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Gene expresssion mediated by *cis*-acting sequences of the *Krüppel* gene in response to the *Drosophila* morphogens *bicoid* and *hunchback*

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The initial expression of the gap gene *Krüppel* (*Kr*) occurs in a precisely bounded central region of the *Drosophila* blastoderm embryo. According to genetic analysis, the spatial limits of the *Kr* expression domain are controlled by the morphogenetic activities of the anterior organizer gene *bicoid* (*bcd*) and the anterior gap gene *hunchback* (*hb*). Using gene fusion analysis, we assayed for *cis*-acting sequences of the *Kr* gene which mediate transcriptional activation and localized gene expression in response to *trans*-acting factors. A 730 bp *Kr* control element drives gene expression in place of the endogenous *Kr* central domain. This *cis*-acting element, *Kr*730, is composed of *bcd* and *hb* responsive sequences. They map into regions of mutiple *hb* and *bcd* protein *in vitro* binding sites. A 142 bp core fragment containing one low affinity *hb* and five medium to strong *bcd* protein binding sites drives gene expression in a *Kr*-like location in the centre of the embryo. Our results show that this fragment represents a target for the redundant activator/repressor system provided by the anterior morphogens *bcd* and *hb*.

Key words: anterior organizer system/*cis*-acting elements/*Drosophila* blastoderm/gap gene regulation/*Kr trans*-acting factors

Introduction

The longitudinal body pattern of the *Drosophila* larva derives from the activity of a cascade of segmentation genes which subdivides the early embryo into a series of repeating segment precursors (Akam, 1987; Ingham, 1988 for reviews). The localized expression of the segmentation genes depends on a hierarchical series of regulatory interactions. The first step in this process is the establishment of positional information by maternal factors which organize the spatial domains of expression of the gap class of zygotic segmentation genes (Nüsslein-Volhard *et al.*, 1987). Gap gene proteins form broad and overlapping concentration gradients in the syncytial blastoderm embryo (Gaul and Jäckle, 1989; Pankratz *et al.*, 1989; Stanojevic *et al.*, 1989; Hülskamp *et al.*, 1990) which provide spatial cues along the antero-posterior axis of the embryo for the regulation of subordinate segmentation gene expression (Frasch and Levine, 1987; Howard, 1988; Ingham and Gergen, 1988; Stanojevic *et al.*, 1989; Pankratz *et al.*, 1990; Warrior and Levine, 1990). The recurring theme of the segmentation hierarchy is a progressive refinement in the patterns of segmentation and homeotic gene expression that ultimately establish the correct number, size and polarity of segments (Akam, 1987; Ingham, 1988 for reviews).

The establishment of thoracic and abdominal segments depends on the activities of the gap genes *hunchback* (*hb*), *Krüppel* (*Kr*) and *knirps* (*kni*) (Nüsslein-Volhard and Wieschaus, 1980; Lehmann, 1988; Gaul and Jäckle, 1989). *hb* is necessary for the establishment of the head and the thoracic region, *Kr* for the thoracic and anterior abdominal region and *kni* for most of the abdomen. Expression of the *Kr* gene can be first observed at the syncytial blastoderm stage in the central region of the embryo (Knipple *et al.*, 1985; Gaul *et al.*, 1987). The regulation of *Kr* expression at this early stage involves the activity of at least two genes, the maternal anterior organizer gene *bicoid* (*bcd*) and the gap gene *hb* (Frohnhöfer and Nüsslein-Volhard, 1986; Gaul and Jäckle, 1987; Hülskamp *et al.*, 1990).

bcd is the active component of the maternal anterior pattern organizer system which provides an anterior to posterior protein concentration gradient. Based on genetic arguments it has been proposed that the *bcd* protein (BCD) acts as a morphogen which regulates a set of target genes (Frohnhöfer and Nüsslein-Volhard, 1986; Driever and Nüsslein-Volhard, 1988a,b; Struhl *et al.*, 1989). The only identified direct target gene of *bcd* that responds to discrete concentration values of the BCD gradient is *hb* (Schröder *et al.*, 1988; Driever and Nüsslein-Volhard, 1989; Struhl *et al.*, 1989; Driever *et al.*, 1989).

Zygotic *hb* expression depends on the direct interaction of BCD with *hb cis*-regulatory elements upstream of the *hb* proximal promotor (Driever *et al.*, 1989). These sequences contain several *in vitro* BCD binding sites of different binding affinities. They define a threshold concentration within the BCD gradient above which the *hb* gene becomes activated (Driever and Nüsslein-Volhard, 1989; Driever *et al.*, 1989). The above action of BCD on *hb* results in zygotic *hb* expression in the anterior half of the embryo. However, there is also a *bcd*-independent source of *hb* activity that is provided maternally. Due to the activity of the posterior organizer system (Nüsslein-Volhard *et al.*, 1987), the maternal *hb* protein (HB) becomes localized in the anterior region of the embryo as well (Tautz, 1988; Hülskamp *et al.*, 1989; Irish *et al.*, 1989; Struhl *et al.*, 1989). The maternally and zygotically derived *hb* proteins share an identical amino acid sequence (Tautz *et al.*, 1987) and they form a steep concentration gradient, which in contrast to the BCD gradient (Driever and Nüsslein-Volhard, 1988a,b), is restricted to the anterior half of the embryo (Tautz, 1988). Genetic evidence suggests that the spatial pattern of initial *Kr* expression is critically dependent on the BCD and HB gradients (Gaul and Jäckle, 1987; Hülskamp *et al.*, 1990). Low levels of each of *hb* and *bcd* activities initiate *Kr* expression in the central region of the embryo (Hülskamp *et al.*, 1990), while high levels prevent *Kr* expression in more anterior positions (Gaul and Jäckle, 1987; Hülskamp *et al.*, 1990). A possible direct

interaction of the two gene products with the *Kr* gene has not been established yet.

Recent analysis of the *Kr cis*-regulatory region revealed two separate *cis*-acting control units, termed CD1 and CD2, which drive fusion gene expression in the authentic *Kr* central domain at the syncytial blastoderm stage (Hoch *et al.*, 1990). Genetic evidence suggests that the control of these two elements depends on the same set of *trans*-acting factors, suggesting that they mediate localized gene expression by the same basic mechanism (Hoch *et al.*, 1990). Here we use gene fusion analysis to assay systematically for *hb* and *bcd* regulatory sequences of the CD1 control unit. Our results provide evidence for a direct interaction of BCD with *Kr cis*-regulatory sequences. This points to *Kr* as being a second target gene which is directly regulated by the morphogenetic BCD gradient. Moreover, we have identified a 142 bp core sequence within the CD1 element which contains a low affinity HB in addition to multiple BCD *in vitro* binding sites. This 142 bp core element mediates gene expression in a central region of the embryo in response to *hb* and *bcd* activities.

Results

Two *cis*-acting control elements of the *Kr* gene, CD1 and CD2, contain the sequence information for the correct activation and the localized expression of *Kr* in wild type embryos (Hoch *et al.*, 1990). The CD1 element is contained within a 1.2 kb DNA fragment which maps to about 3–4 kb upstream of the *Kr* transcription start (BNc1.2HZ, Figure 1; details in Hoch *et al.* 1990). To delimit further the sequences sufficient to activate gene expression in the *Kr* central domain, and eventually to search for the corresponding *trans*-acting factors responsible, we inserted deletion forms of this fragment (Figure 1) upstream of a hsp70–*LacZ* (HZ) fusion gene construct (Hiromi and Gehring, 1987; see also Materials and methods). The resulting *Kr*–HZ fusion genes (Figure 1) were then inserted into the *Drosophila* genome by P element-mediated germ line transformation (Rubin and Spradling, 1982) and the expression patterns of β-galactosidase (β-gal) were monitored in the transgenic embryos by antibody staining. The precise limits of the expression domains are variable depending on the staining

