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The Gradient Morphogen *bicoid* Is a Concentration-Dependent Transcriptional Activator

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Summary

The *bicoid* (*bcd*) protein is expressed in an anteroposterior gradient in early Drosophila embryos and controls the zygotic activation of the segmentation gene *hunchback* (*hb*) in a broad but precisely bounded anterior domain. Here we show that the *hb* gene contains multiple regulatory elements that mediate transcriptional activation in response to *bcd* protein. Further, we demonstrate that the resulting patterns of expression in vivo depend critically on both the *bcd* gradient profile and the number and quality of these *hb* elements. Finally, we show that these same elements mediate *bcd*-dependent transcriptional activation in yeast and that this interaction requires distinct DNA binding and activating regions in the *bcd* protein. Our results argue that *bcd* protein normally binds and activates the *hb* gene in a concentration-dependent fashion, thereby allowing the gradient of *bcd* protein to dictate where the *hb* gene is initially turned on in early embryos. They also suggest that the *bcd* gradient has the instructive capacity to activate other subordinate control genes by the same mechanism, each in a distinct spatial domain according to its affinity for *bcd* protein.

Introduction

The precise patterning of embryonic tissues derives in part from the ability of cells to select particular developmental paths based on their relative position. Yet, the nature and mode of action of such "positional information" (Wolpert, 1969) has proven elusive. One notion which has received considerable attention for many years is that this information is provided by gradients of diffusible, "form-generating" molecules, or *morphogens* (Turing, 1952). Localized sources of diffusible, unstable morphogens could generate stable, continuous gradients. Such gradients might provide a series of concentration thresholds that dictate distinct developmental outcomes as a function of distance from the source.

Embryological and genetic experiments on insect embryos have provided considerable evidence that the body pattern is organized by morphogen gradients emanating from localized sources at both ends of the egg (Sander, 1959, 1960, 1976; Nüsslein-Volhard et al., 1987). This view has received compelling support from the analysis of the *bicoid* (*bcd*) gene in Drosophila. Transcripts of the *bcd* gene are synthesized in the nurse cells during oogenesis and then transported to the egg cell where they are trapped at the prospective anterior pole (Frigerio et al., 1986; Berleth et al., 1988). Following fertilization, these transcripts serve as a localized source of *bcd* protein which accumulates rapidly at the anterior pole and diffuses posteriorly generating a stable concentration gradient (Driever and Nüsslein-Volhard, 1988a, 1988b). Finally, the graded distribution of *bcd* protein has been shown to exert a dominant organizing influence on the development of anterior body pattern (Frohnhöfer and Nüsslein-Volhard, 1986, 1987; see also Driever and Nüsslein-Volhard, 1988b).

How does the changing concentration of a single molecular species dictate different developmental outcomes? It is already clear that the *bcd* gradient organizes the spatial expression of regulatory molecules that ultimately determine the number, size, sequence, and polarity of the body segments (e.g., Frohnhöfer and Nüsslein-Volhard, 1987; Tautz, 1988; reviewed in Ingham, 1988). However, its molecular role in this process is uncertain.

One possible mechanism is suggested by the presence of a homeobox domain in the *bcd* coding sequence (Frigerio et al., 1986; Berleth et al., 1988). Proteins containing homeobox domains are thought to act primarily as transcription factors, binding directly to particular DNA sequences and activating or repressing transcription (e.g., Jaynes and O'Farrell, 1988; Thali et al., 1988; Han et al., 1989; reviewed in Scott et al., 1989). Hence, graded *bcd* protein may directly control the spatial expression of a number of target genes by binding and regulating their transcriptional activity in a concentration-dependent fashion. A likely target for such a direct interaction is the segmentation gene, *hunchback* (*hb*; Nüsslein-Volhard and Wieschaus, 1980; Lehmann and Nüsslein-Volhard, 1987; Bender et al., 1987). The *bcd* protein is required for the early activation of *hb* gene expression in the anterior half of the embryo (Tautz et al., 1987; Tautz, 1988; Schröder et al., 1988; Driever and Nüsslein-Volhard, 1989). Moreover, this event is essential for the *bcd* gradient to organize anterior body pattern: embryos lacking the *hb* gene develop like embryos with diminished *bcd* function (Lehmann and Nüsslein-Volhard, 1987; Bender et al., 1987; Frohnhöfer and Nüsslein-Volhard, 1986). Finally, Driever and Nüsslein-Volhard (1989) have recently shown that the *bcd* protein can bind to a number of *hb* DNA sequences in vitro. Some of these sites fall within portions of the *hb* gene that appear to be required for *bcd*-dependent transcriptional activation to occur (Schröder et al., 1988; Driever and Nüsslein-Volhard, 1989); hence, these sites might directly mediate the patterned expression of the *hb* gene controlled by the *bcd* gradient.

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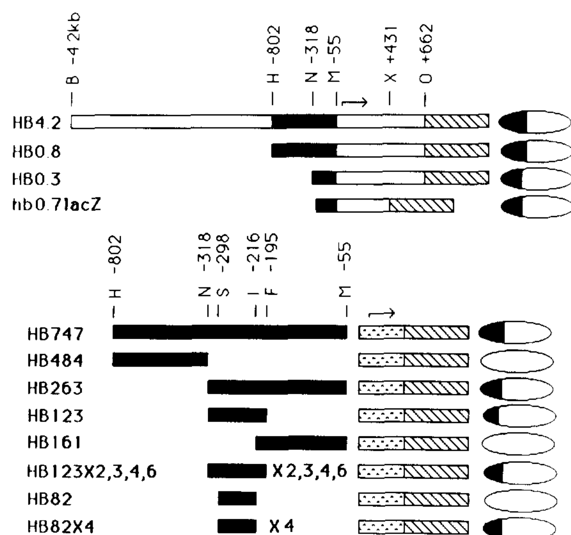

Figure 1. Mapping Regulatory Elements Responsible for Anterior Activation of the *hb* Gene

Two sets of constructs were used to define *hb* regulatory elements necessary and sufficient for activating anterior gene expression during early embryogenesis (see Experimental Procedures). The first set (top) includes three *hb:lacZ* hybrid genes in which the *hb* gene is truncated at an NcoI (O) site ~75 amino acids downstream of the start of translation and fused in-frame to the *lacZ* coding sequence. All three derivatives activate anterior *lacZ* expression, indicating the existence of regulatory elements within the interval bounded by the NheI (N) and NcoI sites. A related derivative *hb0.7lacZ* described by Schröder et al. (1988) is also shown; this construct, which contains *hb* sequences between the SalI (S) and XbaI (X) sites, also activates anterior gene expression in a similar domain, indicating the presence of regulatory elements within this shorter fragment. In the second set of constructs (bottom), fragments from the putative regulatory domain of the *hb* gene immediately upstream of the TATA box and transcriptional start site were inserted as single or multiple copies just in front of the TATA box and transcriptional start site of the *hsp70* gene, the transcripts from this gene give rise to an *hsp70:lacZ* fusion protein (see Experimental Procedures). The behavior of these various constructs in early embryos is indicated to the right (see Figure 2). Open bars: *hb* genomic DNA. Filled-in bars: *hb* upstream regulatory domain. Diagonal hatching: *lacZ* coding sequence. Dots: *hsp70* TATA box, transcriptional start site, and first seven amino acids of coding sequence. B, H, N, M, X, O, S, I, and F: restriction enzymes BamHI, HindIII, NheI, MluI, XbaI, NcoI, SalI, NciI, and FspI, respectively.

Here we test whether direct interactions between *bcd* protein and *hb* DNA control the localized activation of the *hb* gene observed in vivo. Our approach has been to define *cis*-acting regulatory sequences in the *hb* gene that are capable of activating gene expression in vivo in response to *bcd* protein. We then show that these elements, like the endogenous *hb* gene, respond only where the concentration of *bcd* protein exceeds particular thresholds. Finally, we establish that these same elements can mediate *bcd*-dependent gene activation in a completely heterologous system, yeast, and provide evidence that *bcd* protein contains distinct regions capable of binding these *hb* sequences and activating transcription. Based on these results, we argue that the *bcd* protein normally binds distinct target sites in the *hb* gene and that its affinity for these sites determines the minimum concentration of *bcd* protein necessary to activate *hb* transcription. We suggest that this direct interaction is reponsible for the ability of the *bcd* gradient to specify where the *hb* gene is expressed in early embryos, and may constitute a general mechanism by which the *bcd* gradient governs the patterns of expression of other developmental control genes.

