 shows the expression pattern of the 8.8 kb BRE. Like the β-galactosidase staining pattern, RNA expression is first detected in a uniform domain from 10 to 50% egg length (RNA pattern not shown, for the protein data see Qian *et al.*, 1991). Parasegmental stripes appear at gastrulation and are initially one parasegment wide, becoming broader than a parasegment only toward the end of germ band extension. A major difference between the *in situ* hybridization pattern and the protein pattern is that, at the end of germ band extension, the stripes begin to fade, first weakening in the center to give thin *engrailed*-like stripes. Later even the *engrailed*-like stripes fade leaving only a faint, uniform expression within the BRE expression domain. After germ band retraction, there remains only a very weak expression in the lateral ectoderm, extending into the head region. This late pattern appears at earlier stages with the smaller *bxI* construct or with the 500 bp BRE module. In contrast to the RNA pattern, the protein pattern remains in the form of pair-rule stripes and persists after germ band retraction (Qian *et al.*, 1991). The RNA data make it clear that, like the *Ubx* RNA, the hybrid reporter

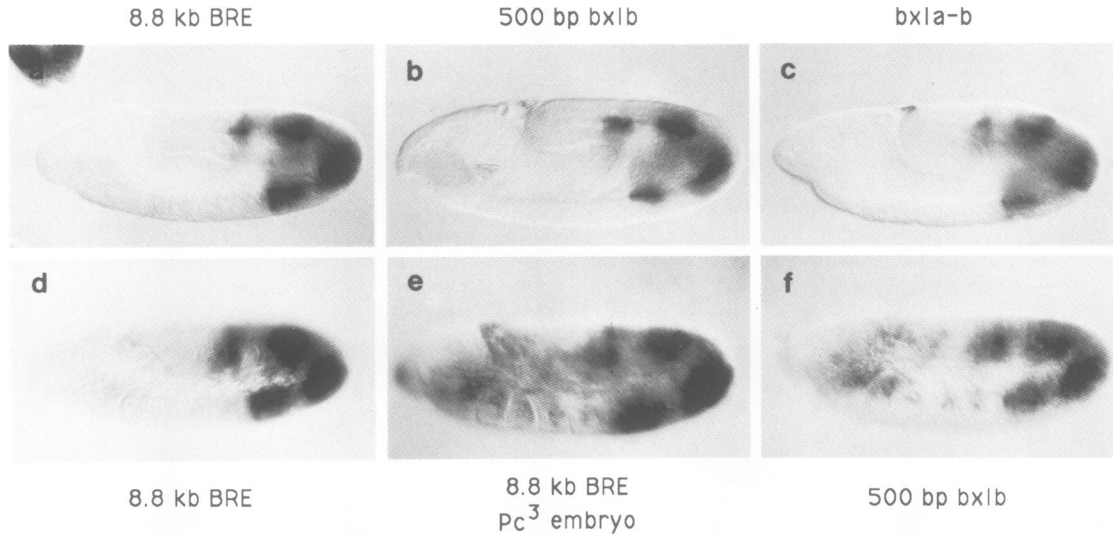

Fig. 3. Expression patterns of the 500 bp BRE module and larger constructs. (**a**), (**b**) and (**c**) show the expression patterns of the 8.8 kb BRE, the 500 bp BRE module (*bxIb*) and the *bxIa-b* element at the germ band extension stage. At this stage, expression of the 500 bp module is predominantly mesodermal (arrowhead in b). Expression of *bxIa-b* extends to the ventral ectoderm but not as much as that of the 8.8 kb BRE element. (**d**), (**e**) and (**f**) show the breakdown of maintenance. At the beginning of germ band retraction, the Pc^3 mutation (e) relaxes the 8.8 kb BRE pattern (compare with d) so that ectopic expression appears anterior of PS6, extending into the head region, as well as in cells within the normal expression domain that do not normally express. The expression of the *bxIb* module (f) in wild-type embryos shows anterior ectopic transgressions similar to those of the 8.8 kb element in Pc^3 embryos. At least part of this ectopic expression resembles the basal pattern (Bienz *et al.*, 1988) that stains cells of the peripheral nervous system.

gene transcript turns over rather rapidly and that the persistent protein pattern does not reflect the continued activity of the reporter gene at those stages but is due to the stability of the bacterial β-galactosidase. The inability of the BRE to maintain expression once the initial activators have ceased to act indicates that it does not respond to the *trithorax* group of genes, responsible for positive maintenance. Both the RNA and the protein data suggest that the BRE is activated in stepwise fashion, first by a ubiquitous activator, then by a pair-rule gene and finally by a segment polarity gene. In the rest of this paper, we will present data from anti-β-galactosidase staining instead of *in situ* hybridization since they are faster to obtain and since our focus is on the early stages of BRE expression, when RNA and protein data are comparable.

Alone, the 500 bp BRE module activates expression predominantly in the mesoderm (Figure 3b) and at a lower level in the lateral ectoderm. Its overall activity is lower than that of either the 1.7 kb *bxI* fragment (see map in Figure 1) or the intact 8.8 kb *bx* construct. Although the 8.8 kb construct expresses strongly in the mesoderm, it also expresses at a high level in the ventral and lateral ectoderm and its pattern is much richer than that of the BRE module (compare Figure 3a and 3d with 3b, and see also Qian *et al.*, 1991). Thus, the sequences flanking the BRE module enhance BRE activity and, in particular, are required for high level expression in the ectoderm. The effect of the flanking sequences is progressive: addition of 170 bp from the immediate right of the BRE module is sufficient to expand the ectoderm expression detectably (construct *bxIb-Pst* in Figure 1, pattern not shown); the addition of a 700 bp *Sal*–*Apa*I fragment from the left of the BRE module (construct *bxIa-b* in Figure 1) gave stronger ectoderm expression, though still less pronounced than that of the entire 8.8 kb DNA (compare Figure 3a with 3c).

Another important effect of the flanking sequences is to stabilize the expression pattern. The expression of the 8.8 kb BRE–*Ubx*–*lacZ* fusion gene is clearly more stable than that of smaller BRE constructs. Its anterior boundary is maintained until after germ band retraction, with slight variations between different transformed lines. After stage 14 (Campos-Ortega and Hartenstein, 1985), weak ectopic expression appears gradually anterior of PS5 and extends into the head region until the entire embryo becomes β-galactosidase positive. The smaller constructs show ectopic anterior expression much earlier. For example, the ectopic expression of the *bxI* construct appears at about stage 11, at the beginning of germ band retraction; the 500 bp BRE module shows still stronger and earlier ectopic expression (Figure 3f). Part of this ectopic expression corresponds to the basal pattern activated by the *Ubx* promoter, characterized by staining of the cells of the peripheral nervous system (Bienz *et al.*, 1988; Qian *et al.*, 1991). The ectopic expression displayed by the smaller constructs is very similar to that produced by the 8.8 kb BRE in embryos homozygous for a *Pc* mutation. Figure 3e shows that in such *Pc*-deficient embryos, ectopic expression of the 8.8 kb BRE construct begins earlier and the normal pattern itself is appreciably weaker. The weakening probably results from the negative effects of the posterior homeotic genes *abd-A* and *Abd-B*, since the lack of *Pc* activity also relaxes the restriction of anterior expression of these two genes, which normally down-regulate *Ubx* in PS7–13 (Struhl and White, 1985; Wedeen *et al.*, 1986). The similarity between the expression of the intact 8.8 kb BRE in the Pc^- embryos and of the smaller BRE constructs in normal embryos suggests the presence of multiple sequences that, to a lesser or greater extent, interact with products of the *Pc* group genes and contribute to maintain the correct pattern of *Ubx* expression.