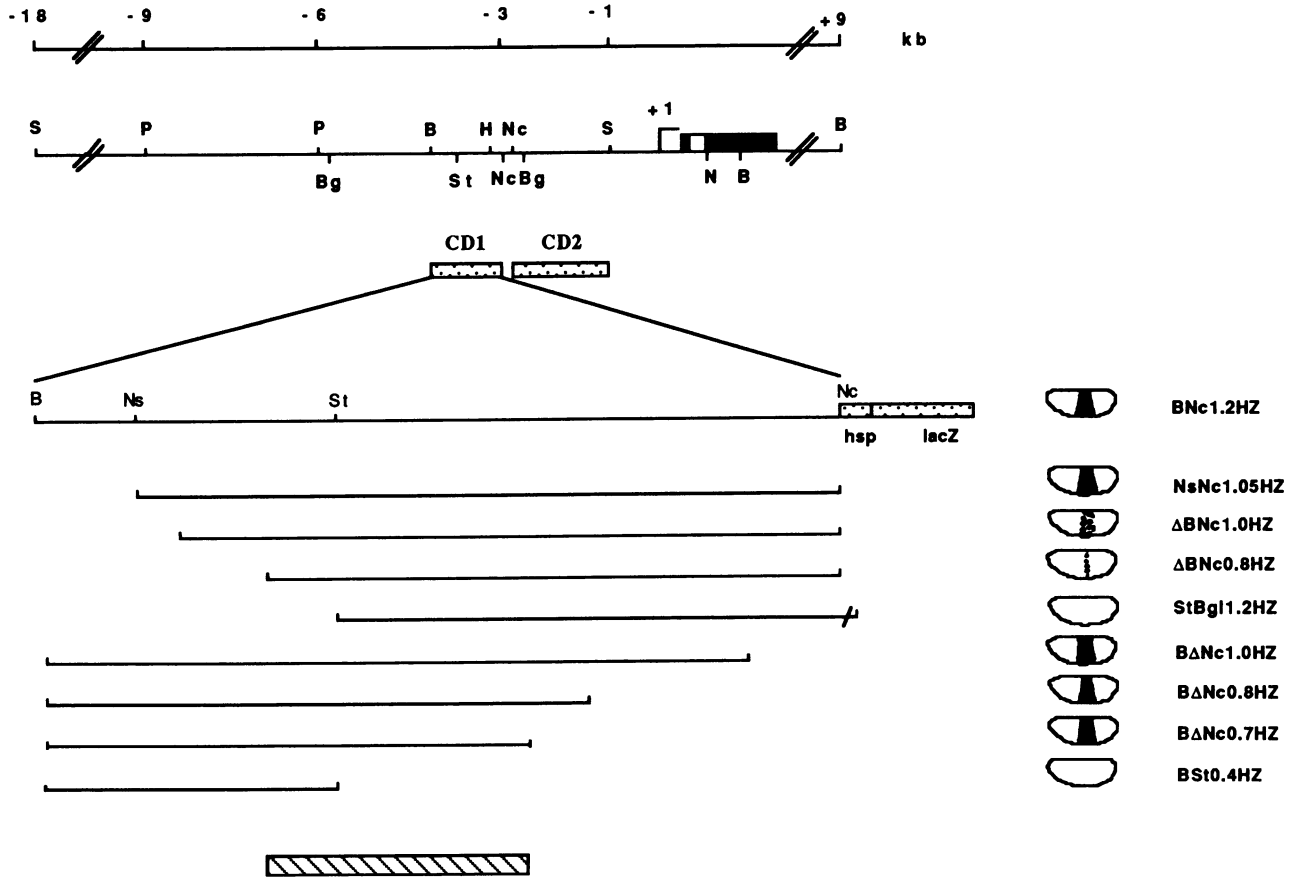


Fig. 1. Deletion analysis of the CD1 element. The physical map of the *Kr* gene (below the kb marker; top line) covers ~27 kb of DNA. The arrow marks the transcription start site followed by the open reading frame (black bar) interrupted by an intron (open bar). For details see Rosenberg *et al.* (1986). Diagnostic restriction sites are indicated as B, *Bam*HI; *Bgl*II; H, *Hin*dIII; N, *Not*I; Nc, *Nco*I; Ns, *Nsi*I; P, *Pst*I; S, *Sal*I and St, *Stu*I. CD1 and CD2 designate the location of the *Kr cis*-acting elements (stippled boxes at the top) which mediate central expression in the blastoderm embryo (Hoch *et al.*, 1990). Various 5′ and 3′ deletions of a 1.2 kb *Bam*HI–*Nco*I *Kr* upstream fragment which covers the CD1 element are shown in the middle part of the figure (see Materials and methods). The designation of the derived fusion gene constructs is shown on the right. Characters refer to restriction sites, numbers to the length of the DNA fragment in kb, Δ refers to Bal31 deletions, and HZ to the P element vector HZ50PL (Hiromi and Gehring, 1987; see Materials and methods). The blastoderm expression patterns of the corresponding fusion gene constructs in wild type embryos are shown schematically on the right. Strong intensity of the expression domains is marked in black, weaker intensity is stippled. The hatched box at the bottom points to the 400 bp region which seems to be essential for correct spatial regulation of CD1 element-mediated expression (see text).

conditions applied. Alterations of the reporter gene expression domains such as shifts or expansions (see below) were therefore only scored if they could be observed in several transgenic lines of a single reporter gene construct under constant staining conditions (see Materials and methods).

Deletion analysis of the CD1 element

Deletions of up to 300 bp from the 5′ end of the 1.2 kb CD1 element had no significant effect on the spatial limits of gene expression, but the level of expression appeared to be decreased (constructs ΔBNc1.0HZ and ΔBNc0.8HZ; see Figure 1). However, when additional sequences up to the diagnostic *Stu*I restriction site (Figure 1) were removed from the 5′ region of the CD1 element, no gene expression could be detected (construct StBgl1.2HZ; see Figure 1 and Hoch *et al.* 1990). Deletions of up to 470 bp from the 3′ end led to no obvious change of expression in comparison with CD1 element-mediated gene expression (constructs BΔNc1.0HZ; BΔNc0.8HZ and BΔNc0.7HZ; see Figure 1). No expression was observed with constructs which lack sequences downstream of the *Stu*I site (construct BSt0.4HZ). These results suggest that the 400 bp sequence indicated by the hatched bar in Figure 1 contains the core sequences that determine the spatial localization of CD1-mediated gene expression. We note that additional 5′ sequences seem to be required for the normal level of gene expression. These sequences are contained within the BΔNc0.7HZ construct, which is expressed in a pattern and at levels indistinguishable from CD1-mediated gene expression (Figure 1). We refer to this sequence as the *Kr*730 element.

In order to ensure that the *Kr*730 element has not lost the target sites for mediating *hb* and *bcd* activities, we monitored the expression patterns of the *Kr*730−HZ fusion gene in *bcd* and *hb* mutants. In addition, we examined its expression in embryos in which the maternal HB is uniformly distributed along the anterior−posterior axis (Tautz, 1988). Such embryos can be obtained from females which are homozygously mutant for *nanos* (*nos*), an essential component of the maternal posterior organizer system (Nüsslein-Volhard *et al.*, 1987; Lehmann, 1988).

In comparison with wild type embryos (Figure 2a), *Kr*730−HZ expression expands and shifts anteriorly (Figure 2b) in *hb* mutant embryos. In embryos from *nos* mutant mothers, *Kr*730−HZ expression expands posteriorly (Figure 2c), while the expression domain of the *Kr*730−HZ fusion gene in embryos from *bcd* mutant mothers is shifted towards anterior and the level of expression is reduced (Figure 2d). Thus, the *Kr*730−HZ expression patterns in the different mutant embryos show the same alterations as has been observed for the *Kr* protein expression pattern (Gaul and Jäckle, 1987) or the CD1-dependent gene expression patterns in the corresponding mutant background (Hoch *et al.*, 1990). This finding indicates that the *Kr*730 DNA contains target sites for direct or indirect interaction with BCD and HB.

Multiple BCD and HB in vitro binding sites in Kr730 DNA

bcd and *hb* encode sequence-specific DNA binding proteins containing a homeodomain (Frigerio *et al.*, 1986) and the zinc finger motif (Tautz *et al.*, 1987) respectively, and have been shown to bind DNA (Driever and Nüsslein-Volhard, 1989; Treisman and Desplan, 1989; Stanojevic *et al.*, 1989).