Results

Identification of *hb* Regulatory Elements Capable of Activating Anterior-Specific Gene Expression in Early Embryos

The *hb* gene contains a single coding sequence under the control of two promoters (Tautz et al., 1987; Schröder et al., 1988). The distal promoter is active first during oogenesis and later in two narrow stripes during the final (14th) nuclear division cycle preceding cellularization of the blastoderm. Expression of *hb* via this promoter does not depend on *bcd* function (Tautz, 1988). In contrast, the proximal promoter is silent during oogenesis, but is activated during the 11th or 12th nuclear cycle in a restricted domain encompassing the anterior 45% of the embryo; transcripts from this promoter persist until the end of the blastoderm stage and the onset of gastrulation. Zygotic activation of the proximal promoter is one of the first transcriptional events detected in early embryos and is completely dependent on *bcd* function (Tautz et al., 1987; Schröder et al., 1988).

Deleted forms of the *hb* gene containing as little as 300 bp upstream of the proximal promoter are capable of responding to *bcd* and generating a pattern of early zygotic gene expression similar to that of the normal gene (Schröder et al., 1988; Figure 1). To determine which sequences are sufficient to activate this early pattern of expression, we inserted short DNA fragments from the *hb* promoter region into a position just upstream of the TATA box and transcriptional start site of an *hsp70:lacZ* (*HSZ*) reporter gene (Lis et al., 1983; Garabedian et al., 1986; Hiromi and Gehring, 1987; see legend to Figure 1). The resulting *HB:HSZ* genes were then inserted into the Drosophila genome by P element–mediated transformation, and the expression of *lacZ* protein was monitored in early embryos.

As shown in Figures 1 and 2, a 747 bp segment of the *hb* upstream region (*HB747*) contains regulatory elements capable of activating anterior expression of the *HSZ* reporter gene. Subdividing the initial 747 bp segment into distal (*HB484*) and proximal (*HB263*) fragments revealed that only the proximal 263 bp fragment could activate anterior *lacZ* expression (here and below, failure to detect *lacZ* activity corresponds to a level of expression that is at least 20-fold lower than that associated with any of the *HB:HSZ* genes giving a positive response—see Experimental Procedures). When this 263 bp fragment was further subdivided into overlapping distal (*HB123*) and proximal (*HB161*) fragments, only the distal 123 bp fragment retained regulatory activity. Finally, when this 123 bp fragment was truncated by deleting 20–30 bp from both ends (*HB82*), the resulting 82 bp fragment failed to activate detectable *lacZ* expression. Thus, the ability of the intact 747 bp upstream region to activate anterior expression

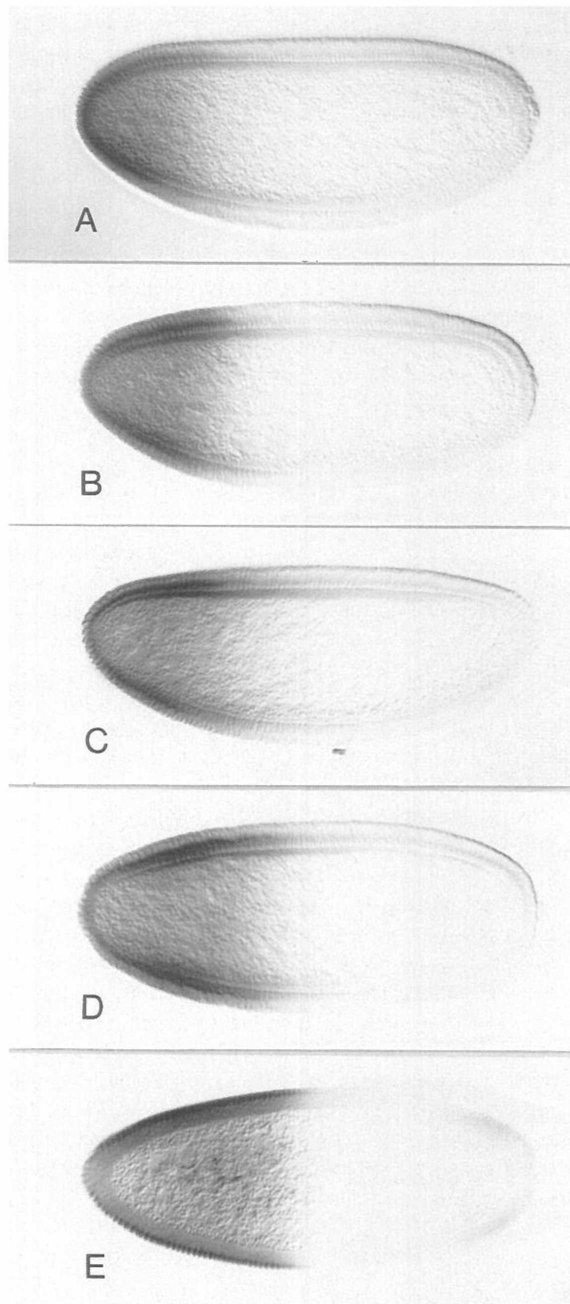

Figure 2. Anterior Gene Activation Mediated by Regulatory Elements from the *hb* Gene

(A), (B), (C), and (D) show, respectively, the patterns of *lacZ* expression generated by the *HB:HSZ* genes *HB123*, *HB123X2*, *HB263*, and *HB747* relative to the expression of *hb* protein by the endogenous gene (E). Note that the posterior boundary of expression moves progressively posteriorly as the either the number of copies of a fragment or the size of the fragment increases. Note also that the boundary of *lacZ* expression is not as sharp as that of the endogenous *hb* protein. This difference may reflect (i) that the *hb* mRNA or protein is less free to diffuse from its site of synthesis than products of the *lacZ* gene, or (ii) that the activation of the *hsp70:lacZ* gene is less tightly controlled than the endogenous *hb* gene. All of the embryos are on or just over the verge of completing cellularization and beginning gastrulation.

of the *HSZ* gene depends on a 123 bp core region that is normally situated around 200 bp upstream of the proximal start site of *hb* transcription.

Multiple *hb* Elements Contribute to the Pattern of Gene Expression in the Anterior Embryo

Both the endogenous *hb* gene as well as the various *HB:HSZ* hybrid genes described above are initially expressed in broad domains extending from the anterior pole to characteristic posterior limits along the body. As shown in Figure 2, the boundaries are quite sharp, *lacZ* expression generally declines from high uniform levels anteriorly to undetectable levels posteriorly over an interval of approximately 10%–15% egg length (EL). Note though that *lacZ* expression is somewhat less sharp than that of the endogenous *hb* gene, which falls from high to undetectable levels over an interval of around 5%–8% EL (Figure 2E). This difference may reflect the ability of *lacZ* gene products to diffuse more readily than *hb* transcripts or protein (a distinct possibility since *hb* protein, unlike *lacZ* protein, is generally trapped in nuclei). It is also likely that the boundary of endogenous *hb* gene expression is sharper because it is controlled by additional regulatory interactions that the *HB:HSZ* genes are not subject to (e.g., activation of the distal *hb* promoter; Schröder et al., 1988). Nevertheless, the boundaries of *lacZ* expression derived from the different *HB:HSZ* genes are sufficiently sharp to allow them to be distinguished from one another.

Although the core regulatory region (*HB123*) appears both necessary and sufficient for activating anterior *lacZ* expression, the extent of the response, especially the posterior boundary of expression, clearly depends on additional regulatory elements. Thus, the core 123 bp fragment activates *lacZ* expression in the anterior 20%–30% of the embryo; however, the 263 and 747 bp fragments lead to expression in domains of approximately 30%–40% and 35%–45%, respectively, approaching that of the endogenous *hb* gene, which extends to around 44%–50% EL (Figure 2). The role of the additional regulatory elements outside the 123 bp core is unclear at present: although they clearly augment the response of elements within the core, they do not appear to be capable of functioning without it (Figure 1).