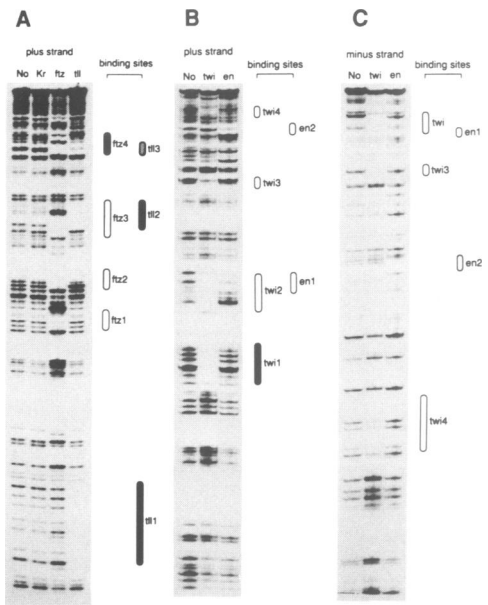

Fig. 4. Footprints of *ftz*, *tll*, *twi* and *en* proteins. (**A**) *ftz* and *tll* footprints on the plus strand of the 500 bp BRE module (corresponding to the sequence shown in Figure 5); (**B**) *twi* and *en* footprints on the plus strand of the 500 bp module; (**C**) *twi* and *en* footprints on the minus strand of the 500 bp module. Lanes labeled No are controls with no protein extract added. The *Kr* lane in (A) is another control using *Kr* expressing extract which does not bind to *bx* DNA. The positions of the footprints are indicated by the boxes to the right of the gels. The degree of shading gives a rough estimation of the binding affinity for each site.

hb, tll, ftz, en, twi bind to the BRE module

Genetic analysis indicates that *hb*, *ftz* and *tll* are the best candidates for direct regulators of *Ubx* expression (Ingham and Martinez-Arias, 1986; White and Lehmann, 1986; Irish *et al.*, 1989; Reinitz and Levine, 1990). *hb* binds to the BRE and PBX elements (Qian *et al.*, 1991; Zhang *et al.*, 1991) and we have shown that the *hb* binding sites in the BRE are essential for setting the anterior boundary of BRE expression, proving that *hb* is a direct regulator of *Ubx in vivo*. We have also shown that, just as for the endogenous *Ubx* gene, *tll* is needed to establish the posterior boundary of BRE expression, while *ftz* is required for the enhancement in the even-numbered parasegments (Qian *et al.*, 1991). The transition from the pair-rule stripes to the *en*-like stripes shown by the *in situ* hybridization data (Figure 2) suggests that the segment polarity gene *en* might be the activator for the late pattern.

The *bx* region is necessary for correct *Ubx* expression in the abdominal somatic mesoderm that gives rise to the larval abdominal musculature (Hooper, 1986). In the entire *abx/bx* regulatory region, the BRE element is the only enhancer directing strong embryonic mesoderm expression (Simon *et al.*, 1990; Qian *et al.*, 1991). This is first detectable as an enhancement of expression in the mesoderm anlage at the beginning of ventral furrow formation. This expression increases gradually as gastrulation proceeds and the mesoderm becomes the predominant site of expression at the germ band extension stage. The mesodermal preference is particularly pronounced with constructs containing the 500 bp BRE module (Figure 3b), indicating that mesodermal factors have target sites within its sequence. The likely candidates for activators of mesoderm expression are the products of *twist* (*twi*) and *snail* (*sna*), zygotic genes activated in the ventral region of the embryo by the maternal morphogen *dorsal* (Jiang *et al.*, 1991; Pan *et al.*, 1991; Thisse *et al.*, 1991). Genetic studies indicated that the *twi* product, a helix−loop−helix DNA binding protein (Thisse *et al.*, 1988), is required to activate mesoderm-specific genes while the *sna* product, a zinc finger DNA binding protein (Boulay *et al.*, 1987), prevents ectoderm and neurectoderm genes from being expressed in the mesoderm (Kosman *et al.*, 1991; Leptin, 1991).

Consistent with these considerations, DNase I footprinting analyses reveal that, in addition to *hb*, *tll*, *ftz*, *en* and *twi* bind to the 500 bp BRE module (Figure 4). Figure 5 summarizes the binding sites of these proteins and their sequences. *tll* binds to three high affinity footprinting sites. *ftz* has two low affinity (sites 1 and 2) and two medium affinity sites (sites 3 and 4). DNase hypersensitivity indicates the presence of three additional very weak *ftz* binding sites on the 5′ side of footprint 4 (Figure 5 and data not shown). *twi* has two high affinity sites (sites 1 and 2) and two weak sites (sites 3 and 4, Figure 4). Finally, *en* binds to two rather weak sites, one overlapping *ftz* site 3 and the other overlapping one of the very weak *ftz* sites. It is evident from Figure 5 that the binding sites of these regulators are closely clustered or overlap extensively, suggesting functional interactions. No binding sites for *sna* protein were detected.

tll sets the posterior boundary

The *tll* product is a member of the retinoic acid receptor family and has been shown to have sequence-specific DNA binding activity (Pignoni *et al.*, 1990; Hoch *et al.*, 1992; Pankratz *et al.*, 1992). We have shown that deleting the *hb* binding sites not only resulted in the anterior shift of the BRE expression pattern but also relaxed its posterior boundary, implying that the deletion also removed at least some *tll* binding sites in the enhancer (Qian *et al.*, 1991). The positions of the *tll* footprints confirmed this. The arrangement of *tll* binding sites in the 500 bp BRE module is such that *tll* site 3 overlaps with *hb* site 3 as well as *ftz* site 4 (Figure 5). The deletion that removed *hb* site 3 (Qian *et al.*, 1991) also removed much of *tll* site 3. The resulting change in the expression pattern suggests that the deletion frees *ftz* site 4 from repression both in the anterior half of the embryo and in the posterior terminal region. This result confirms that both *hb* and *tll* repress BRE directly. The *tll* footprints shown in Figure 4 were obtained with a putative *tll* DNA binding domain expressed in bacteria (expression plasmid from J.A.Lengyel, see Hoch *et al.*, 1992). Although the full length, bacterially expressed *tll* protein binds weakly to *bx* DNA in immunoprecipitation assays, we have not yet been able to obtain footprints with it, perhaps because it is expressed poorly in bacteria compared with the DNA binding domain (not shown). It is also possible that efficient *tll* binding to DNA requires some unknown ligand to expose the DNA binding domain, or that it must complex with other proteins as is the case for some of the steroid hormone receptors to which *tll* bears homology (Glass *et al.*, 1989; Liao *et al.*, 1990).

Regulation by ftz

The *ftz* product is a homeodomain protein that has been shown to stimulate transcription *in vitro* (Ohkuma *et al.*,