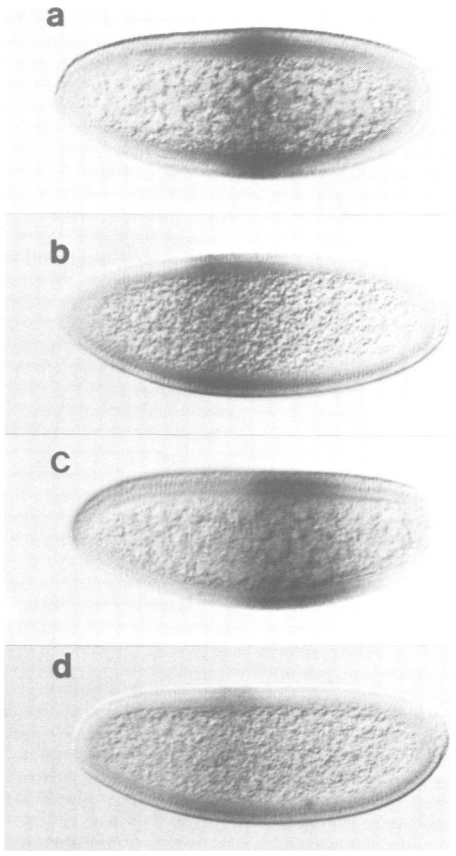


Fig. 2. β-gal expression patterns of the fusion gene construct BΔNc0.7HZ (*Kr*730 element) in maternal effect and gap mutant embryos. (**a**−**d**) Anti-β-gal antibody staining of whole mount embryos carrying the fusion gene construct BΔNc0.7HZ. (**a**) Expression pattern of the fusion gene construct BΔNc0.7HZ (see Figure 1) in wild type embryos. The β-gal expression domain is localized in a region of the authentic *Kr* central domain (compare with Figure 6c). (**b**) Expression pattern in embryos mutant for the gap gene *hb*. The expression domain is expanded and slightly shifted towards anterior. (**c**) Expression pattern in embryos derived from *nos* mutant mothers. The expression domain is expanded towards posterior. (**d**) Expression pattern in embryos derived from *bcd* mutant mothers. The expression domain is shifted towards anterior. The embryos are shown in the following orientation: dorsal is up and anterior is to the left.

As a first step to see whether there might be a direct interaction of BCD and HB with the *Kr* gene, we searched for corresponding binding sites on *Kr*730 DNA.

The ability of BCD and HB to bind to *Kr*730 DNA and the location of their potential target sequences were analysed by *in vitro* footprinting techniques (Galas and Schmitz, 1978) using an *Escherichia coli*-derived BCD homeodomain and a full size *E.coli* HB. As shown in Figures 3 and 4, the BCD homeodomain protects four areas of different sizes within *Kr*730 DNA. Within the protected areas six BCD binding sites which vary slightly from the consensus sequence established by Driever and Nüsslein-Volhard (1989) can be found. HB binds to multiple sites within *Kr*730 DNA, all of them matching the consensus sequence for HB binding (Stanojevic *et al.*, 1989; Treisman and Desplan, 1989). Interestingly, the BCD and HB binding sites seem to be clustered into two areas around the diagnostic *Stu*I restriction site, which splits the *Kr*730 DNA into two pieces. As determined by *in vitro* footprinting analysis, the fragment upstream of the *Stu*I restriction site contains one high, one

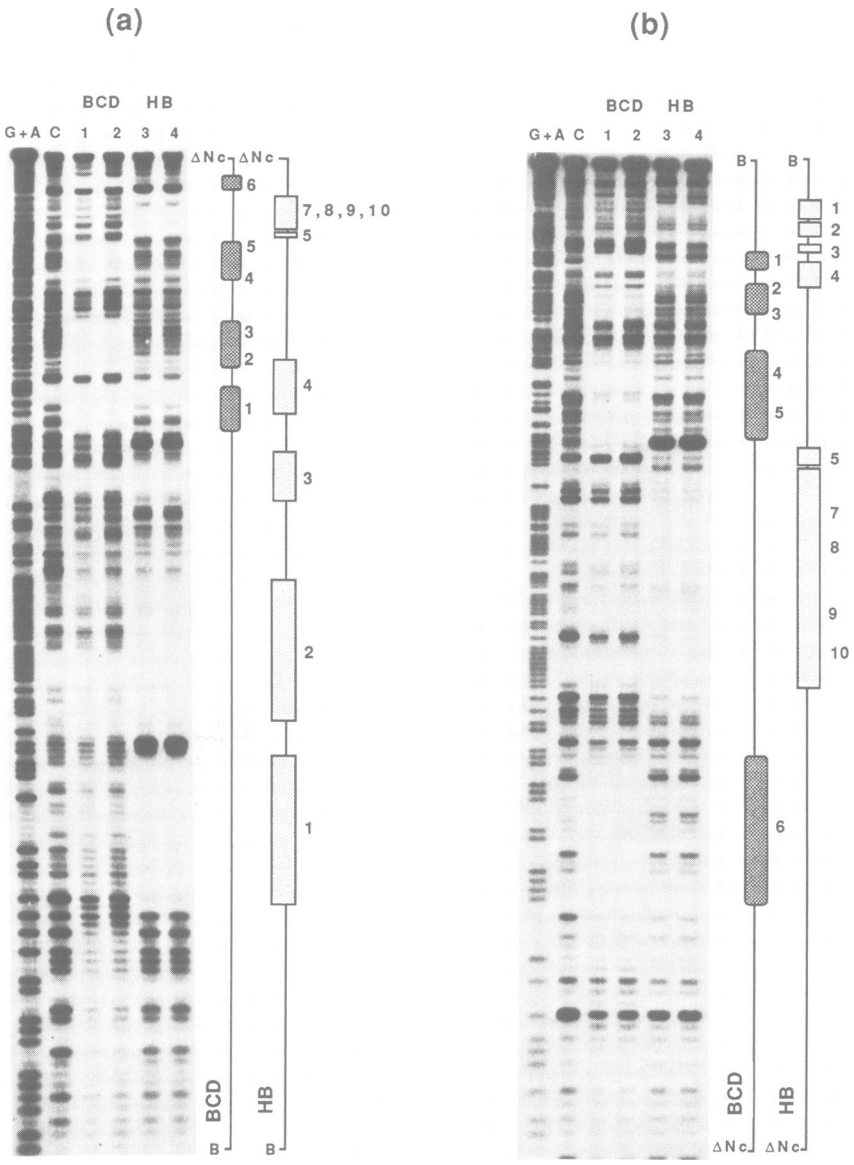


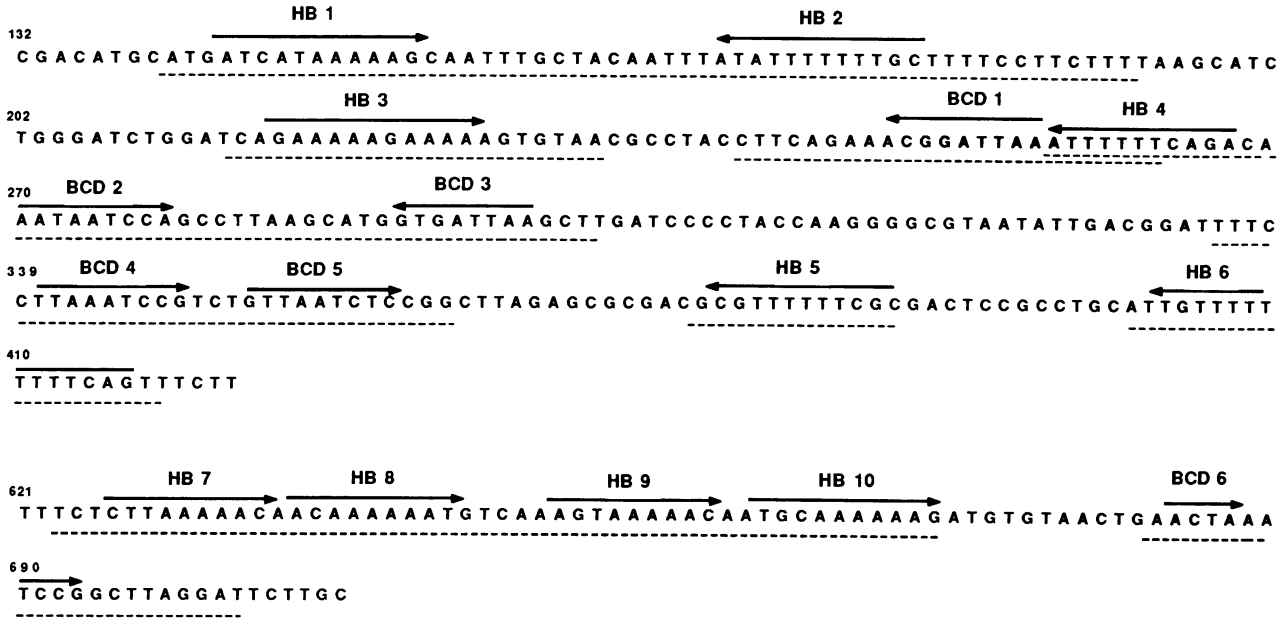
Fig. 3. BCD homeodomain and HB binding to the *Kr*730 element. Footprinting experiments showing the binding sites for BCD and HB on the *Kr*730 element. (**a**) − strand; (**b**) + strand. For the binding reaction, a shortened fragment (see Materials and methods) was incubated in each (a) and (b) with 7.5 μg bacterial extract containing either BCD homeodomain protein (lanes 1 and 2), 7.5 μg extract with HB (lanes 3 and 4) or with 7.5 μg control extract containing the T7 expression vector alone wihtout *bcd* or *hb* coding sequences (lanes C). Lanes G+A indicate in (a) and (b) deoxyguanosine and deoxyadenosine sequences of the corresponding footprinted strand. At the right side of both footprinted strands, the orientation of the original *Kr*730 element is indicated. B stands for *Bam*HI and ΔNc for Δ*Nco*I (see construct BΔNc0.7HZ in Figure 1). In addition, an arbitrary numeration and the location of either BCD or HB binding sites on the *Kr*730 element are shown with stippled boxes (see below: BCD, HB). For the location, orientation and sequences of the protected regions, see Figure 4.