Eukaryotic promoters are often composed of several functionally redundant elements, the number and potency of these elements determining the response of the promoter to putative transcriptional activators. We therefore tested whether the partial or negligible activities of the more severely truncated forms of the *hb* regulatory region could be enhanced by using multiple copies of these fragments. In the first case, we generated transgenic flies bearing constructs carrying two, three, four, and six tandem copies of the 123 bp core fragment in front of the *HSZ* gene (*HB123X2*, *X3*, *X4*, *X6*). The posterior boundary of expression generated from these constructs shifted significantly more posteriorly as the number of 123 bp fragments rose, approaching the pattern observed for one copy of the 263 bp fragment (e.g., Figures 2C and 2D). The level of *lacZ* expression also appeared to increase as the number of 123 bp fragments rose. Note however that the ap-

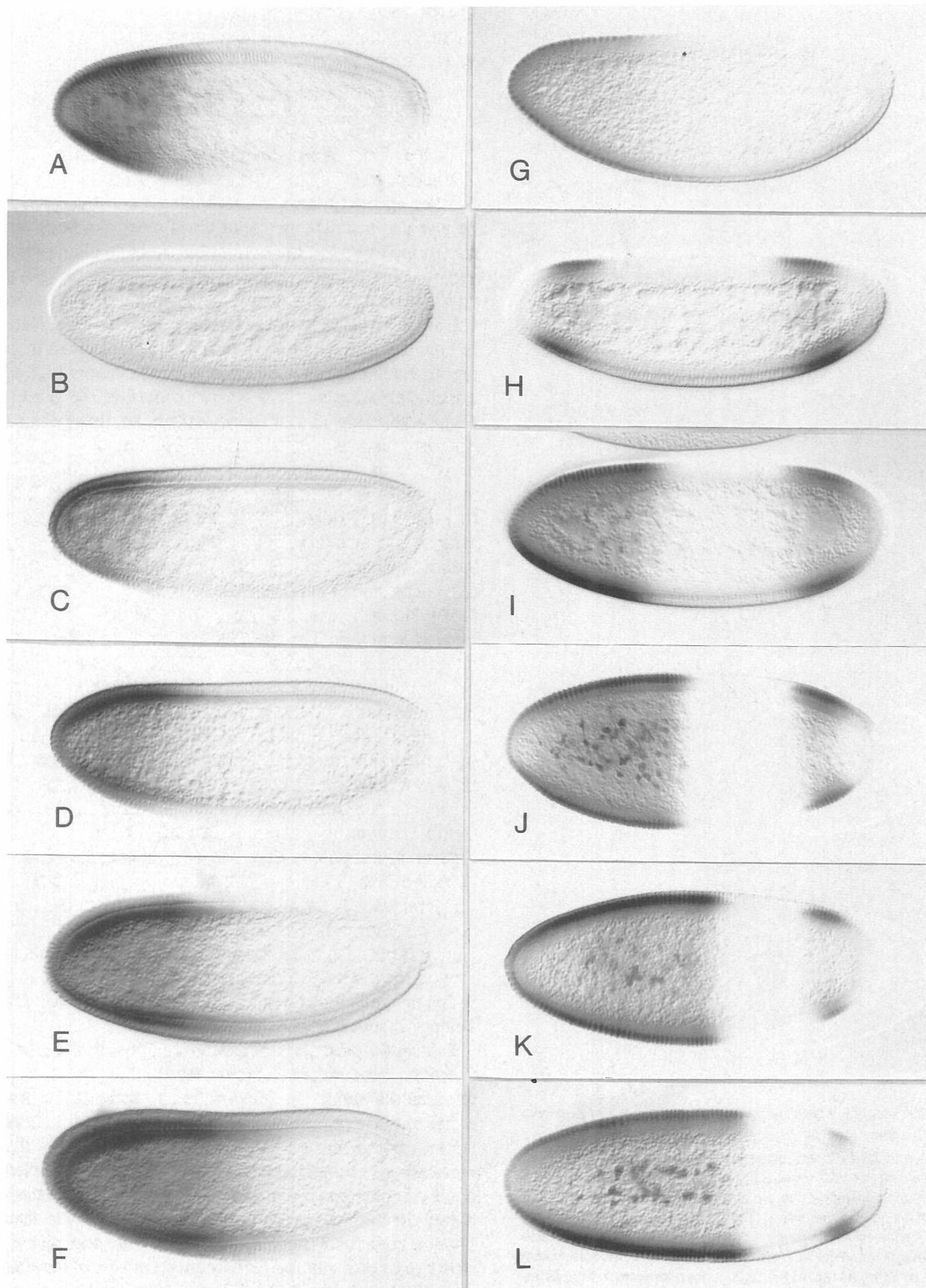

Figure 3. The *bcd* Gradient Controls the Pattern of Activation of Both the *HB:HSZ* and *hb* Genes

(B), (C), (D), (E), and (F) show the pattern of *lacZ* expression generated by the *HB263* gene in embryos derived from mothers carrying zero, one, two, four, and six copies of the *bcd* gene; similarly (H), (I), (J), (K), and (L) show the patterns of endogenous *hb* protein expression in their sibling embryos, respectively. Note that the boundaries of expression of both genes shift posteriorly as the *bcd* gene dosage rises. Note also that the *HB263* gene is not active in embryos derived from mothers lacking functional copies of the *bcd* gene (B), despite the fact that such embryos express the endogenous *hb* gene under independent genetic control via a different promoter (G, H). (G) shows an earlier syncytial blastoderm derived from a mutant *bcd* embryo expressing *hb* protein derived solely from maternal *hb* transcripts. By the late cellular blastoderm stage (H), this maternally

parent posterior shifts in the boundary of *lacZ* expression are unlikely to be due simply to the increasing levels of expression, as shown below in the case of *HB123* and *HB123X2* genes in *vasa exuperentia* embryos. In the second case, we examined derivatives containing four copies of the more severely truncated 82 bp fragment (*HB82X4*). Although one copy of *HB82* has no detectable activity, four copies activate a pattern of anterior expression similar to that of two copies of the 123 bp fragment (*HB123X2*; data not shown). These results indicate that the activity of the 747 bp regulatory domain depends on the collective behavior of a series of partially redundant elements, some of which can substitute for others when present in multiple copies. Further, they suggest that the 123 bp core region, which is both necessary and sufficient for activation, may itself depend on at least two component elements, one of which can substitute for the other when present in multiple copies.

Dependence of the *hb* Anterior Activation Elements on the Concentration of *bcd* Protein

To test whether the posterior limits of expression of the *HB:HSZ* genes as well as the endogenous *hb* gene are dictated by the concentration gradient of *bcd* protein, we examined the distribution of their products in embryos derived from mothers carrying zero, one, two, four, or six copies of the *bcd* gene.

Embryos derived from mothers carrying no functional copies of the *bcd* gene fail to activate *hb* transcription from the proximal (anterior-specific) promoter although they do express *hb* protein derived from maternal and zygotic mRNAs transcribed from the distal promoter (Tautz et al., 1987; Tautz, 1988; Schröder et al., 1988; Figures 3G and 3H). Such embryos fail to express detectable *lacZ* protein from *HB:HSZ* genes such as *HB747* and *HB263* (e.g., Figure 2B), indicating that *bcd* protein is required for these regulatory elements to mediate *lacZ* expression.

As previously shown (Driever and Nüsslein-Volhard, 1988b), the concentration of *bcd* protein at any point along the antero-posterior axis of the embryos is roughly proportional to the number of the *bcd* gene copies present maternally. In embryos derived from mothers carrying one, two, four, or six copies of the *bcd* gene, the domains of *hb* and *HB:HSZ* expression spread posteriorly as the number of copies increases from one to six (e.g., Figure 3). Equivalent results were obtained with the *HB263*, *HB123*, *HB123X2*, and *HB82X4* genes in which *HSZ* expression is regulated by elements within the 263, 123 or 82 bp fragments of the *hb* upstream region (see Figure 1). Note, however, that at any given *bcd* gene dosage, the posterior boundaries of expression of these different genes were distinct, displaying the same rank order observed in wild-type embryos (Figure 2). Thus, the boundaries delimiting the domains of *hb* and *HB:HSZ* expression appear to be defined by specific concentration thresholds of *bcd* protein: these threshold concentrations are generated at progressively more posterior positions in embryos derived from mothers with increasingly higher numbers of copies of the *bcd* gene.