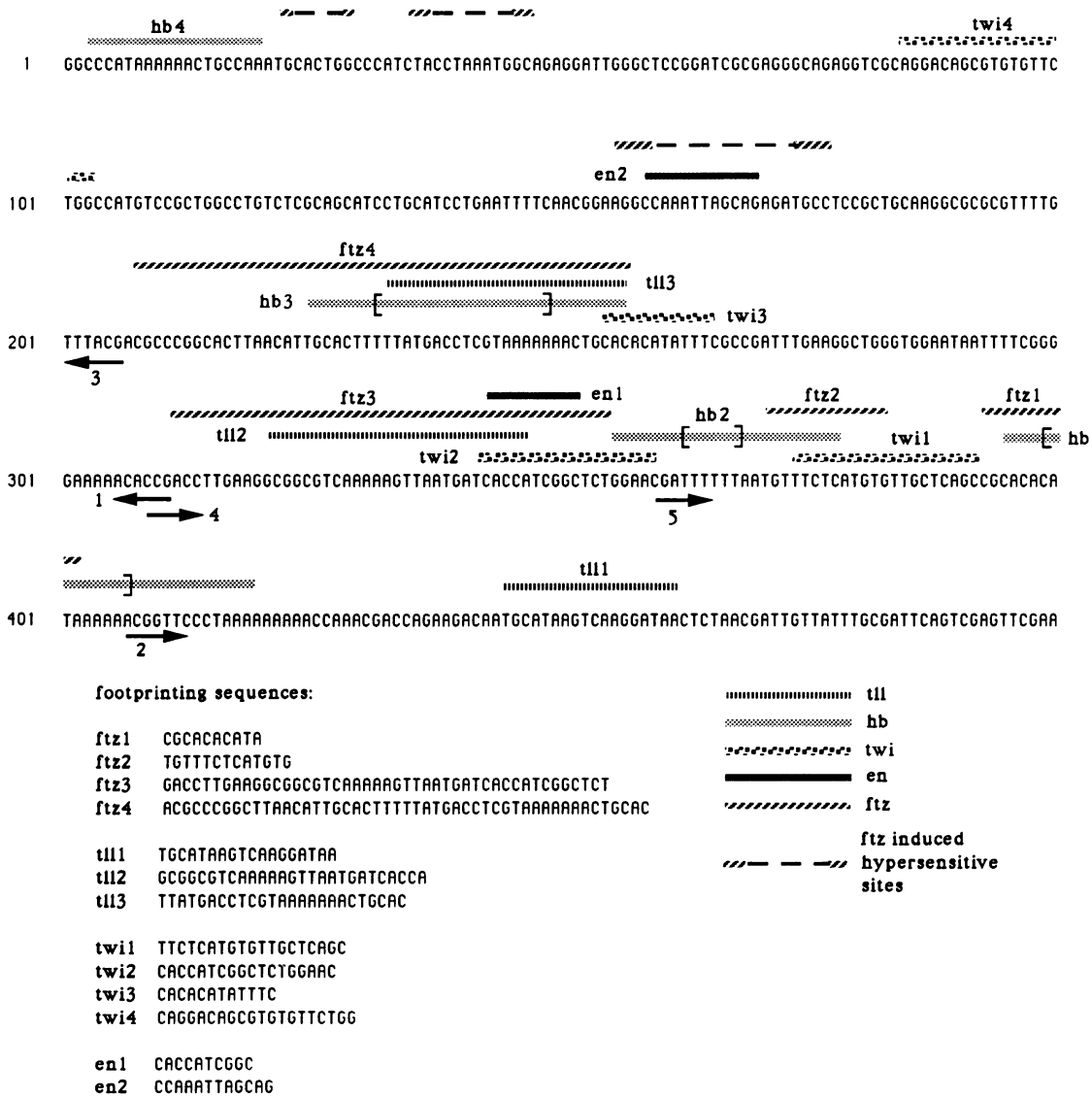

Fig. 5. Sequence of the 500 bp BRE module with *hb*, *ftz*, *tll*, *twi* and *en* footprinting sites. The binding sites for different proteins are marked by bars with different patterns identified in the lower right corner of the figure. There are four sites for *hb*, *ftz* and *twi*; three for *tll*; two for *en*; in addition, *ftz* protein induces three hypersensitive regions (gel not shown). The sequences of the *ftz*, *tll*, *twi* and *en* footprints are summarized below. The brackets in *hb* binding sites 1, 2 and 3 mark the sequences deleted in the *bxIΔhb1−3* element (Qian *et al.*, 1991). Arrows below the sequence represent primers used in PCR reactions to make internal deletions (see Materials and methods). The pair of primers 1 and 2 was used to delete *ftz* sites 1−3 (*bxIΔftz1−3*); the pair of primers 2 and 3 was used to delete all four *ftz* sites (*bxIΔftz1−4*); the pair 3 and 4 for *ftz* site 4 deletion (*bxIΔftz4*); and the pair 3 and 5 for *ftz* site 3−4 deletion (*bxIΔftz3−4*).

1990) and bind to a variety of sequences with the consensus TCAATTAAAT (Desplan *et al.*, 1988; Hoey and Levine, 1988). Of the four footprint sequences presented in Figure 5, only a TTAAT in the middle of site 3 agrees with part of the TCAATTAAAT consensus sequence. The other three sites, and especially site 4, have no obvious resemblance to the consensus sequence or to one another. The *ftz* binding sites are so clustered with those of *hb* and *tll* that they partially overlap or are closely associated with them (Figure 5). We assessed the functionality of these *ftz* sites by a series of deletions as well as by a mutational analysis of site 4.

When a 96 bp DNA spanning *ftz* sites 1, 2 and 3 (construct *bxIΔftz1−3*, Figures 1 and 5) was removed, the resulting element, containing only *ftz* site 4, displays almost normal initiation of expression until early germ band elongation, giving a pattern whose anterior border is set at ~50% egg length and whose expression is enhanced in the even-numbered parasegments (Figure 6A, a and b). However, towards the end of germ band elongation, the pattern starts to differ from the BRE pattern in that the enhancement in the even-numbered parasegments is less pronounced than normal and each stripe becomes split into two narrower bands (Figure 6A, c). The pattern is also less stable and ectopic expression appears between stripes and spreads into the anterior part of the embryo. Similarly, when a 103 bp deletion removes *ftz* site 4 and the sequence between sites 3 and 4, the pattern directed by the resulting *bxIΔftz4* construct resembles the wild-type BRE pattern at early stages and is followed by the resolution of the even-numbered stripes into narrower *en*-like stripes at the end of germ band extension (Figure 6B, a and b).