medium and three low affinity BCD binding sites. In addition, it contains six HB binding sites. Two of them bind HB with high affinity, two with medium and two with low affinity (see Figure 4b). The fragment downstream of the *Stu*I site contains one BCD binding site of medium affinity flanked by four medium affinity HB binding sites (Figure 4b). The location, orientation and sequences of the binding sites within *Kr*730 DNA are shown in Figures 4 and 5a.

The Kr-like expression pattern depends on sequences encompassing BCD and HB binding sites

The *Kr*730 element gives rise to high level gene expression in place of the authentic *Kr* central domain. The *Bam*HI−*Stu*I subfragment of *Kr*730 DNA (construct BSt0.4HZ; see Figure 1) which contains five out of six BCD and six out of ten HB *in vitro* binding sites (see Figure 5a) failed to direct expression of the hsp−*LacZ* fusion gene. However, mutiple copies of the *Bam*HI−*Stu*I fragment in front of the hsp−*LacZ* reporter gene construct or a single fragment in front of a reporter gene construct containing the strong *Kr* basal promoter in combination with the *lacZ* gene (M.J.Pankratz and H.Jäckle, unpublished result) drive gene expression in a central region of the embryo (Figure 6a). Two and four tandemly arranged fragments in front of the *Kr*−*LacZ* reporter (constructs 2BSKrZ and 4BSKrZ; Figures 5b and 6b) increased the level of gene expression and sharpened the borders of the expression domain. These results show that the *Bam*HI−*Stu*I fragment is able to mediate low level gene expression in a central domain of the embryo. We termed this subfragment of *Kr*730 DNA

(a)



(b)

	HB	BCD
weak sites	HB 4,6	BCD 2,3,4
medium sites	HB 3,5,7,8,9,10	BCD 5,6
strong sites	HB 1,2	BCD 1

Fig. 4. Location and orientation of BCD and HB binding sites on *Kr*730 DNA. (**a**) The complete sequence of regions containing binding sites for BCD and HB is shown. The numeration of the *Kr*730 element from 1 to 730 indicates the actual size of the fragment in bp but is arbitrary concerning its location in the *Kr* upstream region. bp 1 starts at the *Bam*HI site, the 5′ end of the *Kr*730 element (see also Figure 5a). Protected regions are indicated below the sequence with a stippled bar. The orientation of consensus sequence sites for BCD and HB binding (Driever *et al.*, 1989; Stanojewic *et al.*, 1989; Treisman and Desplan, 1989) are indicated above the sequences by solid arrows. Note the two clusters of HB and BCD binding sites 5′ and 3′ of the diagnostic *Stu*I restriction site (see text) at position 440. Numbers above the solid arrows indicate a numeration of BCD or HB sites within the *Kr*730 element from 5′ to 3′. (**b**) Designation of weak, medium and strong HB and BCD binding sites according to the footprinting experiments. Note that all of the BCD binding sites deviate slightly from the consensus sequence determined by Dreiver *et al.* (1989) but still have conserved the base pairs shown to be important for BCD binding (Hanes and Brent, 1991). The BCD sites of *Kr* do not have the spacing of 100 bp that seems to be important for *hb* gene regulation (Driever *et al.*, 1989). In fact, the BCD binding sites in the 5′ cluster of the *Kr*730 element lie close together with a mean distance of 20–30 bp from each other, except for binding site 6, which is ~300 bp apart. We note that some of the BCD binding sites of the *hb* proximal promotor (Driever *et al.*, 1989) lie also close together. Fragments encompassing this region seem to have important regulatory functions for *hb* gene expression (Struhl *et al.*, 1989).

'BS-element'. We note that aside from the quantitative aspects of gene expression, this element gives rise to an expression domain which is positioned slightly anterior to the authentic *Kr* central domain (compare Figure 6a with 6c) or the one mediated by the *Kr*730-element (compare Figure 2a with 6a). This observation indicates that sequences in the 3′ region of the *Kr*730 element are needed for the correct positioning of the expression domain.

The BS element mediates bcd and hb activities

To test whether the activation of the BS element containing fusion gene constructs is dependent on the concentration gradient of BCD, we placed the BS–KrZ constructs in the background of embryos that derived from females containing none to six copies of the *bcd* gene in their genome. As shown previously, the concentration of BCD along the anterior–posterior axis of the embryo is roughly proportional to the number of gene copies present in the female (Driever and Nüsslein-Volhard, 1988b). Extra copies of the *bcd* gene lead to a shift of the endogenous *Kr* domain towards the posterior pole of the embryos (compare Figure 6c and 6d). Similarly, the 4BSKrZ expression domain is shifted towards posterior in embryos that derive from females containing an increased copy number of the *bcd* gene (see Figure 6b and f). Furthermore, the 4BSKrZ reporter gene is not expressed in embryos which lack the maternally derived *bcd* gene product (Figure 6e). These results demonstrate that the activation and the spatial aspects of BS element-dependent gene expression are critically dependent on the BCD concentration *in vivo*.

The expression domains of the fusion gene constructs containing one and multiple copies of the BS element are