Further evidence that the various *HB:HSZ* genes respond to different minimum thresholds of *bcd* protein has been obtained by examining their activity in embryos derived from females lacking both the *exuperantia* (*exu*) and *vasa* (*vas*) gene functions. Loss of both gene functions leads to low levels of *bcd* protein throughout the embryo (Frohnhöfer and Nüsslein-Volhard, 1987; Driever and Nüsslein-Volhard, 1988a); note, however, that *bcd* expression is not uniform, as previously reported, but rather forms a shallow antero-posterior gradient extending to the posterior pole (Figure 4B). Under these conditions we find that the *HB263* derivative gives rise to anterior *lacZ* expression, as does the *HB123X2* derivative which carries two copies of the core fragment (Figures 4D and 4E). Note that the boundaries of expression of both genes are less distinct than in wild-type embryos and that the domain of *lacZ* expression generated by the *HB263* gene extends more posteriorly than that of the *HB123X2* gene. In contrast, no expression is obtained from the *HB123* derivative carrying only a single copy of the core (Figure 4C). Hence, the generally low level of *bcd* protein present anteriorly in these embryos appears to be above the threshold necessary for triggering expression of the *HB263* and *HB123X2* derivatives, but below that for triggering the *HB123* derivative. The restricted anterior expression obtained from the *HB263* and *HB123* derivatives differs from that of the endogenous *hb* gene, which is expressed at ubiquitously high levels in *vas exu* embryos (Figure 4F), suggesting that these two genes are refractory to low levels of *bcd* protein sufficient to activate the endogenous gene.

These results establish that both the endogenous *hb* gene as well as the regulatory elements identified upstream of the proximal transcriptional start site respond to the *bcd* protein gradient in a concentration-dependent fashion. Moreover, they indicate that the minimum concentration required to activate gene expression depends on the number or quality of the regulatory elements.

The *hb* Anterior Activation Elements Do Not Respond in an Autoregulatory Fashion to *hb* Protein

The close association between the patterns of endogenous *hb* and *lacZ* expression observed in these experiments raises the possibility that the *hb* regulatory elements we have defined do not respond directly to *bcd* protein, but rather are the targets of the *hb* protein that

derived protein has dissipated. However, these mutant embryos now activate stripes of *hb* protein at both ends, under the control of the terminal determinant system (Tautz, 1988; see Nüsslein-Volhard et al., 1987). The embryo in (A) is derived from parents carrying two copies of the *bcd* gene, but lacks zygotic *hb* gene activity (see Experimental Procedures); nevertheless, it shows the normal pattern of *lacZ* expression obtained from the *HB263* gene. Finally, note that in (I), (J), (K), and (L), increasing numbers of copies of the *bcd* gene are also associated with progressively more posterior positions of the posterior domain of zygotic *hb* gene expression. Whether this posterior shift of the terminal stripe reflects a direct response to the changing *bcd* gradient profile is unknown.

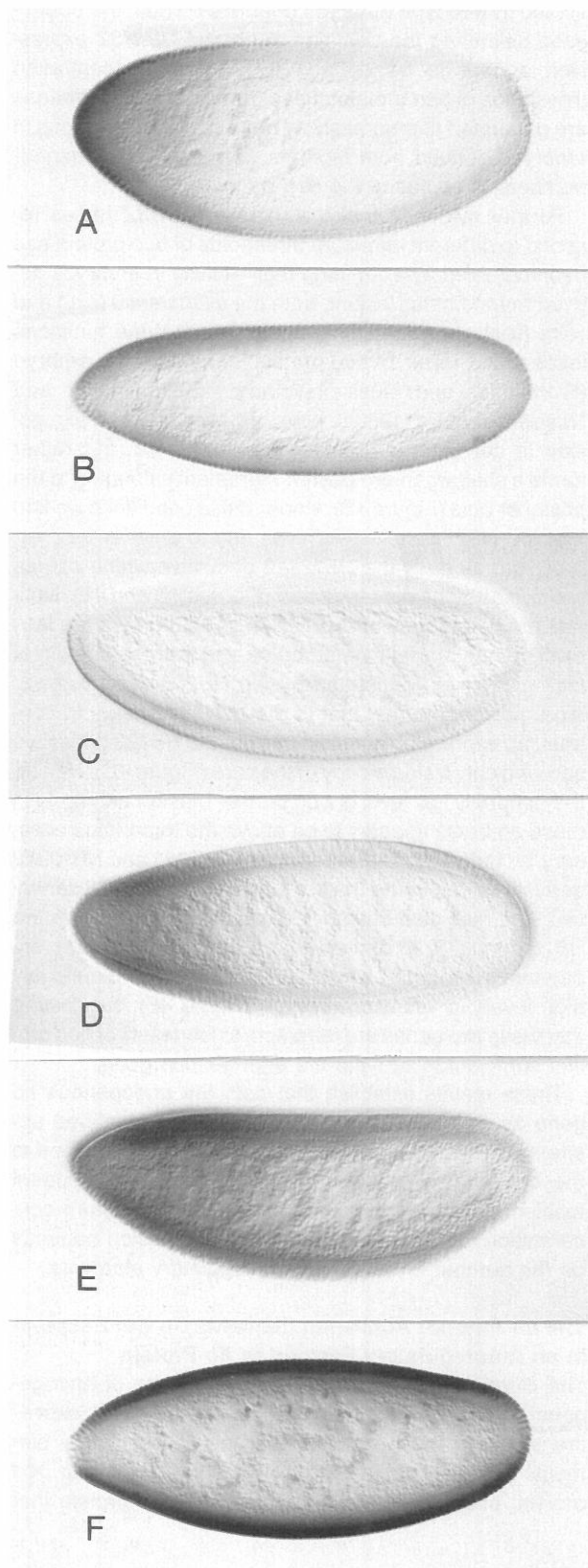

Figure 4. Expression of *HB:HSZ* and *hb* Genes in *vasa exuperantia* Mutant Embryos

(A) and (B) show the *bcd* protein gradient in wild-type and *vasa exuperantia* (*vas exu*) mutant embryos, respectively. Note that in wild-type embryos, the gradient peaks at high levels at the anterior pole and drops dramatically to undetectable levels midway down the body; however, in *vas exu* embryos, relatively low levels of *bcd* protein are detected throughout, forming a shallow gradient with decreasing *bcd* protein toward the posterior pole. (C), (D), (E), and (F) show the patterns of expression of the *HB123*, *HB123X2*, *HB263*, and endogenous *hb* genes in *vas exu* embryos (to be compared with A, B, C, and E in Figure 2). The generally low level of *bcd* expression is insufficient to activate the *HB123* gene. In contrast, both the *HB123X2* and *HB263* genes are activated anteriorly, the domain of expression of the *HB263* gene spreading significantly posterior to that of *HB123X2*, as in wild-type embryos. Note that the boundaries of expression of these two genes are less well defined than in wild-type embryos (Figures 2B and 2C), presumably because of the more gradual decline in *bcd* protein concentration. Finally, note that the endogenous *hb* protein is expressed uniformly throughout the embryo, indicating that even the relatively low level of *bcd* protein present at the posterior end is sufficient to activate the gene fully.

stimulates its own expression in an autoregulatory fashion. Two results argue strongly against this possibility. First, as described above, *bcd*$^-$ embryos fail to activate *lacZ* expression under the control of the putative *bcd*-dependent regulatory elements (Figure 3B). Yet, these embryos express *hb* protein derived from both maternal and zygotic transcripts of the distal *hb* promoter in anterior and posterior domains of early embryos (Figures 3G and 3H), indicating that *hb* protein is not sufficient in itself to trigger these anterior-specific elements. Second, *hb*$^-$ embryos that express the *bcd* protein gradient but lack any zygotic *hb* gene function activate normal patterns of *lacZ* expression from derivatives such as *HB263* (Figure 3A). These embryos do contain moderate levels of *hb* protein derived from maternal transcripts; nevertheless, this maternally derived protein is unlikely to be responsible for triggering *lacZ* expression as it is also present in *bcd*$^-$ embryos that do not express any of the *HB:HSZ* genes. Thus, the *hb* regulatory elements defined here appear to respond specifically to the distribution of *bcd* rather than *hb* protein.

The *hb* Anterior Activation Elements Mediate Transcriptional Activation by the *bcd* Protein in Yeast

The experiments described above demonstrate that *bcd* protein regulates the activation of both the endogenous *hb* gene and a series of *HB:HSZ* genes via regulatory elements normally situated immediately upstream of the proximal *hb* promoter. To test whether this regulation results from a direct interaction between *bcd* protein and these *cis*-acting regulatory elements, we tried to determine whether these elements can mediate *bcd*-dependent transcriptional activation in yeast.