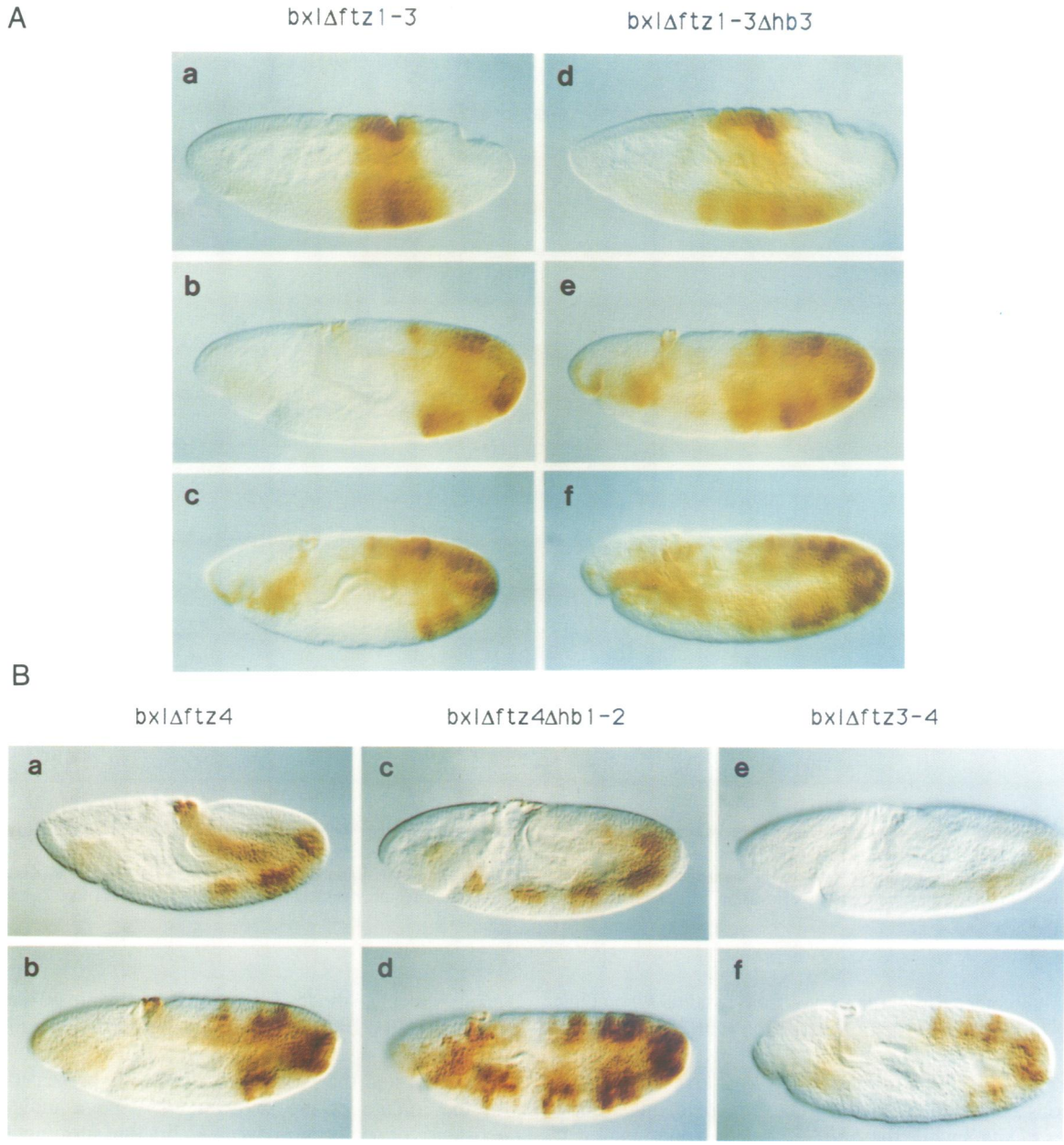

Fig. 6. *ftz* binding sites are essential for pair-rule expression. (**A**) Removal of *ftz* sites 1, 2 and 3 causes early loss of pair-rule expression. The pattern given by *bxIΔftz1−3* is comparable with that of the wild-type BRE at early gastrulation (**a**) and germ band extension stages (**b**). At the time of segment formation (**c**), the *bxIΔftz1−3* pattern starts to differ from the wild-type by resolving each of the first three stripes into two narrower bands (f). (**d**), (**e**) and (**f**) show the effect of deleting the *hb* (and *tll*) consensus sequence in the remaining *ftz* site 4. Now both the anterior and posterior borders, set by *hb* and *tll*, are relaxed, giving a new stripe at PS4 and an additional half stripe at PS12. The new stripes are weaker than the normal stripes or than the ectopic stripes obtained with our previous deletion construct *bxIΔhb1−3* (Qian *et al.*, 1991). The difference suggests that some partial repression still exists which, in the absence of the other *ftz* binding sites, cannot be completely overcome by *ftz* binding at site 4. (**B**) Progressive loss of *ftz* binding sites leads to increasing *engrailed*-like stripes. The expression of *bxIΔftz4* (**a** and **b**) shows that *ftz* sites 1−3 are able to direct a *ftz*-like pattern but the stripes are more *engrailed*-like than the wild-type pattern (Figure 3a). Removal of *hb* sites 1−2 from *bxIΔftz4* (construct *bxIΔftz4Δhb1−2*) relaxes the anterior boundary (**c** and **d**). Removal of *ftz* site 3 from *bxIΔftz4* (construct *bxIΔftz3−4*) converts the largely *ftz*-like stripes to *en*-like stripes (**e** and **f**).

These changes indicate that *ftz* site 4 and one or more of *ftz* sites 1−3 are functional on their own and are important for continued and high level BRE expression in the pair-rule fashion. The results also show that these *ftz* sites are independently regulated by the *hb* and *tll* binding sites that overlap with them. The importance of such overlap was further demonstrated when we partially deleted the *hb* binding sequences in these two constructs without impairing the *ftz* binding sites (constructs *bxIΔftz1−3Δhb3* and *bxIΔftz4Δhb1−2*, Materials and methods). Entirely consistent with our previous *hb* site deletion experiment, the removal of the overlapping *hb* sites resulted in a relaxation of the anterior border of the expression domain in the case of *bxIΔftz4Δhb1−2* (Figure 6B, c and d), and of both the anterior and posterior boundaries in the case of *bxIΔftz1−3Δhb3* (Figure 6A, d, e and f). *bxIΔftz4Δhb1−2* displays a stronger anterior shift than *bxIΔftz1−3Δhb3*, suggesting that the remaining *ftz* site 4 in *bxIΔftz1−3Δhb3*

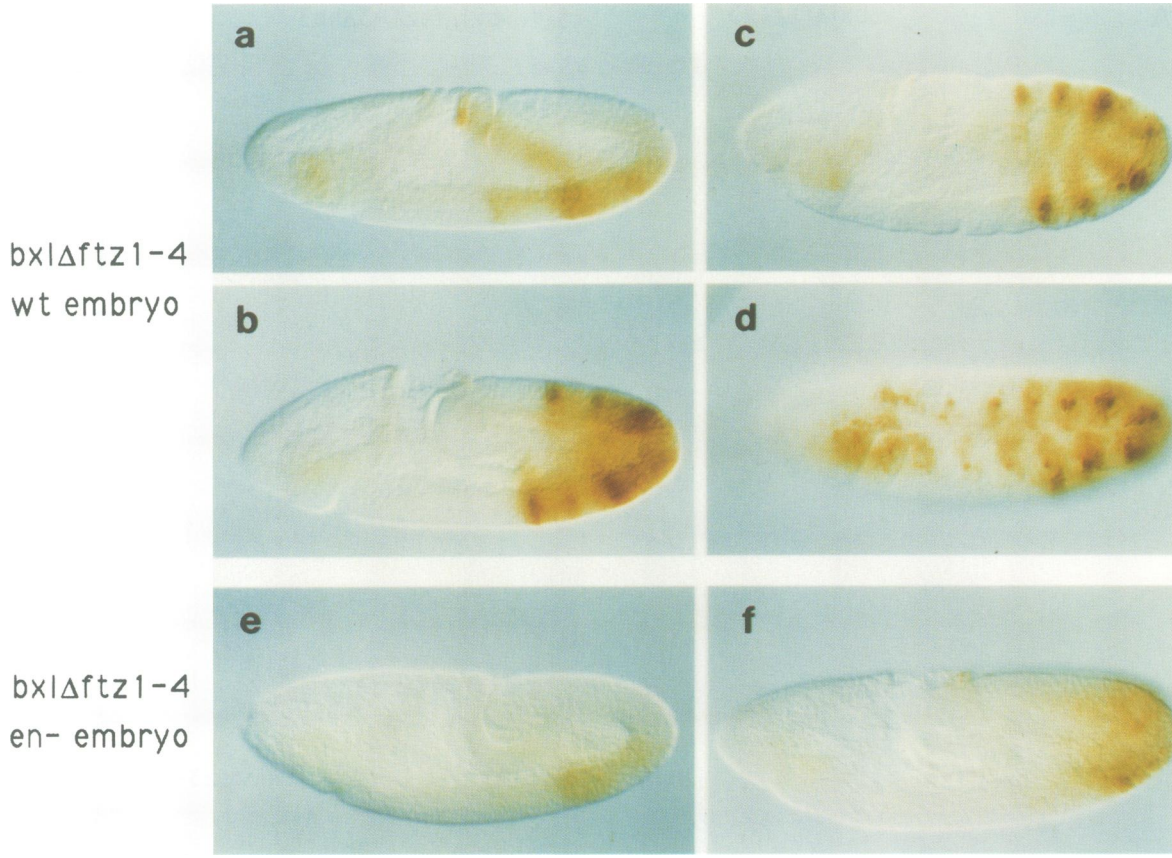

Fig. 7. Removal of *ftz* sites leaves *en* stripes. The expression of the deletion construct *bxlΔftz1−4* shows slight pair-rule modulation at early gastrulation (**a**). As germ band extends, the expression quickly evolves into an *en*-like stripe pattern (**b** and **c**). Anterior ectopic expression appears by the time the germ band starts to retract and segmental grooves appear (**d**). In *en*$^{-}$ embryos [homozygous *en*^{B}, (**e** and **f**)], the stripe pattern is replaced by a low level of homogeneous expression, indicating that the stripe generation requires *en* activity and that a ubiquitous factor is involved in the activation.

is either still under partial repression from residual *hb* binding activity or needs synergistic interaction with *ftz* sites 1−3 to be fully active in anterior regions. In contrast, *bxlΔftz1−3Δhb3* but not *bxlΔftz4Δhb1−2* gives a posterior shift, consistent with the fact that the *Δhb3* deletion partially removes *tll* site 3 while the *Δhb1−2* deletion does not touch *tll* sites 1 and 2. This indicates that *tll* site 2, which overlaps *ftz* site 3, and perhaps *tll* site 1 (see Figure 5) effectively block *ftz* sites 1−3 from activating the promoter in the posterior terminal region.