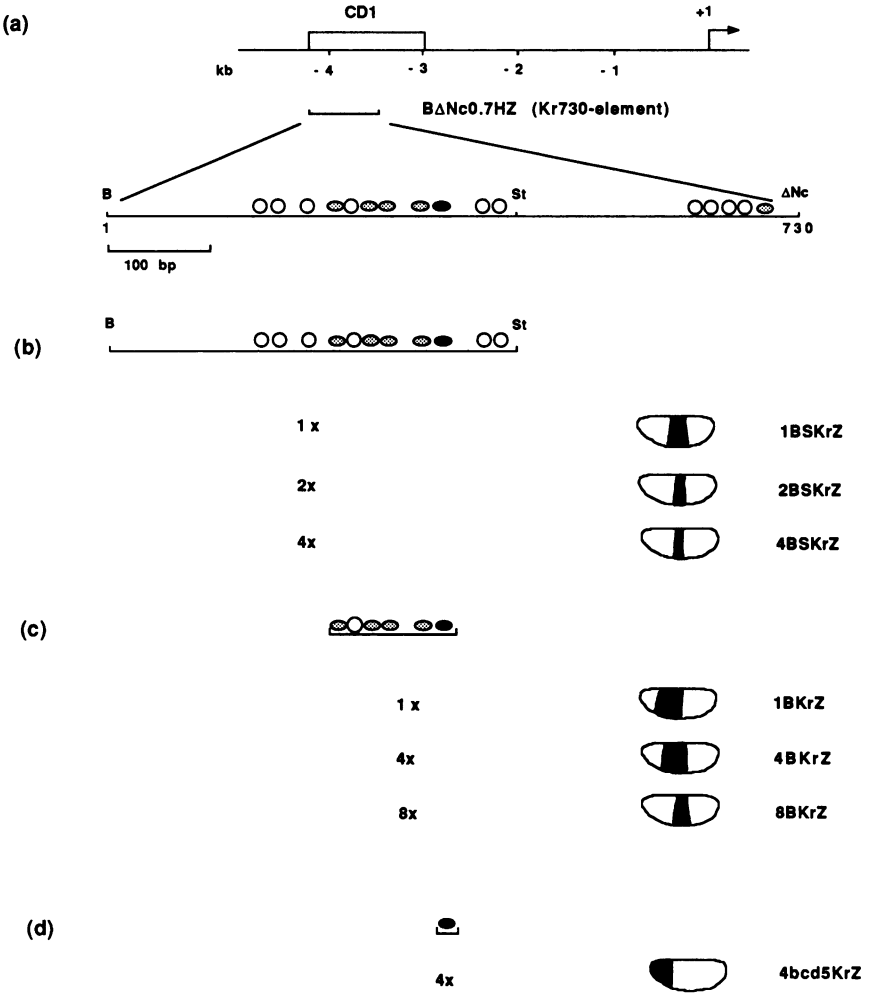


Fig. 5. Reporter gene constructs driven by sub-elements of the *Kr*730 element. (**a**) The physical map of the *Kr* upstream region is shown on top. The arrow marks the transcription start site of the *Kr* transcription unit and the box indicates the location of the CD1 element. The *Kr*730 element is indicated below. Diagnostic restriction sites are indicated as B (*Bam*HI) and St (*Stu*I). ΔNc refers to the 3′ end of the *Kr*730 element (see Materials and methods, and Figure 1). Enlarged below is a map of the 440 bp *Bam*HI−*Stu*I subfragment (BS element) of the *Kr*730 DNA containing HB and BCD binding sites (see Figure 4). The location of binding sites for HB (stippled circles) and BCD (ovals) are indicated (see also Figures 3 and 4). The black oval marks BCD binding site 5. The designation of constructs and their schematic expression patterns in wild type embryos are shown on the right side. (**b**) 1×, 2× and 4× indicate the number of copies of the BS element in the P element vector KrZ (see materials and methods); (**c**) Location of the 142 bp B element which is derived from the BS element. 1×, 4× and 8× indicate the number of copies of the B element in front of the KrZ fusion gene. (**d**) Location of the bcd5 sequence within the B element. The 4bcd5KrZ fusion construct contains four copies of the BCD binding site number 5 (see Figure 4; sequence see text) in tandem in front of the KrZ fusion gene. The expression pattern mediated by this construct in early wild type embryos is shown schematically on the right.

expanded and shifted slightly towards anterior in *hb* mutant embryos (compare Figures 6g and 7c with 6b and 6a) which derive from heterozygous *hb*/+ parents. Such embryos contain half the maternal *hb* gene dose but no zygotic *hb* activity. In embryos laid by *nos* females, in which high concentrations of maternal HB are uniformly distributed throughout the entire embryo (Tautz, 1988), no BS element-dependent gene expression can be observed in early embryos (data not shown) and only a faint staining can be detected in the central region of these embryos at late cellular blastoderm just before gastrulation starts (compare Figure 6b with 6h).

Taken together, these results demonstrate that transcriptional activation of gene expression from the BS element critically depends on *bcd* activity. In addition, *hb* can override *bcd*-dependent activation by repression at high levels of maternal *hb* activity indicating that the BS element contains target sites for *hb*-dependent repression. We noted, however, that the domain of BS-mediated gene expression is not only expanded, but also shifted slightly anteriorly in embryos which lack zygotic *hb* activity. This subtle effect indicates that it is possible to activate gene expression by the BS element in a position of low HB concentrations in the embryo as has been argued on the basis of earlier genetic results (Hülskamp *et al.*, 1990). This conclusion is further underlined by the observation that the expression domain is shifted posteriorly in embryos that derived from *nos* heterozygous mothers (data not shown). In such embryos, a low level of maternal HB is ectopically present in the posterior region (Tautz, 1988).

hb and bcd-responsive sequences within the BS element

In order to test whether it is possible to localize *bcd* activating and *hb* repression function, we isolated the cluster of five BCD *in vitro* binding sites from the high affinity HB binding sites of the BS element. The corresponding 142 bp fragment was fused in one or several copies to the *Kr* promoter−*lacZ*

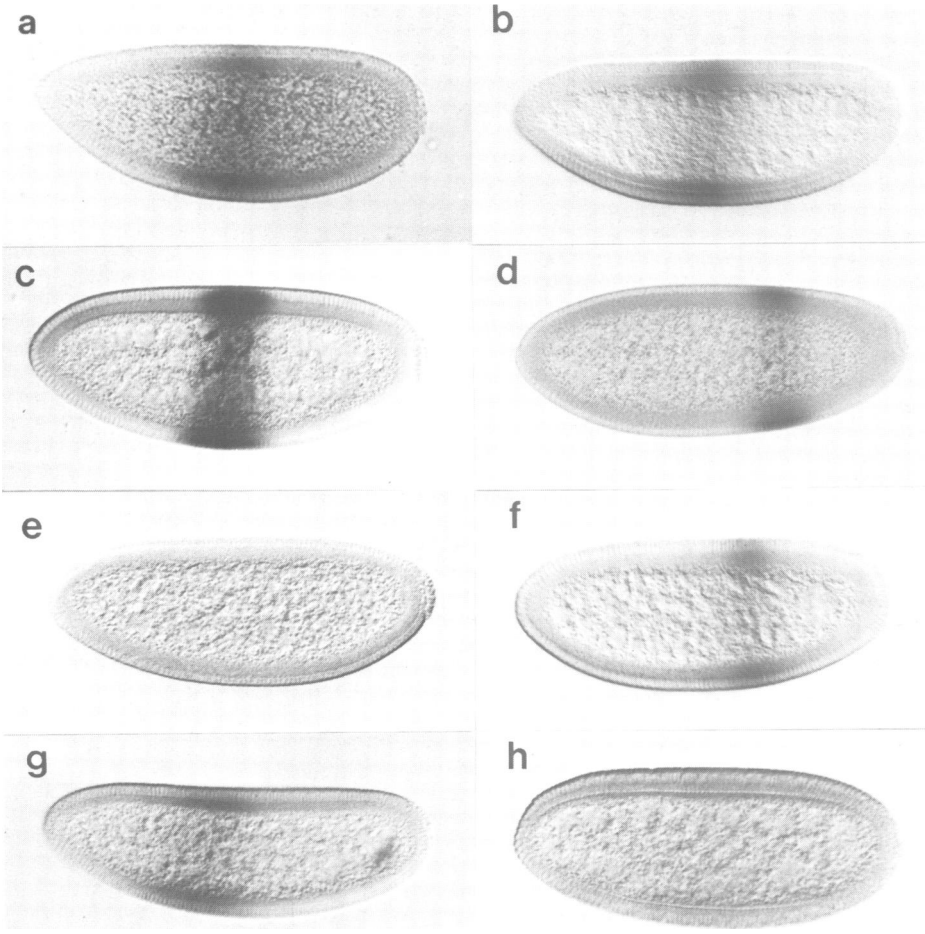


Fig. 6. BS-mediated expression patterns in wild type embryos and in embryos with altered *hb* and *bcd* activity. Anti-β-gal antibody stainings of whole mount embryos harbouring BS-dependent fusion genes (see also Figure 5b). (**a**) 1BSKrZ in wild type embryos. Note that the expression domain is slightly anterior to the *Kr* wild type central domain (compare with c) and has borders that fade out. This makes it difficult to determine their exact position. The expression domain extends roughly from ~42% to 55% egg length (EL; 0% EL is the posterior pole). (**b**) Expression pattern mediated by the 4BSKrZ construct in wild type embryos. Multiplying the BS element results in a sharpening of the borders of the expression domain which extends now from ~45% to 53% EL. (**c**) *Kr* expression pattern in wild type blastoderm embryos revealed by anti-Kr antibody staining. (**d**) *Kr* central domain in blastoderm embryos derived from females with six copies of the *bcd* gene, as revealed by anti-Kr antibody staining. Note that increasing the BCD concentration leads to a posterior shift of the endogenous *Kr* central domain. (**e**) Expression pattern of the 4BSKrZ fusion gene in embryos derived from *bcd* mutant mothers and (**f**) from mothers which carry six copies of the *bcd* gene. Activation and spatial localization of the BS-mediated expression pattern depends on *bcd* activity. Increasing the BCD concentration leads to a shift of the expression domain towards posterior as in the case of the endogenous *Kr* central domain. (**g**) Expression mediated by the 4BSKrZ fusion gene in *hb* mutant embryos. Note that the expression domain expands and shifts towards anterior, compared with its location in wild type embryos (see b). (**h**) Expression pattern of 4BSKrZ in a late blastoderm embryo derived from a *nos* mutant mother where high levels of maternal *hb* activity are ectopically present throughout the embryo (Tautz, 1988). A weak expression can be detected in the central region of the embryo. No activation of the BS fusion genes could be detected in early blastoderm embryos derived from these mothers (data not shown). Orientation of embryos: dorsal up and anterior left.

fusion gene (see Figure 5a). The expression patterns mediated by these constructs in wild type embryos closely resemble the BS element-mediated gene expression in *hb* mutant embryos (compare Figures 7a and 7c; 7d and 6h). This suggests that the 142 bp fragment, which we termed the 'B-element', may have lost the major *hb*-responsive target sites which mediate repression of gene expression in the wild type embryo. We noted, however, that the 4BKrZ and 8BKrZ reporter genes, in which the B element is present in multiple copies, seem still to respond to altered *hb* activity. In *hb* mutant embryos the expression domain of the 8BKrZ fusion gene is slightly shifted towards the anterior (Figure 5c). This indicates that the B element is still able to respond weakly to *hb* activity. This response might be mediated by the low affinity HB *in vitro* binding site which is present on the B element.