A series of fragments derived from the upstream regulatory region of the *hb* gene, including those used in the *HB263*, *HB161*, *HB123*, and *HB82* derivatives tested in Drosophila, were fused just upstream of the yeast *his3* TATA element and structural gene (see Figure 5). The resulting *HB:HIS3* hybrid DNAs (*Y263*,*Y161*, *Y123*, *Y102*, *Y85*, and *Y82*) were integrated into the yeast genome such that they replaced the normal *his3* chromosomal locus. Yeast cells carrying these target genes were then transformed with DNA constructs in which various forms of the

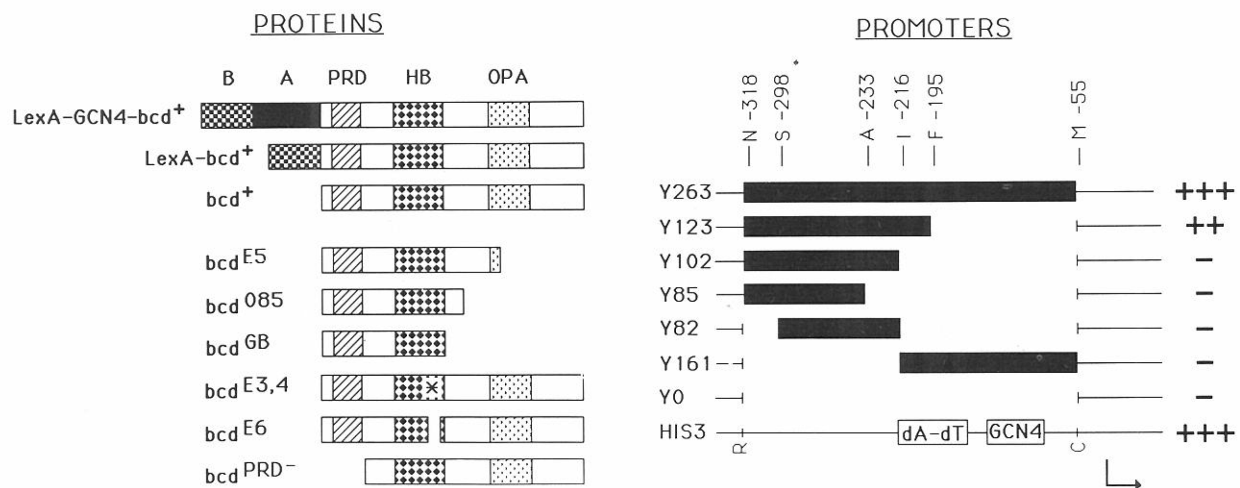

Figure 5. Structures of Proteins and Promoters Tested in Yeast

Left panel: protein structures. The *bcd* protein is 494 amino acids in length and contains a PRD repeat (PRD; amino acids 12–41), a homeobox (HB; amino acids 97–156) and an opa repeat (OPA; amino acids 261–304). The wild-type protein was expressed unaltered or fused to the binding domain of the bacterial *lexA* protein (B; amino acids 1–87) or to both the *lexA* binding domain and the transcriptional activation domain of the yeast *GCN4* protein (A; amino acids 12–144; numbering as in Hope and Struhl [1986]). Also shown are the structures of the mutant *bcd* proteins: these proteins were each expressed as *lexA-bcd* and *lexA-GCN4-bcd* derivatives identical to the *lexA-bcd*+ and *lexA-GCN4-bcd*+ derivatives diagrammed at top except for the mutant lesion. As described in the text, Experimental Procedures, and the legend to Figure 7, the bcd^{GB}, bcd^{085} and bcd^{E5} mutations are amber mutations that truncate the protein at amino acids 157, 183, and 263, respectively; the bcd^{E3} and bcd^{E4} mutations change amino acids 127 and 131, the bcd^{E6} mutation causes an in-frame deletion of amino acids 125–135, inclusive, and the bcd^{PRD-} deletion removes the first 53 amino acids of the protein. The results presented in the text, Table 1, and Figure 6 show that the region of the protein between the homeobox domain and the beginning of the opa repeat (amino acids 157–263) is required for transcriptional activation (amino acids 157–263). Its sequence is as follows: AspGlnHisLysAspGlnSerTyrGluGlyMetProLeuSerProGlyMetLysGlnSerAspGlyAspProProSerLeuGlnThrLeuSerLeuGlyGlyGlyAlaThr-ProAsnAlaLeuThrProSerProThrProSerThrProThrAlaHisMetThrGluHisTyrSerGluSerPheAsnAlaTyrTyrAsnTyrAsnGlyGlyHisAsnHisAlaGlnAlaAsnArg-HisMetHisMetGlnTyrProSerGlyGlyGlyProGlyProGLySerThrAsnValAsnGlyGlyGlnPhePheGlnGlnGlnGln (Berleth et al., 1988; the amino acids changed to stop codons by the bcd^{GB}, bcd^{085}, and bcd^{E5} mutations are shown in italics). Right panel: promoter structures. The bottom line shows the wild-type *his3* promoter region, including the poly(dA-dT) element, the *GCN4* binding site, the transcriptional start site (Struhl, 1986), and EcoRI (R) and SacI (C) restriction sites positioned 447 and 84 bp upstream of the start site. The *Y0* gene is derived from the wild-type gene by deleting all the sequences between the EcoRI and SacI sites. The *Y82*, *Y85*, *Y102*, *Y123*, *Y161*, and *Y263* genes were constructed by inserting the appropriate fragments from the *hb* upstream regulatory region (shown in black) in place of the deleted *his3* sequences, just upstream of the *his3* TATA box and transcriptional start site (see Experimental Procedures: N, S, A, I, F, and M = restriction enzymes NheI, SalI, BalI, NciI, FspI, and MluI, respectively). All of the *Y* series genes were inserted into the *his3* chromosomal locus by gene replacement, and the resulting cells were transformed with *YCp88* plasmids in which the the various *bcd* proteins are expressed under the control of the constitutive *ded1* promoter (Hope and Struhl, 1986). The phenotypes of strains carrying the different *Y* series alleles and expressing either *bcd*+ or *lexA-bcd*+ protein were tested by assaying their ability to grow in the presence of aminotriazole (see Experimental Procedures and Table 1); +++ = growth equivalent to a strain containing the wild-type *his3* gene; ++ = slower than wild-type, but easily detectable growth; − = no growth.

bcd coding sequence were placed under the control of the yeast *ded1* promoter (Hope and Struhl, 1986). The resulting strains were assayed for *his3* expression by their abilities to grow in medium containing aminotriazole; the degree of aminotriazole resistance is directly related to the level of *his3* mRNA (Hill et al., 1986; Struhl, 1987).

Yeast cells expressing intact *bcd* protein activate *his3* expression when the 263 bp *hb* fragment is present just upstream of the *his3* TATA box (*Y263*); activation is not observed in control cells containing *Y263* but lacking *bcd* protein (Figure 6). The degree of aminotriazole resistance indicates that the level of *bcd* activation through *Y263* is roughly equivalent to that achieved by the transcriptional activator *GCN4* through its target site in the normal *his3* promoter (Hill et al., 1986). As in flies, activation by *bcd* protein is also mediated by the 123 bp core fragment (*Y123*), but appears less strong than that mediated by the 263 bp fragment. In addition, both the 161 bp fragment adjacent to the core domain and the more severely truncated 82 bp core fragment fail to respond.

Thus, fragments of the *hb* regulatory domain that normally dictate anterior gene expression in fly embryos are capable of mediating *bcd*-dependent transcriptional activation in yeast. Moreover, there is a good correlation between the subset of fragments that are active in each organism, suggesting that similar if not identical molecular interactions are occurring in both.