When we removed an additional 103 bp, including *ftz* site 4 and the sequence between *ftz* sites 3 and 4 from *bxlΔftz1−3*, the pair-rule pattern was abolished, except for a slight enhancement in even-numbered parasegments at the beginning of germ band extension (constructs *bxlΔftz1−4*, Figure 7a−d). This weak early modulation may be due to three very weak *ftz* sites that remain in the element and are detected as DNase hypersensitive sites (Figure 5). Expression from this construct is detectable at the blastoderm stage, though much weaker and in a somewhat narrower domain than before. As the germ band extends dorsally, the expression evolves into an *en*-related pattern with narrow stripes in every parasegment. A somewhat more surprising aspect of the *bxlΔftz1−4* enhancer is that, even in the absence of the strong *hb* binding sites 1, 2 and 3, the anterior border remains at ~50% egg length at blastoderm and at PS6 at later stages. We interpret this to mean that these three *hb* sites regulate the *ftz*-dependent stripes while *en*-like stripes are controlled by other *hb* sites. A fourth strong *hb* footprint is found near the *ApaI* end of the BRE module (Figures 1c and 5) and the presence of DNase hypersensitive sites in the middle of the *bxlΔftz1−4* element indicates the existence of one or more weak *hb* binding sites (not shown). We suppose that when *hb* sites 1, 2 and 3 are mutated without removing the *ftz* binding sites associated with them, more anterior *ftz*-dependent stripes are produced. When these *ftz* sites are also removed, *hb* site 4 and other weaker *hb* sites still exert firm control over the remaining activators. When *ftz* site 3 was removed from the *bxlΔftz4* element by a 54 bp deletion (*bxlΔftz3−4*, Figures 1c and 5), the pair-rule modulation is again abolished, giving rise to an *en*-like pattern within the BRE expression domain (Figure 6B, e and f). This result confirms that *ftz* site 3 is functional. The initial weak pair-rule modulation of *bxlΔftz3−4* lasts a bit longer than that of *bxlΔftz1−4*, an effect attributable to the presence of *ftz* sites 1 and 2 in this construct.

The deletion analysis strongly argues that *ftz* sites 3 and 4 are functional and that their combined activity, plus the contribution of *ftz* sites 1 and 2, constitutes the functional core of the BRE enhancer. This conclusion is further supported by site-directed mutagenesis of *ftz* site 4 of *bxlΔftz1−3*. Reasoning that the FTZ protein might recognize near-consensus sequences, we mutated three TAAs in *ftz* site 4 and one of the two CACTT sequences (Figure 5 and

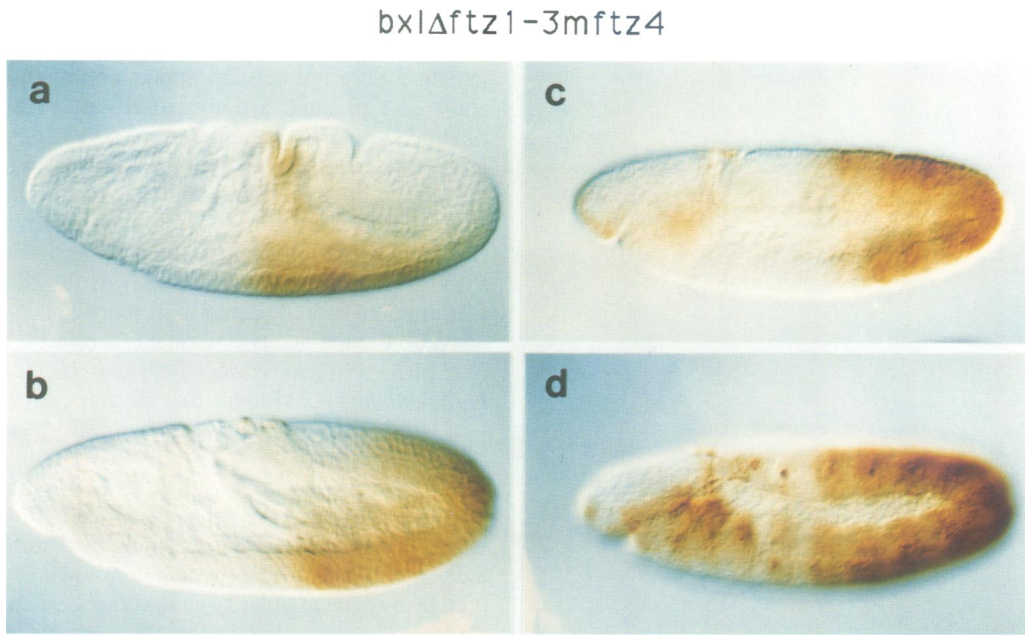

Fig. 8. Site-directed mutagenesis confirms that *ftz* site 4 is functional. We mutated seven nucleotides in the 50 bp *ftz* site 4 of the *bxIΔftz1−3* element, affecting TAAT and CACTT motifs. These changes abolished *in vitro* binding of *ftz* to this site (data not shown) and converted the pair-rule modulation of the *bxIΔftz1−3* pattern into a uniform expression at early stages (**a** and **b**) on which a faint *en*-like pattern is superposed at later stages (**c** and **d**). The loss of pair-rule modulation shows that binding of *ftz* to this site is responsible for the *ftz*-like pattern. The strength of the uniform expression compared with that of the *bxIΔftz1−4* element (Figure 7e and f) suggests that ubiquitous activators have targets in the region between *ftz* sites 3 and 4.

Materials and methods). Assayed *in vitro*, *ftz* site 4 of the mutated *bxIΔftz1−3mftz4* no longer binds to *ftz* (not shown). As predicted, the expression pattern of the mutated *bxIΔftz1−3mftz4* closely resembles that of *bxIΔftz1−4*. Expression begins at a low level, as does that of *bxIΔftz1−4*, with slight pair-rule modulation, then it becomes stronger but rather uniform within PS6−12. As the germ band extends, the uniform pattern evolves into *en*-like stripes like those of *bxIΔftz1−4* (Figure 8, compare with Figure 7a−d). In some of the embryos, the *en* stripes are detectable in earlier stages as thin stripes superimposed on the uniform staining. The loss of *ftz* binding and of pair-rule expression resulting from the mutation of *ftz* site 4 proves that this site is functional.

A slight but significant difference between the expression of *bxIΔftz1−3mftz4* and *bxIΔftz1−4* is that the latter evolves into *en*-like stripes early and the inter-stripe expression level is low while the former has stronger uniform expression that evolves into the *en*-like stripes rather late. This suggests that sequences either within *ftz* site 4 or, more likely, between sites 3 and 4 contain binding sites for a uniformly distributed activator. Such a ubiquitously present factor might be responsible for the initial homogeneous activation of the BRE as well as the uniform BRE and *Ubx* expression in *ftz* null embryos. As will be discussed when we examine the *en* input, additional site(s) for the ubiquitous factor are likely to reside outside this 103 bp interval between the 5′ ends of the *ftz* sites 3 and 4. Thus, there could be two or more binding sites for the ubiquitous activator, just as there are multiple sites for *ftz*, *en*, *twi*, *hb* and *tll*. The resulting expression pattern would depend on the relative contribution from each of these factors. If the ubiquitous activator predominates, the pattern will be rather uniform; if there are more *en* binding sites, the pattern will be more *en*-like, etc.