Fusion genes containing one or several copies of the B element are not expressed in *bcd* mutant embryos (see e.g. Figure 8a), and multiple copies of the B element (as in 4BKrZ or 8BKrZ; see Figure 7d–g), led to increased gene expression and to a shift of the expression domain towards posterior in wild type embryos. Furthermore, the expression pattern from each of the three constructs is dynamic and varies in a stage-specific manner. As shown in Figure 7e, 8BKrZ expression is initiated at syncytial blastoderm stage in a position slightly posterior to the *Kr* central domain. During the blastoderm stage, the expression domain shifts anteriorly (Figure 7f) and it reaches a final

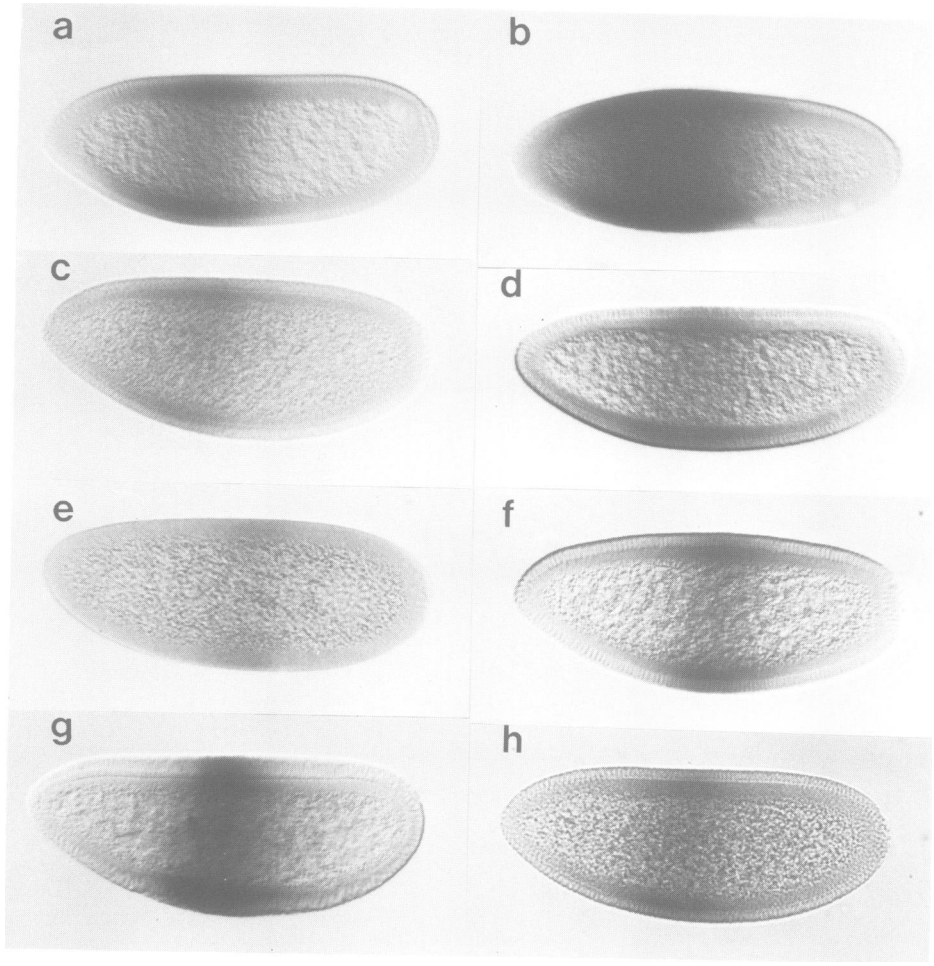


Fig. 7. B element-mediated expression patterns. (**a**–**h**) Antibody stainings of whole mount embryos with anti-β-gal antibody. (**a**) Expression pattern of the 1BKrZ fusion gene in wild type embryos. Note that the expression pattern looks very similar to that of the 1BSKrZ fusion gene in *hb* mutant embryos (compare with c). (**b**) Embryo harbouring the same construct as in (a) but overstained. Note that due to a prolonged staining time, the boundaries of the expression domain are shifted (compare a and b; the attempts to standardize the staining patterns for comparisons of the expression domains in different embryos are described in Materials and methods). (**c**) For comparison with (a): expression pattern of the 1BSKrZ fusion gene in *hb* mutant embryos. (**d**–**g**) Expression patterns of wild type embryos harbouring KrZ fusion gene constructs driven by multiple copies of the B element (d) 4BKrZ (e–g) 8BKrZ. The location of the expression domain varies in a stage-specific manner, as shown e.g. for the 8BKrZ construct (see text). Note that the increased number of B element copies in front of the reporter gene construct leads to a more posterior localization of the expression domain in the embryo. (**h**) Expression pattern of the 8BKrZ fusion gene in *hb* mutant embryos. Note the slight anterior shift (compare with f) of the expression domain indicating a response to altered *hb* activity (see also text).

position slightly anterior to the endogenous *Kr* central domain at around gastrulation (see Figure 7g). This temporal pattern of 8BKrZ expression correlates with a decrease of BCD concentration along the longitudinal axis of the blastoderm embryo with time (Driever and Nüsslein-Volhard, 1988).

To test further whether the activation of constructs containing the B element is dependent on the concentration gradient of BCD, we placed the 8BKrZ construct in embryos which derived from females containing none to six copies of the *bcd* gene in their genome. No expression is observed in embryos which lack *bcd* activity (Figure 8a). In embryos laid by females containing one, two, four or six wild type copes of *bcd*, the expression driven by the 8BKrZ construct increases and the posterior limit of the expression domain spreads posteriorly (Figure 8a–c). These results argue that both B element-mediated gene expression and the spatial limits of the gene expression domain are mainly dependent on the BCD concentration.

Gene expression mediated by the B element does not occur in the anterior region of wild type embryos or in embryos which lack zygotic *hb* activity. Furthermore, the domain of B element-mediated gene expression shifts towards posterior when the level of *bcd* activity is increased in embryos that derive from females containing extra copies of the *bcd* gene (see above). In such embryos, the anterior region in which BKrZ gene constructs are not expressed expands posteriorly, following the area in which the concentration of BCD is high. This indicates that B element-mediated expression is repressed in a BCD-dependent manner. In an effort to identify a BCD-dependent *Kr* repressor, we monitored B element-mediated gene expression in embryos mutant for the gap gene *giant* (*gt*) (Petschek *et al.*, 1987; Reinitz and Levine, 1990; Kraut and Levine, 1991; Eldon and Pirrotta, 1991), and the three recently identified gap-like genes, *orthodenticle* (*otd*), *button head* (*btd*) and *empty spiracles* (*ems*), that act in the anterior region of the embryo and may be controlled by higher BCD concentration values than *hb* (Dalton *et al.*, 1989; Cohen and Jürgens, 1990; Finkelstein and Perrimon, 1990). No obvious alteration of the B element-mediated gene expression could be observed in these mutant