Distinct Domains in the *bcd* Protein Are Required for DNA Binding and Activation

The ability of *bcd* protein to activate transcription in yeast via the *hb* regulatory elements strongly suggests that the protein is capable of directly binding specific sites in the *hb* DNA and activating the yeast transcriptional machinery. If so, it should contain structural domains capable of performing each of these molecular interactions. We therefore tested whether such functional domains exist by assaying the putative DNA binding and transcriptional activating abilities of mutant *bcd* proteins in which particular

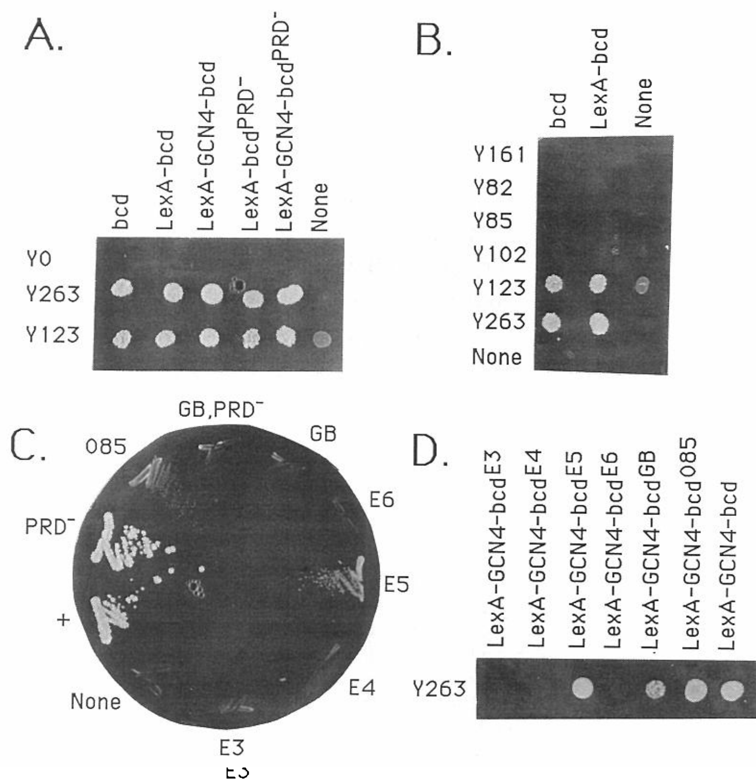

Figure 6. Phenotypic Analysis in Yeast Cells

(A) Activation of the *HB:HIS3* genes *Y123* and *Y263* by intact *bcd* protein and its *lexA-bcd* and *lexA-GCN4-bcd* derivatives. Here, as in (B) and (D), 10^4 cells from strains containing the indicated *HB:HIS3* gene and *bcd* protein derivative (see Figure 5) were spotted on plates containing 20 mM aminotriazole and incubated for 2 days. Control strains lacking either *hb* or *bcd* sequences fail to grow under these conditions except for the strain containing *Y123* which grows slowly in the absence of *bcd* protein (the *cis*-acting sequences involved in mediating this *bcd*-indendent expression appear to map between the NciI and FspI sites in the *hb* DNA, but have not been investigated further); note, however, that *Y123* strains expressing *bcd* protein grow much better under these conditions, indicative of *bcd*-dependent activation. (B) *Cis*-acting *hb* sequences required for mediating *bcd*-dependent transcriptional activation. Note that only the *Y263* and *Y123* genes give a positive response. (C) Relative abilities of *lexA-bcd* protein and various *bcd* mutant derivatives to activate the *Y263* gene. In this case, strains containing the *Y263* gene and the indicated *lexA-bcd* derivative were streaked on a plate containing 20 mM aminotriazole and incubated for 3 days (*GB, PRD⁻* is a *lexA-bcd*^{GB} derivative that also lacks the PRD repeat). (D) Relative abilities of *lexA-GCN4-bcd* and its *bcd* mutant derivatives to activate the *Y263* gene. Note that the *bcd*^{E3}, *bcd*^{E4}, and *bcd*^{E6} derivatives, all of which have lesions in the homeobox, render the corresponding *lexA-GCN4-bcd* fusion protein incapable of activating expression mediated by the *hb* regulatory sequences, though these proteins are capable of mediating expression via the *lexA* binding site (Table 1).

regions are absent, altered, or replaced by known binding and activation domains derived from other proteins.

The *bcd* protein is known to contain at least three distinct structural motifs, a "PRD-repeat" encoding a repeating his-pro polymer at the amino-terminal end, a homeobox domain in the middle, and an "opa-repeat" encoding a polyglutamine repeat near the carboxy-terminal end (Frigerio et al., 1986; Berleth et al., 1988; Figure 5). At least one of these, the homeobox domain, has been implicated in site-specific DNA binding (reviewed in Scott et al., 1989). We first determined the molecular lesions responsible for nine *bcd* mutations (Frohnhöfer and Nüsslein-Volhard, 1986; Driever and Nüsslein-Volhard, 1988a) as described in Figure 7 and Experimental Procedures. Of these mutations, two are missense codons within the homeobox (*bcd*^{E3}, *bcd*^{E4}), one is an in-frame deletion of part of the homeobox (*bcd*^{E6}), and three are amber codons truncating the protein 2, 28, and 108 amino acids downstream from the homeobox domain (*bcd*^{GB}, *bcd*^{085}, and *bcd*^{E5}). In addition, we also generated a *bcd* coding sequence lacking the entire PRD-repeat (Experimental Procedures).

The protein-coding regions from the seven mutations described above as well as the wild-type gene were then fused to the DNA-binding domain of the bacterial *lexA* protein (Brent and Ptashne, 1985) alone or in conjunction with the transcriptional activation domain of the yeast *GCN4* protein (Hope and Struhl, 1986; Figure 5). The resulting hybrid proteins were then tested for their ability to activate the *HB:HIS3* target gene *Y263* as well as a second target gene composed of the *lexA* binding site, a yeast TATA element, and the *lacZ* structural gene (*LEXA:LACZ*; Table 1). *lexA-bcd* or *lexA-GCN4-bcd* derivatives capable of activating the *HB:HIS3* gene must have a region capable of recognizing and binding to the *hb* regulatory sequences, whereas *lexA-bcd* derivatives activating the *LEXA:LACZ* gene must contain a transcriptional activation function.

bcd⁺ Derivatives with or without the PRD Repeat

As shown in Table 1, the *lexA-bcd*⁺protein activates expression from both the *HB:HIS3* and the *LEXA:LACZ* target genes. The *lexA-GCN4-bcd*⁺protein behaves similarly except that it activates *lacZ* expression more efficiently. As proteins containing only the *lexA* binding domain do not activate transcription from equivalent target genes (Brent and Ptashne, 1985; Hope and Struhl, 1986), the intact *bcd* protein appears to contain a region that is functionally analogous to the activation domain of the *GCN4* regulatory protein. However, *bcd* appears to be a relatively weak activator compared with *GCN4* protein: when both proteins are fused essentially intact to the *lexA* binding domain, the *lexA-bcd* derivative stimulates transcription of the *LEXA:LACZ* target gene about 6-fold less efficiently than *lexA-GCN4* derivative (Table 1). *lexA-bcd*^{PRD} and *lexA-GCN4-bcd*^{PRD} fusion proteins lacking the PRD-repeat near the N-terminus of *bcd* appear indistinguishable from their wild-type counterparts suggesting that this region is not essential for DNA binding or transcriptional activation mediated by the *hb* regulatory sequences in yeast.