These results indicate that most, if not all, of the four *ftz* binding sites are functional. This, plus the lack of strong homology between them, suggests that even *in vivo* the FTZ protein can bind potentially with rather low specificity. Since not all *in vitro* binding sites are functional in regulating transcription, we suppose that, *in vivo*, the target specificity of the *ftz* homeoprotein may not be defined solely by the binding sequence. Additional interactions, perhaps with the ubiquitously expressing activator(s), might be required to enhance binding or to make the bound *ftz* protein functional as a transcriptional regulator. This conclusion is supported by the fact that the TCAATTAAAT consensus sequence by itself cannot confer *ftz*-dependent pair-rule stripe expression *in vivo* (Vincent *et al.*, 1990), and that although *ftz* binds to the *bxII* and *bxIII* DNA fragments (Qian *et al.*, 1991) more efficiently than to the BRE module itself *in vitro* (data not shown), these fragments are silent in the reporter gene constructs.

Competitive binding of the hb and ftz to the BRE module

It is clear from the distribution of the binding sites of *hb*, *tll*, *ftz*, *en* and *twi* in the enhancer sequence (Figure 5), that the products of these five early pattern-forming genes do not bind independently of one another. In particular, the significant overlap of the binding sites for the repressors *hb* and *tll*, and the activators *ftz* and *twi* suggests that the repressors may prevent the binding of the activators or that the binding of the repressors interferes with the proper function of the activators, or that both mechanisms are operating. In a competitive footprinting assay, we found that the *hb* product is able to interfere with the binding of the *ftz* product to *ftz* sites 1, 2 and 4 (Figure 9) or at least to alter the extent of the *ftz* footprint to the point that it cannot

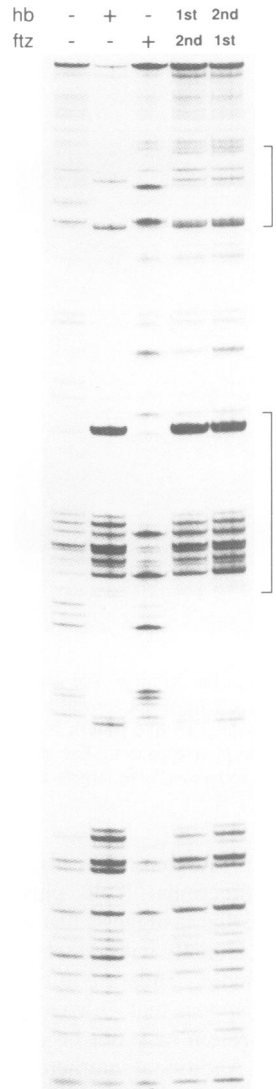

Fig. 9. Competitive binding of *hb* and *ftz* to the BRE module *in vitro*. The mutual interference of *hb* and *ftz* was assayed by incubating one protein with the DNA probe for 20 min before adding the second protein. DNase I digestion followed after a further 20 min. The lanes are marked to indicate reaction with no added protein, with a single protein or with both proteins in either order of addition. Regardless of the order of addition, *hb* is able to convert the *ftz* protection pattern to the *hb* pattern (best demonstrated by *ftz* sites 2 and 4, marked by brackets). This suggests that *hb* not only prevents binding of *ftz* to an overlapping site but also displaces pre-bound *ftz*.

be distinguished from the footprint produced by *hb* alone. This interference is apparent regardless of the order of addition of the two proteins, suggesting that *hb* protein can actually displace the previously bound *ftz* product. Although the assay is rather crude and the relative amounts of active proteins used may not reflect the *in vivo* situation, the result shows that competitive binding can be demonstrated *in vitro* and is a simple and likely explanation for the antagonistic interaction of *hb* and *ftz in vivo*. Similar competition between *bicoid* and *knirps* products for binding to a regulatory element of the *Krüppel* (*Kr*) gene has been shown to be important for the spatial control of *Kr* expression (Hoch *et al.*, 1992) and competition between *bicoid* and *hb* and *Kr* products is important for defining the borders of *eve* stripe 2 (Small *et al.*, 1992). The competitive binding explanation does not exclude other types of interaction. For example, *ftz* site 3 does not overlap appreciably with *hb* site 2; *twi* site 4 and *en* site 2 are not closely linked to the *hb* or *tll* binding sites. However, the repressors can apparently still silence the effect of activators binding to these sites, implying that they either interfere with the function of the bound activators or block the binding of other co-activators. Repression through competition and interference argues for a short-range effect of *hb* as shown, for example, by the fact that *hb* site 4 could not repress the *ftz*-dependent activation in the anterior segments seen with the constructs *bxIΔhb1−3* (Qian *et al.*, 1991), *bxIΔftz1−3Δhb3* and *bxIΔftz4Δhb1−2* (Figure 6).

Input from en and twi

The *in situ* RNA hybridization data show that BRE expression is activated in succession by a uniform activator, then by a pair-rule gene, which, as shown above is *ftz*, and then by a segment polarity gene at late germ band extension stages. The contribution of the segment polarity gene accounts for the observed wider-than-one parasegment stripes of the protein pattern, and is consistent with the *en*-like stripes produced by the truncated elements such as *bxIΔftz3−4*, *bxIΔftz1−4*, *bxIΔftz1−3mftz4* (Figures 6, 7 and 8), in which the *ftz* binding sites are removed or mutated. The most likely activator of BRE in the *en* expressing domain is naturally *en* itself. Several pieces of evidence support *en* as a direct regulator: (i) when the *bxIΔftz1−4* element is introduced into an en^- background, the *en*-like stripes are replaced by a weak, uniform pattern of expression (Figure 7e and f); (ii) the BRE module contains two target sites for *en* binding (Figures 4 and 5), the stronger of the two, *en* site 2, is included in the *bxIΔftz1−4* element, accounting for the *en*-like pattern it produces; and (iii) the expression pattern of the *bxIΔftz4* is more *en*-like than that of the *bxIΔftz1−3*, consistent with the fact that *bxIΔftz4* has two *en* sites while *bxIΔftz1−3* has only one. The weak, uniform expression of the *bxIΔftz1−4* in en^- embryos suggests the presence of binding site(s) for the ubiquitous activator in this truncated element, similar to those postulated to be in the interval between *ftz* sites 3 and 4 (see above). The *en* site 2 of the *bxIΔftz1−4* construct is not closely linked to either *hb* or *tll* binding sites yet the *en*-dependent pattern is repressed by both gap gene products. This might be because *en* binds only weakly to the enhancer and can be competed out by nearby *hb* and *tll* proteins whose binding is too weak to be detected in the condition used for footprinting, or because *en* activation depends on other factors whose binding is blocked by the remaining *hb* site 4 and *tll* site 1. An alternative that we cannot strictly exclude yet, is that, despite the *en* footprints, it is not *en* but another factor dependent on *en* that is directly responsible for the late *en*-like pattern. One paradox of the *en* dependence model is that, in the absence of *ftz*, BRE produces a uniform pattern instead of the expected *en*-like stripes. A plausible explanation for this is that, since *ftz* is required for *en* expression, in a ftz^- embryo only the odd-numbered *en* stripes remain. Since expression of BRE in odd *en* stripes is considerably weaker, the remaining stripes in ftz^- embryos might be more difficult to discern. In fact, a weak pattern is sometimes observable in the BRE expression of ftz^- embryos.