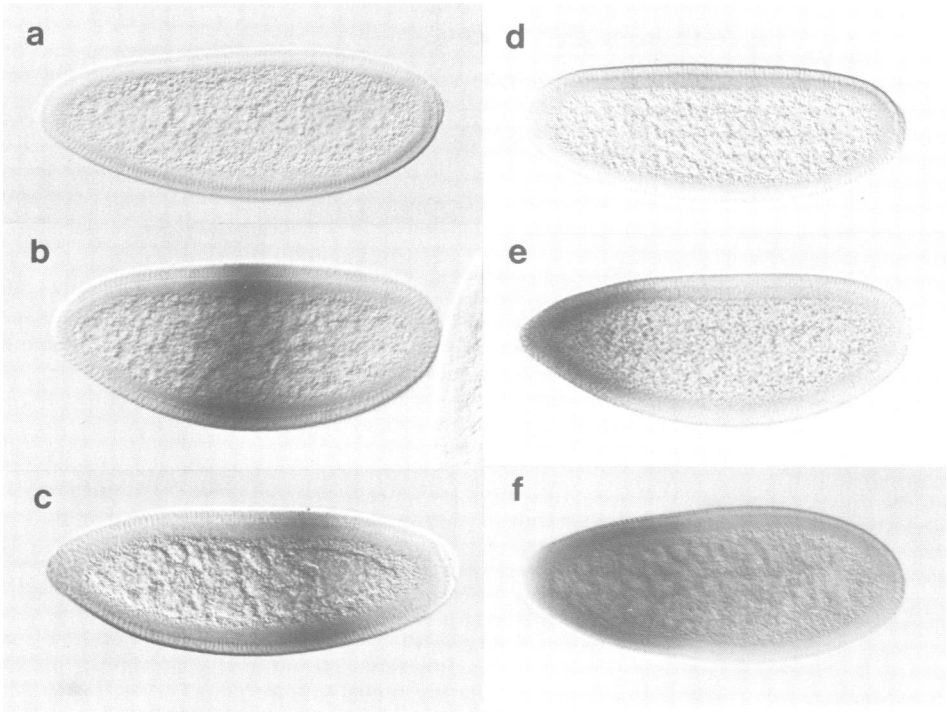


Fig. 8. Dependence of the B element and 4bcd5KrZ fusion genes on *bcd* activity. Anti-β-gal antibody stainings of whole mount embryos harbouring the 8BKrZ fusion gene construct (**a**–**c**) or the 4bcd5 fusion gene (**d**–**f**) (see also Figure 5b). Embryos shown in (**a**) and (**d**) derived from homozygous *bcd* E1 mutant mothers (no functional *bcd* gene). Embryos shown in (**b**) and (**e**) were laid by from wild type mothers (two copies of *bcd* gene), and the embryos shown in (**c**) and (**f**) derived from mothers harbouring six copies of the *bcd* gene. Increasing the number of *bcd* gene copies in the female leads to a posterior shift of the expression domains in embryos carrying the corresponding fusion gene constructs. This shows that the B–KrZ as well as the 4bcd5–KrZ-mediated expression domains are dependent on *bcd* activity. Note that in these staining patterns the expression domain expands posteriorly, thereby shifting the posterior boundary. Since in (e) and (f) only one border of the expression domain is shifted, care was taken to ensure that this shift towards posterior (compare e and f) was not due to overstaining as exemplified in Figure 7b (see Materials and methods).

embryos (data not shown). This finding leaves the possibility that the maternal HB complement in zygotic *hb* mutant embryos and BCD-dependent *hb* activity in embryos that derive from females with an increased copy of the *bcd* gene are sufficient to provide the repression observed.

Activation of gene expression by a single BCD binding site

The experiments described so far have delimited the *bcd*-responsive element to a 142 bp sequence containing a series of five BCD *in vitro* binding sites. To test further whether BCD is able to interact directly with this *Kr cis*-regulatory element *in vivo*, we made fusion gene constructs containing multiple copies of a single BCD *in vitro* binding site. Fusion gene constructs containing four tandem copies of a single BCD *in vitro* binding site of the sequence AGATC**TGTTAATCTCC**GGATCC ('bcd5-element'), 4bcd5KrlacZ, give rise to spatially restricted expression within the anterior third of the wild type embryo at early blastoderm stage (Figure 8e). No expression was observed in the absence of *bcd* activity (Figure 8d). In embryos from females containing increasing copies of the *bcd* wild type gene, the posterior limit of the 4bcd5-mediated expression domain expands posteriorly as the number of *bcd* wild type gene copies in females is increased from one to six copies (see also Materials and methods). This dosage-dependent posterior shift of the 4bcd5-mediated expression domain is exemplified by comparison of the expression domains shown in Figure 8e and f. These results show that the *in vitro* BCD binding site no.5 of the B element can mediate transcriptional activation *in vivo*, and that the posterior extent of the gene expression domain along the longitudinal axis is critically dependent on the concentration of BCD.

Discussion

The *Kr*730 core fragment of the CD1 control unit of the *Kr* gene drives reporter gene expression in place of the authentic *Kr* expression domain at blastoderm stage. The patterns of *Kr*730-mediated gene expression in the various mutant embryos, in which the protein gradients of BCD and HB have been altered, indicate that *Kr*730 DNA contains the essential target sites for a direct or indirect interaction with the two anterior morphogens in the early *Drosophila* embryo (Frohnhöfer and Nüsslein-Volhard, 1986; Hülskamp *et al.*, 1990). Here we focus on their interactions with this *Kr cis*-acting element, but we would like to emphasize that additional components of the pattern forming system of *Drosophila*, such as the terminal system (Nüsslein-Volhard *et al.*, 1987; Weigel *et al.*, 1990b), and posteriorly acting gap genes (Gaul and Jäckle, 1989; Eldon and Pirrotta, 1991; Kraut and Levine, 1991) are likely to interact with these *Kr cis*-acting sequences as well.

Interaction of HB with Kr cis-acting sequences

The BS element is a subfragment of the *Kr*730 DNA and contains multiple high and medium affinity HB binding sites. This BS element contains target sequences which are essen-

tial for mediating HB-dependent repressor function, as indicated by the expression patterns in *hb* mutant embryos as well as in embryos derived from homozygously *nos* mutant mothers. The ability to mediate this repressor function is lost almost completely when sequences containing the high and low affinity HB *in vitro* binding sites are deleted from the BS element. This suggests that HB-dependent repressor function might be mediated by one or several of these binding sites.

Given the observation that the expression of the BS element is absent in *nos* mutant embryos, where maternal HB is distributed throughout the embryo (Tautz, 1988), we can roughly estimate that the HB concentration value which overrides BS-mediated gene activation must be in the range of high maternal HB concentration. This concentration value, as estimated by antibody staining, is always present in the anterior portion of the wild type embryo where HB forms a steep concentration gradient (Tautz, 1988). The anterior expansion of the BS-mediated gene expression domain in embryos which lack zygotic *hb* activity is then consistent with the argument that the anterior border of BS-mediated gene expression is set by repression at high HB concentration.

In addition to setting the anterior border, HB could also be involved in setting the posterior border of BS element-mediated gene expression in a region in the embryo of low HB concentration. There is an anterior shift of the posterior border of the BS expression domain in zygotic *hb* mutant embryos, where a lowered HB gradient is formed by the maternally derived *hb* activity. Furthermore, in embryos derived from *nos* heterozygous females, where the HB gradient is extended towards posterior (Tautz, 1988) the expression domain is shifted posteriorly. These results suggest that HB may also act as an activator. However, the activation function is less obvious than the repressing function of HB since there is no BS element-mediated expression in *bcd* E1 embryos, where low levels of maternal HB are still present (Tautz, 1988). This observation suggests that BCD is an essential component for the activation of BS-mediated gene expression (see below), leaving open the possibility that BCD and HB may act cooperatively to increase the sensitivity of the response to limiting amounts of HB.

The BS element mediates reporter gene expression in the embryo slightly more anterior than the *Kr*730 element. Furthermore, BS-mediated gene expression is not observed in *nos* embryos (in which high maternal *hb* activity is present throughout the embryo), whereas the *Kr*730 element can be activated in the posterior region of these embryos (Figure 2c). Since the *Kr*730 element has four additional HB and one additional BCD sites, these binding sites could be involved in determining the values of HB concentration where activation and repression can occur, thereby fixing the spatial limits of the expression domain in a more posterior position.

Interaction of BCD with Kr cis-acting sequences

Multiple copies of a single BCD binding site within the BS element (the bcd5 sequence) mediate reporter gene expression in the anterior third of wild type embryos. The alterations of the expression patterns in embryos containing different levels of BCD clearly argue that BCD activates gene expression by interaction with this target sequence *in vivo*. This finding provides strong molecular evidence that *Kr* is an immediate target gene of BCD and it is reminiscent of *bcd*-dependent activation of fusion gene constructs containing single BCD binding sites of a 300 bp enhancer/promoter region of the *hb* gene (Driever *et al.*, 1989; Struhl *et al.*, 1989). With *hb* control elements, the fusion genes are activated above a critical threshold concentration of the BCD gradient (Driever *et al.*, 1989; Struhl *et al.*, 1989). The position in the embryo at which *hb* is activated is determined by the affinity of BCD binding site(s) in the *hb* enhancer/promoter region (Driever *et al.*, 1989).