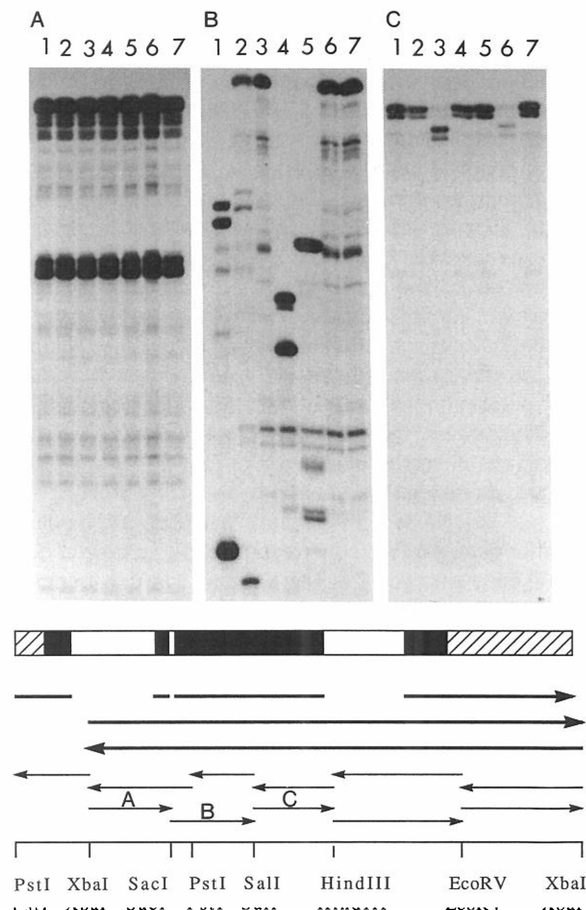

Figure 7. Mapping *bcd* Mutations

Labeled sense or antisense transcripts of mutant alleles were generated as described in Experimental Proceduress, hybridized to labeled complementary RNA probes, the hybrids digested with RNAase to cleave mismatches, and the products displayed by electrophoresis and autoradiography. The *bcd* transcription unit is diagrammed with the 5′ end to the left (introns = open boxes; exon coding regions = solid boxes; exon noncoding regions = hatched boxes). The bold arrows represent the unlabeled transcripts: the processed sense transcript was purified from homozygous or heterozygous mutant embryos and the sense and antisense genomic transcripts were synthesized in vitro from cloned DNAs. The various labeled wild-type probes are shown as fine arrows; results using probes A, B, and C hybridized to antisense mutant genomic transcripts are shown in (A), (B), and (C). For each panel, lanes 1–7 correspond to mutants bcd^{E3}, bcd^{E4} bcd^{E5}, bcd^{GB}, bcd^{085}, bcd^{111}, and bcd^{2-13}, respectively. None of the mutations map within the segment covered by probe A and all lanes appear identical (the prominent lower band is probably due to a polymorphism; other minor bands in all panels are due either to polymorphisms or background cleavage at correctly matched positions—the simulataneous analysis to several different mutants serves to identify such bands). In (B), lanes 1, 2, 4, and 5 show novel bands while the remaining lanes show the wild-type pattern. In (C), lanes 3 and 6 show novel bands while all other lanes have the wild-type pattern. All mutations were further characterized by sequencing the appropriate regions.

bcd Homeobox Mutations

All three *lexA-GCN4-bcd*hybrid proteins containing mutations in the homeobox domain (bcd^{E3}, $bcd^{E4}$and bcd^{E6}) fail to activate the *HB:HIS3* gene yet they are capable of activating the *LEXA:LACZ* gene. These mutations therefore selectively eliminate the ability of their *lexA-GCN4-bcd* derivatives to interact with the *hb* target sequences, consistent with the view that the homeobox domain is required for DNA binding. Note, however, that the *lexA-bcd* derivatives of these mutant proteins, which should in principle be able to bind the *lexA* target sites and activate transcription, fail to activate both the *LEXA:LACZ* and *HB:HIS3* genes. This negative result may reflect a simple instability of the mutant proteins in yeast, though their *lexA-GCN4-bcd* derivatives are sufficiently stable to bind and activate the *LEXA:LACZ* target gene. An alternative possibility is that the *bcd* DNA binding domain increases transcriptional activation mediated by the *lexA* DNA binding domain, especially as the *GCN4* and *jun* DNA binding domains have very similar effects (Hope and Struhl, 1986; Struhl, 1987).

bcd Carboxy-Terminal Truncations

Derivatives of the three amber mutations (bcd^{GB}, bcd^{085}, and bcd^{E5}), all of which truncate the *bcd* protein downstream of the homeobox, retain at least some ability to activate the *HB:HIS3* gene. Of these, the bcd^{GB} mutation is most revealing. As shown in Table 1, the *lexA*-bcd^{GB} protein fails to activate either the *HB:HIS3* or *LEXA:LACZ* target genes, whereas the *lexA-GCN4*- bcd^{GB} protein activates both. Thus, the bcd^{GB} mutation appears to delete a region required for transcriptional activation which is located carboxy-terminal to the homeobox domain while retaining the ability to recognize and interact with the *hb* target sequences. Activity of the *lexA*-bcd^{GB} protein can be restored by adding the *GCN4* activation domain, suggesting that the carboxy-terminal region of *bcd* deleted by the bcd^{GB} mutation is functionally analogous to an activation domain. The remaining amber mutations, which leave the homeobox domain intact but truncate the coding sequence 28 (bcd^{085}) and 108 (bcd^{E5}) amino acids downstream, behave differently from the bcd^{GB} mutation in that they retain the ability to activate partially both the *LEXA:LACZ* and *HB:HIS3* genes when fused only to the

Table 1. Phenotypes of *lexA-bcd* and *lexA-GCN4-bcd* Derivatives in Yeast

	lexA-bcd Derivatives		*lexA-GCN4-bcd* Derivatives	
bcd Allele	*his3*	*lacZ*	*his3*	*lacZ*
wt	+ + +	80	+ + +	120
PRD⁻	+ + +	50	+ + +	ND
E3	–	1	–	70
E4	–	8	–	50
E5	+ +	130	+ + +	250
E6	–	4	–	50
085	+	30	+ + +	120
GB	–	15	+ +	90

Levels of activation of the *HB:HIS3 (Y263)* and *LEXA:LACZ* genes mediated by the *lexA-bcd* and *lexA-GCN4-bcd* derivatives are shown for each *bcd* allele tested. *his3* levels were determined by relative aminotriazole resistance (see Figure 6) and are indicated as follows: + + + = growth equivalent to a strain containing the wild-type *his3* gene; + + = slower than wild-type, but easily detectable growth; + = barely detectable growth; – = no growth. *lacZ* levels were measured in a strain *KY330* by standard enzymatic assay and were normalized to the level mediated by *lexA-GCN4* (450 U; Hope and Struhl, 1986) in cells analyzed in parallel. ND = not determined.

lexA binding domain (Table 1). These results show that the amino-terminal portion of the *bcd* protein, including the homeobox domain, is capable of recognizing and binding to the *hb* target sequences, while the carboxy-terminal domain is required for transcriptional activation. It is noteworthy that the carboxy-terminal domain, particularly the region between the homeobox and opa repeat, is not acidic (Berleth et al., 1988; see legend to Figure 5), in contrast to the transcriptional activation regions of other proteins (Hope and Struhl, 1986; Ma and Ptashne, 1987; Struhl, 1988). However, it does have an unusually high serine and threonine content, suggesting the possibility that it may be acidified by phosphorylation. In this regard, it is noteworthy that *bcd* protein has recently been shown to be phosphorylated during early Drosophila embryogenesis (Driever and Nüsslein-Volhard, 1989).

The phenotypic properties of these mutant proteins in Drosophila (Frohnhöfer and Nüsslein-Volhard, 1986, 1987; Driever and Nüsslein-Volhard, 1988a) correlate with their activities in yeast. All three homeobox mutations as well as two amber mutations immediately downstream of the homeobox domain (bcd^{GB} and bcd^{085}) act as strong hypomorphic or amorphic alleles. Conversely, the bcd^{E5} mutation, which encodes a less severely truncated activation domain, behaves as a weak hypomorphic allele having more apparent function in both yeast and flies. As in the case of the target sequences, the correspondence between the phenotypes assayed in each organism supports the argument that similar molecular interactions underlie *bcd*-dependent transcriptional activation in both.

Discussion

The key attribute of a morphogen gradient is that different concentrations of a single molecular species can elicit distinct molecular and ultimately cellular behaviors. Hence, to understand how gradients organize pattern we need to determine the mechanisms by which morphogens act on their immediate targets in a concentration-dependent manner. Preliminary analyses of embryos lacking the *bcd* gradient suggest that *bcd* protein is associated with transcriptional activation of the *hb* gene (Tautz, 1988; Schröder et al., 1988; Driever and Nüsslein-Volhard, 1989), transcriptional repression of the *Krüppel* gene (Gaul and Jäckle, 1987), and possibly, translational repression of ubiquitous transcripts of the *caudal* gene (Macdonald and Struhl, 1986; unpublished data; Mlodzik and Gerhing, 1987a, 1987b). Here we confirm and extend the recent results of Driever and Nüsslein-Volhard (1989) showing that at least one of these responses, transcriptional activation of the *hb* gene, almost certainly results from a direct interaction between *bcd* protein and *hb* DNA. Further, we establish that the concentration dependence of this interaction determines where the *hb* gene is activated in the intact embryo. The interaction between graded *bcd* protein and the *hb* gene therefore serves as a valid starting point to examine the molecular mechanisms by which morphogen gradients control pattern.