Somatic mesoderm is the major tissue of BRE-directed expression. The presence and the positions of *twi* binding sites in the BRE module make it very likely that *twi* directly activates mesodermal expression of BRE. In *twi* null embryos at the beginning of ventral furrow formation, the normal enhancement of BRE expression in the mid-ventral region (Figure 2c of Qian *et al.*, 1991) is undetectable, leaving only weak expression in the presumptive mesoderm (not shown). The lack of mesoderm enhancement of BRE in *twi* mutant embryos could be due to either the lack of *twi* or the consequent absence of mesoderm (Leptin and Grunewald, 1990; Leptin, 1991). Therefore, the conclusive proof that *twi* acts directly on BRE will require mutation of the *twi* binding sites. Mesodermal BRE expression maintains the pair-rule type of modulation, that is, only the even-numbered parasegments display intense expression (Figure 3b) while the interstripe regions remain low in β-galactosidase activity. Since *twi* expression itself has no pair-rule modulation (Leptin, 1991), mesodermal activation is either inhibited in odd-numbered parasegments by some repressor or requires a co-activator in addition to *twi* itself. A simple interpretation would be that the joint action of *ftz* and *twi* is necessary to enhance expression in the mesoderm.

Discussion

Pattern formation

The *Ubx* gene has a complex pattern of expression. The task of establishing the full *Ubx* pattern is divided among several regulatory modules scattered over some 100 kb, each responsible for either even- or odd-numbered parasegments and for different parts of the pattern within each segment. In addition to the BRE and the ABX elements, located ~30 and 50 kb downstream of the promoter, there exist at least five autonomous pattern-forming modules in the upstream *bxd* and *pbx* regions (Simon *et al.*, 1990; Müller and Bienz, 1992; V.Pirrotta and D.McCabe, unpublished data). These elements differ in the details of their expression pattern; they may express in the even or in the odd parasegments, they have different distribution of intensities in the different parasegments, produce broader or narrower stripes; some are dorso-ventrally uniform while others are concentrated in the ventral region. In addition, some of these elements, e.g. BRE and PBX, are active in the somatic mesoderm; others, like ABX and BXD, direct CNS expression. Visceral mesoderm expression, lacking a metameric pattern, is directed by separate elements located ~3 kb upstream of the transcription initiation site and by a redundant element in the *bxd* region (Bienz *et al.*, 1988; Irvine *et al.*, 1991).

The molecular dissection of the BRE module reveals the interplay of five regulatory genes responsible for the even-numbered parasegment stripes that constitute the BRE contribution to the overall *Ubx* expression pattern. Although, to be precise, we have not eliminated the possibility that it is not the *ftz*, or *hb*, etc. products that act on BRE *in vivo* but other proteins that are strictly dependent on them, bind at the same sites and are expressed in the same pattern at the same time, we consider this a remote likelihood and conclude that almost surely, the *ftz*, *hb*, etc. products are the direct regulators of BRE. Second site suppression experiments of the type demonstrated by Schier and Gehring (1992), would resolve lingering doubts but would be cumbersome with the large number of regulators acting on the BRE. We interpret our results as showing that the pair-rule protein *ftz* is the primary activator responsible for the metameric pattern; the segment polarity gene *en* expands each stripe and adds further anterior–posterior articulation within it; the proper domain of expression is restricted by *hb* anteriorly and *tll* posteriorly while mesoderm expression is enhanced through concerted action of *ftz* and *twi*. These gene products acting on the BRE module establish the basic pattern of expression but additional sequences flanking the module are important to enhance the level of expression, particularly in the ventro-lateral epidermis, and to help maintain the correct pattern and its boundaries during later embryonic development. Such a picture agrees well with the genetic analysis which showed that the metameric nature of *Ubx* expression is the result of combinatorial action of the gap, pair-rule and segment polarity genes, with *ftz* and *evenskipped* (*eve*) as the primary activators and *hb* and *tll* as the primary negative regulators (Ingham and Martinez-Arias, 1986; White and Lehmann, 1986; Martinez-Arias and White, 1988; Irish *et al.*, 1989; Reinitz and Levine, 1990). The regulatory principles that we have found in the BRE appear applicable to other control modules of *Ubx*, with appropriate modifications. For example, the *ftz* protein has been recently shown to be responsible for the PBX metameric expression (Müller and Bienz, 1992). In the elements that produce odd-numbered stripes, we expect the pair-rule gene *eve* to replace *ftz* as the metameric activator; in some cases additional input from other pair-rule or segment polarity genes might further modulate the anterior–posterior pattern within a parasegment while genes like *sna* might modify the dorso-ventral distribution of expression; we expect that some proneural factor will be involved in turning on or maintaining expression in the precursors of neural tissue. To a first approximation, we suppose that the additive contributions of these several regulatory modules combine to account for most of the parasegmental pattern and intrasegmental detail observed with the intact endogenous *Ubx* gene.

One deviation from the genetic predictions is in the role of the segment polarity gene *en*. The overall expression of the endogenous *Ubx* gene is down-regulated by *en* in the anterior compartment of each parasegment. In contrast, the parasegmental stripe expressed by the 8.8 kb BRE construct is rather uniform within a parasegment and the stripe actually extends into the anterior compartment of the adjacent posterior parasegment. This kind of pattern is common to some (e.g. ABX, PBX) but not other autonomous modules. While *en* seems to have a negative effect on the endogenous *Ubx* gene, it activates the BRE and some but not all of the other isolated modules. One explanation for this apparent difference might be that some regulatory elements are actually repressed by *en* and their cumulative contribution to the overall *Ubx* pattern is much greater than the contribution of the elements that are activated by *en*. An alternative explanation is that some of the elements contain an *en*-dependent silencer able to suppress the response of the promoter in *en*-containing cells.

Promoter competition and pattern maintenance

The presence of several partially redundant control elements raises the questions of how they can all control the same promoter and to what extent the behavior of the isolated elements reflects their activity in the intact gene. To put these

questions in a different form, are the different elements interacting with one another in such a way that their expression in combination is different from the sum of the expression of the separate elements? The expression in the *en* stripes, noted above, might be an example of such an interaction whereby one element might suppress the activity of another in the presence of *en* product. Another example is suggested by the ability of the 8.8 kb BRE element to suppress expression of the basal pattern which is directed by sequences just downstream of the *Ubx* promoter (Bienz *et al.*, 1988). Still another example is given by the results of Müller and Bienz (1991), who found that the anterior expression directed by their BXD element is suppressed when BXD is combined with the ABX or PBX element. Our results show that repression in the anterior part of the embryo is due to *hb* product acting locally to prevent the binding of activators and that *hb* cannot suppress the activation of strong *ftz* binding sites at a distance of 200 bp. How then can *hb* product bound to one element suppress expression directed by another element? A possible explanation is based on competition between the different elements for mutually exclusive interaction with the promoter. According to this model, each module assembles a particular set of activators and repressors and acts as a unit to complex with the promoter and control its activity. The relative ability of the different modules to engage the promoter in a particular cell determines the state of *Ubx* expression in that cell and the complex formed with the promoter could be either transcriptionally active or inactive. We envision that, in the presence of *hb* product, elements such as BRE or PBX are not able to activate the promoter but are still locked in a complex with it, thus excluding activation by other anteriorly active elements such as BXD or the element responsible for the basal pattern. We do not know at present what might determine the order of dominance between regulatory modules: it might be the proximity to the promoter or the affinity for some factor that mediates contact with the promoter or some other property conferred by the flanking sequences. It has been proposed that, once the complex is formed, it is the role of the *Pc* group genes to 'lock' it in so as to maintain the pattern of expression in either an active or silent state when the expression of the segmentation genes fades off. Our *in situ* RNA hybridization results show that in fact, the strong expression activated by the segmentation genes stops after the segmentation gene products have faded off and that the later ectopic expression is dependent on a different set of activators. This suggests that the BRE lacks the ability to respond to the *trithorax* group of genes responsible for positive maintenance. At least in the case of BRE, the function of *Pc*-group genes is to prevent the promoter from being re-activated by non-specific activators. The poorer maintenance of the smaller BRE construct indicates that at least some degree of *Pc*-dependent maintenance is conferred by sequences in the vicinity of the BRE module and that the more flanking sequences are present, the better the maintenance.