A fusion gene construct containing four BCD binding sites of high and low affinities in addition to the medium affinity site bcd5 (the B element; see Figure 5b) drives gene expression more posteriorly than the 4bcd5 fusion gene. No B element-mediated expression can be observed in *bcd* mutants, and the expression domain is shifted in response to changes in the BCD concentration. Similar shifts of the expression domain can also be observed in wild type embryos when the B element is multiplied. This suggests that activation as well as the posterior border of the B element-mediated expression domain is dependent on BCD. As with the *hb* proximal promotor, the affinity of the BCD binding sites and their number in the *Kr* B element may determine a threshold concentration in the BCD gradient above which the reporter gene is activated, thereby setting a posterior boundary of the expression domain. However, variations of the BCD concentration always lead to a concomitant change of the zygotic *hb* protein gradient and possibly of other *bcd*-dependent gene activities as well (Frohnhöfer and Nüsslein-Volhard, 1986; Driever *et al.*, 1989). We note that the B element contains one low affinity HB binding site and responds weakly to *hb* activity in the embryo (see above). Therefore, BCD may not be the factor which sets the posterior limit of the B-mediated expression domain but merely acts as an 'enhancer' of gene expression within the spatial limits that are fixed by *hb* or other BCD-dependent genes.

We do not know how *bcd*-dependent repression of the B element-mediated expression in the region of high BCD concentration is achieved and how this repression controls the anterior boundary of gene expression in the embryo. There is the possibility that BCD may activate one or several target genes such as the gap gene *gt* (Eldon and Pirrotta, 1991; Kraut and Levine, 1991) and the gap-like genes *btd*, *otd* and *ems* (Dalton *et al.*, 1989; Cohen and Jürgens, 1990; Finkelstein and Perrimon, 1990) which in turn repress or prevent transcriptional activation by the B element. However, none of these genes seem to act as a *bcd*-dependent repressor of the *Kr* B element in the anterior-most region of the embryo as revealed by single mutant analysis. We therefore assume that the level of maternal *hb* activity in the anterior pole region might be sufficient to prevent activation of the B element.

Materials and methods

Drosophila strains

The mutant strains such as $bcd^{E:1}$, hb^{7M} and $nos^{1.7}$ have been described in Nüsslein-Volhard *et al.* (1987) and Lehmann (1985). Females containing extra copies of the *bcd* wild type gene were derived from the fly strain $bcd^{+5/+8}$ described in Berleth *et al.* (1988) and Driever and Nüsslein-Volhard (1988b). Mutant embryos were scored as described previously (Hoch *et al.*, 1990).

Construction of fusion genes

Standard procedures (Sambrook *et al.*, 1989) were applied to generate the 5′ and 3′ deletion constructs of the CD1 element (see Figure 1) either by restriction digest or by Bal31 deletion. For this purpose a 1.2 kb *Bam*HI−*Nco*I fragment covering the CD1 element (see Hoch *et al.*, 1990) was inserted into the *Bam*HI/*Eco*RV sites of the Bluescript vector (Stratagene) to give rise to the pBstBN1.2 construct. All further manipulations were done with this DNA.

The shortened CD1 subfragments were inserted into the polylinker of the P element vector HZ50PL (Hiromi and Gehring, 1987), which contains the *hsp*70 basal promoter linked to the bacterial *lacZ* gene and the *rosy* gene as eye colour marker. The natural orientation of the deletion fragments relative to the *Kr* transcribed region was maintained in all *Kr*−HZ constructs described in Figure 1.

The KrZ P element vector (M.J.Pankratz and H.Jäckle, unpublished) is a derivative of the CaSpeR AUG β-gal vector (Thummel *et al.*, 1988) and contains a *Cla*I−*Ssp*I fragment of the *Kr* gene encompassing the *Kr* basal promotor (about −230 to +260) in its polylinker. The various subfragments of the CD1 element, which are contained in one or several copies in the *Kr*−KrZ constructs were generated by restriction digests of the pBstBN1.2 DNA (see above) or by the polymerase chain reaction (PCR). The BS element constructs were gained by *Bam*HI/*Stu*I digest and subsequent self-ligation of the blunt ended fragment for the multiplication. The B element constructs were generated from pBstBN1.2 DNA by PCR with two primers of the sequence 5-CCTACCTTCAGAAAC-3′ and 5′-TCGCGCTCTA-AGCCG-3′ (for their exact location see sequence in Figure 4).

For obtaining the 4bcd5KrZ construct a double-stranded oligonucleotide with the bcd5 binding site sequence 5′-AGATCTGTTAATCTCCGGATCC-3′ was synthesized. It contains a *Bgl*II and a *Bam*HI linker on either side. The binding site was subsequently self-ligated in the presence of the restriction enzymes *Bam*HI and *Bgl*II to generate ligated tandem copies in one orientation only.

Germline transformation

The fusion gene constructs were integrated into the *Drosophila* genome by P element-mediated germline transformation (Rubin and Spradling, 1982). For this purpose the DNA constructs were injected into ry^{506} or w sn^{w} homozygous mutant embryos. Transformant lines were established and their embryonic progeny were assayed for β-galactosidase expression by antibody staining (see below). For each experiment, at least three independent transformant lines were analysed.

Antibody staining of embryos

Anti-β-gal antibodies were a gift of Dr U.Gaul and anti-hb antibody was a gift of Dr D.Tautz. Antibody staining of whole mount embryos was carried out as described (MacDonald and Struhl, 1986) using the Vectastain ABC Elite-horseradish system. The staining intensity was enhanced by the addition of $NiCl_2$ to 0.08%.

We note that the positions of the boundaries of the expression domains are variable depending on staining time, different levels of gene expression and diffusion of the β-galactosidase gene product during the staining procedure (examples shown in Figure 7a and b). In addition, the staining patterns can be variable with different embryonic stages. Therefore, we scored alterations of the expression domains as well as changes in the staining intensities only when clear and reproducible effects were obtained with several, independently transformed lines under constant staining conditions, i.e. we did the stainings to be compared in parallel and with several independent lines at the same time. For the correct assessment of expression boundaries, it is usually not so critical when both the anterior and posterior borders are shifted simultaneously due to, for example, overstaining. However, when only one border is shifted, as in the case of the *bcd* dosage-dependent shift of the 4bcd5−KrZ gene construct (see Figure 8 e and f), the different expansions of the staining domain towards posterior were repeatedly analysed in embryos derived from mothers with one, two, four and six copies of the *bcd* wild type gene in parallel and under various staining conditions. In each series, a dosage-dependent expansion of the expression domain was observed with several transformant lines.

In some of the KrZ fusion gene constructs, an anterior banded expression domain appeared at ~75% EL shortly before the beginning of gastrulation. This expression is probably due to the CaSpeR P element vector and has been described earlier (Weigel *et al.*, 1990a).

Footprinting experiments

To generate the BCD homeodomain bacterial expression vector, we amplified the *bcd* homoebox out of the vector pAR*bcd* (Driever and Nüsslein-Volhard, 1989) by PCR, using a 30mer primer at position 2274−2293 and a 13mer primer at position 2494−2482 of the *bcd* coding region (Berleth *et al.*, 1988). This fragment was then cloned into the blunt ended *Bam*HI site of the bacterial T7 expression vector pET 3b (Studier and Moffat, 1986) to give rise to the pET*bcd* ho vector. The full-length *hb* bacterial expression vector (pET*hb*), kindly provided by Dr Tautz, contains a 2.4 kb *Xba*I fragment, encompassing the coding region of the *hb* gene (Tautz *et al.*, 1987). This fragment was cloned into the *Xba*I site of pET 3b vector. Transformation of bacteria, induction of the expression vectors and preparation of bacterial extracts was performed as described in Kadonaga *et al.* (1987). Footprinting experiments and the Maxam−Gilbert sequencing were done as described by Kadonage *et al.* (1987) except that our fragments were end-filled by the Klenow fragment of DNA polymerase.

To show most of the binding sites for BCD and HB on *Kr*730 DNA in one footprinting experiment, we generated an artificial subfragment of *Kr*730 DNA (see Figure 3) by cutting off 140 bp from its 5′ end at the *Nsi*I site. In addition, we deleted an internal sequence from position 388 bp to 623 bp (numeration see Figure 4) by Bal31 treatment (see above) thereby loosing HB site 6, which was analysed in separate footprinting experiments. The resulting fragment was cloned into the *Eco*RV site of the polylinker of pBst. For end-labelling with Klenow enzyme, the fragment was cut out either with *Xba*I/*Kpn*I (− orientation; see Figure 3a) or with *Pst*I/*Xho*I (+ orientation; see Figure 3b). End-labelling was performed as described in Sambrook *et al.* (1989).

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