Possible role of a general transcription activator

Our results and those of other groups (for example, Schier and Gehring, 1993) show that simple binding by the activator *ftz* is not sufficient for promoter activation. Oligonucleotides with the consensus binding sequence for the *ftz* or *en* products direct neither a *ftz*- nor an *en*-like expression pattern *in vivo* (Vincent *et al.*, 1990) although they may stimulate transcription *in vitro* (Ohkuma *et al.*, 1990). Similarly, we find that although sequences surrounding the BRE module contain very strong *ftz* binding sites they do not direct any expression pattern (Qian *et al.*, 1991). The same is probably true for *en* and *twi* binding sites. Activation might require combinations of factors, for example, a combination of *twi* and *ftz* to produce expression in the mesoderm. Another possibility is that other factors are required to pre-activate the enhancer, for example, by establishing contact with the promoter. In this case, segmentation gene products such as *ftz* or *en* would not work on their own but would be capable of enhancing the action of the activator. Our results support the second hypothesis without excluding the first. In *ftz*$^-$ embryos the BRE enhancer retains a low level of uniform expression (Qian *et al.*, 1991), the *ftz* site 4 mutation led to a uniform expression of the *bxIΔftz1−3mftz4* until late germ band elongation (Figure 8), and even the truncated *bxIΔftz1−4* element retains a weak uniform expression pattern in the absence of *en* product (Figure 7e and f). Such uniform expression could only result from a ubiquitous activator which must have sites of interaction in the BRE module. Footprinting experiments using embryonic nuclear extracts have in fact revealed binding sites for an unknown protein distinct from any of the five gene products that we have analysed in this study. The pre-activation hypothesis might also explain the fact that sequences flanking the BRE module can intensify its activity and expand it to the ectoderm even though alone they are unable to drive any expression. According to this hypothesis, they could become functional, for example exploiting the additional *ftz* binding sites present in the 8.8 kb BRE element, once the BRE module has activated this region or established contact with the promoter.

In contrast to the anterior morphogenetic system, which is positively controlled by the maternal morphogen *bicoid*, posterior morphogenesis in *Drosophila* is largely based on negative regulation. Genetic and molecular analysis of the gap genes *giant* and *knirps* indicates that their expression pattern in the posterior of the embryo is shaped by repressive interactions (Weigel *et al.*, 1990; Reinitz and Levine, 1991; Capovilla *et al.*, 1992; Pankratz *et al.*, 1992) but their initial activation requires the existence of ubiquitous factors. It is possible that common factors are responsible for the early activation of these segmentation genes as well as of *Ubx* and other homeotic genes like *abd-A* and *Abd-B*.

Materials and methods

The procedures for mutant embryo collection, germ line transformation and β-galactosidase staining were described by Qian *et al.* (1991). *In situ* hybridization followed Tautz and Pfeifle (1989).

Footprint analysis

Protein preparation and footprinting analysis were done essentially as before (Qian *et al.*, 1991). The *tll* DNA binding domain expression plasmid was obtained from J.A.Lengyel and has been described in Hoch *et al.* (1992). Since high Mg^{2+} concentration interferes with the binding of *ftz* protein to DNA (Ohkuma *et al.*, 1990), the binding reaction was done in 50 μl binding buffer without Mg^{2+} (100 mM KCl, 35 mM HEPES pH 7.9, 1 mM DTT, 0.1 mM $ZnCl_2$, 12% glycerol and 0.06% NP-40). This Mg^{2+}-free binding reaction worked well for all five bacterially expressed proteins. After incubation, 50 μl of digestion buffer (10 mM $MgCl_2$, 5 mM $CaCl_2$) was added for the DNase reaction as in the original protocol (Qian *et al.*, 1991).

In the competition experiments shown in Figure 9, 40 μg of either the *ftz* or *hb* protein extract was first incubated with the labelled DNA for 20

min on ice, then the same amount of the competing protein extract was added and the mixture was incubated for 20 min more, followed by DNase I digestion and phenol–chloroform extraction. In the control binding reactions with a single protein, after the DNA probe was incubated for 20 min with either the *ftz* or *hb* protein, the same amount of extract from host cells carrying the pET vector without insert was added and the samples were treated in parallel with the competition reactions. Due to its higher level of expression in bacteria, the amount of *ftz* protein used was at least 10 times higher than that of the *hb* protein as estimated by the staining with Coomassie Blue in SDS–PAGE. However, the relative concentrations active *in vivo* are not known and the bacterially produced forms do not necessarily correspond to the forms present in the embryo.

Deletion of ftz binding sites

The following five oligonucleotides were used to delete various *ftz* sites by PCR reactions:

1. TGAGGATCCGGTGTTTTTCCCCGAA
2. TGAGGATCCACGGTTCCCTAAAAAA
3. TGAGGATCCGTAAACAAAACGCGCG
4. TGAGGATCCGACCTTGAAGGCGGCG
5. TGAGGATCCGATTTTTTAATGTTTCTCA

ftz sites 1–3 were deleted by assembling the products of two PCR reactions, reconstructing the *bxI* element without the region containing the three *ftz* sites (Figures 1 and 5). The *bxI* fragment was cloned in the *Sal*I and *Hin*dIII sites of the Bluescript vector and a single-stranded template was used for PCR. The first or 5′ PCR reaction was carried out using the universal M13 sequencing primer and oligonucleotide 1 which primes synthesis from a site immediately 5′ to *ftz* site 3 towards the *Sal*I site in the polylinker. The second or 3′ PCR reaction utilized the M13 reverse primer (which anneals to the 3′ end of the Bluescript polylinker) and oligonucleotide 2 which primes DNA synthesis from immediately 3′ of the *ftz* 1 site towards the *bxI Hin*dIII site. The first PCR product was digested with *Sal* and *Bam*HI, the second product was digested with *Bam*HI and *Hin*dIII and the two products were ligated into a *Sal*I and *Hin*dIII digested Bluescript in a three fragment ligation, reassembling the two halves of the *bxI* sequence minus the *ftz* 1–3 sites (*bxIΔftz1–3*).

The same approach, using different sets of primers and the *bxI*-Bluescript template, was used to make the other deletions. Oligonucleotide 3, which primes from the sequence immediately 5′ of *ftz* site 4 in Figure 4, was used in a 5′ PCR reaction. The product was ligated with the 3′ PCR product of oligonucleotide 2 to give *bxIΔftz1–4* which lacks the entire region from *ftz* site 1 to *ftz* site 4. Oligonucleotide 4, which primes from the sequence immediately 5′ of the *ftz* site 3, was used in a 3′ PCR and the product was ligated with the 5′ PCR product of oligonucleotide 3 to give *bxIΔftz4*. Oligonucleotide 5, which primes from the sequence 4 bp 3′ of *ftz* site 3, was used in a 3′ PCR and the product was ligated with the 5′ PCR product of oligonucleotide 3 to give *bxIΔftz3–4*. Using *bxIΔhb1–3*-Bluescript (Qian *et al.*, 1991) as template in the 5′ PCR reactions that generated *bxIΔftz1–3* gave rise to *bxIΔftz1–3Δhb3*. Similarly, using *bxIΔhb1–3*-Bluescript as template in the 3′ PCR reaction that generated *bxIΔftz4* gave rise to *bxIΔftz4Δhb1–2*.

Mutagenesis of ftz site 4

Mutagenesis was done by the procedure previously used (Qian *et al.*, 1991). Starting with *bxIΔftz1–3*, a two-step mutagenesis changing the 7 nucleotides in *ftz* site 4 (shown below) abolished *ftz* binding *in vitro* and the *ftz*-dependent even-stripe enhancement *in vivo* (Figure 8).

wild-type *ftz* site 4: CGCCCGGCACTTAACATTGCACTTTTTATGA-CCTCGTAAAAAAACTGCAC

mutant *ftz* site 4: CGCCCGGCAGCTGACACTGCACTTTTTCTGACC-TCGCGAAAAAACTGCAC

The mutagenesis targeted two potential consensus motifs: TAAT and CACTT or sequences resembling them.

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