Direct Role for *bcd* in Activating Transcription of *hb*

As described in the introduction, the presence of a homeobox domain in the *bcd* protein suggests that it functions at least in part as a transcriptional regulator which directly binds to and controls the expression of particular target genes. Recent studies, most notably the in vitro footprinting experiments of Driever and Nüsslein-Volhard (1989), support this view by showing that the protein can bind specific sites from the *hb* gene, some of which map to regions of the gene that are necessary for *bcd*-dependent transcriptional activation (see also Schröder et al., 1988). However, as is the case for many other homeobox proteins studied to date (e.g., see Scott et al., 1989), such studies do not establish that the same molecular interactions govern the patterned expression of the endogenous gene observed in vivo.

Here, we take a different approach by first defining the minimal *hb* DNA sequences that are sufficient to mediate anterior gene expression in response to graded *bcd* protein in early embryos. We then show that we can reconstitute *bcd*-dependent transcriptional activation in yeast using just these minimal target sites and the *bcd* protein. Further, we define distinct structural domains in the *bcd* protein which are required for recognizition of the *hb* target DNA and for activation of the yeast transcriptional machinery. These results argue strongly that direct interactions between *bcd* protein and particular *hb* target DNAs are responsible for controlling where the gene is initially activated during normal development. As we describe below, the minimal target site we have defined in vivo and in yeast contains one of the *bcd* binding sites identified by the in vitro footprinting studies (Driever and Nüsslein-Volhard, 1989), adding further support to this conclusion.

Dependence of *hb* Gene Activation on *bcd* Protein Concentration

To activate the *hb* gene in a discrete domain, the graded distribution of *bcd* protein has to provide a critical concentration threshold that distinguishes between all-or-none states of transcriptional activity. The pattern of *hb* gene expression in embryos derived from mothers carrying different numbers of copies of the *bcd* gene clearly shows that activation of the gene is sensitive to the concentration of *bcd* protein (Figure 3 and Results). Similar results were also observed for the *HB:HSZ* genes (see Figure 3), indicating that this concentration dependence is likely to be mediated at the level of the direct interaction between *bcd* protein and its target sites in the *hb* DNA.

Although we have not measured the changes in *bcd* protein concentration that distinguish between all-or-none states of *hb* and *HB:HSZ* gene expression, we can nevertheless estimate how sensitively these genes respond by examining their patterns of expression in embryos derived from females carrying different numbers of copies of the *bcd* gene. Consider first the *hb* gene. As shown in Figures 3I–L, the boundary delineating the pattern of *hb* protein expression shifts posteriorly by approximately 10% EL (egg length) for each 2-fold increase in the number of copies of the *bcd* gene present in the female.

Since changes in *bcd* copy number cause roughly proportional changes in the local concentrations of *bcd* protein (Driever and Nüsslein-Volhard, 1988b), this result suggests that the concentration of *bcd* protein normally falls around 2-fold over intervals of ∼10% EL. This interval is somewhat larger than the interval in which the expression of *hb* protein falls precipitously from high, uniform levels anteriorly to undetectable levels posteriorly (an interval of ∼5%–8% EL). Thus, a 2-fold difference in *bcd* protein concentration appears sufficient to distinguish between on or off states of *hb* gene expression. A similar relationship is also observed for the *HB:HSZ* genes (e.g., Figures 3B-3F): in each case, the shift in the posterior boundary of *lacZ* expression associated with a 2-fold difference in *bcd* copy number spans an interval that is similar to that in which either boundary is defined.

Thus, we infer that approximately 2-fold, or perhaps even smaller, differences in the concentration of *bcd* protein are sufficient to trigger the activation of the *hb* and *HB:HSZ* genes in an all or none fashion. This degree of sensitivity resembles that of the *cI* gene of bacteriophage λ, which responds sharply to few-fold differences in repressor concentration (Meyer et al., 1980).

Setting the Concentration Threshold for Transcriptional Activation

The *HB:HSZ* genes we have generated have identical promoters and coding and flanking sequences, but differ in the size or number of copies of *cis*-acting regulatory regions derived from the *hb* gene. These genes are expressed in broad anterior domains that have different posterior limits (e.g., Figures 2 and 4), each specified by the graded distribution of *bcd* protein (e.g., Figure 3). Thus, the concentration threshold sensed by a particular gene (and hence the posterior boundary of that gene's expression) seems to be determined by the number and quality of the *cis*-acting regulatory elements mediating its response to *bcd* protein. Although we currently know of only one endogenous gene, *hb*, which responds directly to the *bcd* protein, these results suggest that the gradient has the instructive capacity to define many thresholds, each triggering the all or none response of a different gene.

Given our and Driever and Nüsslein-Volhard's (1989) evidence that the *bcd* protein is a transcriptional activator which directly binds *hb* regulatory sites, it is reasonable to propose that the number and affinity of these sites sets the concentration threshold to which a gene responds. Comparison of the boundaries of expression of the various *HB:HSZ* genes supports this possibility. Although elements contained within a critical 123 bp fragment are essential for any response to occur (*HB123*), the minimum *bcd* concentration required to activate the *HB:HSZ* gene containing these elements depends on the presence of auxiliary elements on either side (*HB263*, *HB747*) or additional copies of these same elements (e.g., *HB123X2*; see Figures 2 and 4). In each case, the presence of additional elements renders the gene capable of responding to lower threshold levels of *bcd* protein. It is notable that three of the five *bcd* binding sites detected by in vitro footprinting studies (Driever and Nüsslein-Volhard, 1989) fall within the upstream regulatory region defined here (centered at positions −278, −169 and −64 bp relative to the transcriptional start site of the *hb* gene; see Figures 1 and 5). One of these (−278) is positioned well within the 123 bp core fragment (*HB123*); the other two are present in the adjacent 161 bp fragment which sensitizes the response of the core fragment to lower levels of *bcd* protein (*HB263*) but fails to activate anterior expression on its own (*HB161*). Note, however, that the correlation between binding sites defined in vitro and *cis*-acting regulatory sites defined in vivo is not simple, as indicated by the fact that none of three proximal binding sites defined in vitro appears to be able to function without auxiliary sequences (see also below).

Thus, the number and quality of *cis*-acting regulatory sites present in the *HB:HSZ* genes appears to determine the threshold concentration of *bcd* protein necessary to trigger transcription, suggesting that the net affinity of *bcd* protein for the collection of sites present in each gene dictates the spatial pattern of expression. The *bcd* binding sites defined by the in vitro experiments of Driever and Nüsslein-Volhard (1989) show an approximate correlation with the regulatory sites defined in vivo, which suggests that they may coincide with some of these sites. However, their exact contibution to mediating the response to *bcd* protein remains uncertain.

Cooperative Interactions

Although it is easy to see how the number and affinity of *bcd* binding sites might determine the concentration threshold to which a gene responds, it is more difficult to account for the acute sensitivity with which each gene responds to a particular threshold. One obvious possibility is that *bcd* protein binds cooperatively to its target sites, as is observed in the binding of λ repressor to its tripartite operator (Johnson et al., 1979; reviewed in Ptashne, 1986). Another possibility is that *bcd* protein may interact cooperatively with other transcription factors that bind nearby. One set of results provides evidence for both types of interactions.

When 20–30 bp are deleted from each end of the 123 bp core fragment (*HB123*), the resulting 82 bp fragment (*HB82*; *Y82*) fails to respond to *bcd* protein in both flies and yeast. Yet, this fragment retains the *bcd* binding site defined in vitro (Driever and Nüsslein-Volhard, 1989). Moreover, it can bind *bcd* protein in early embryos, as multiple copies of this fragment (*HB82X4*) respond in a concentration-dependent fashion to the *bcd* gradient. One explanation of this result is that the 82 bp fragment may lack a cryptic *bcd* binding site present in the 123 bp core fragment, but not detected in the footprinting assay. Accordingly, the ability of multiple copies of the 82 bp fragment to restore activity would indicate that cooperative interactions between molecules of *bcd* protein binding at two or more sites are necessary for activation to occur. An alternative possibility is that the smaller fragment may lack the binding site of a general factor present in both flies and

yeast which facilitates the ability of *bcd* protein to bind its target site or activate transcription. In this case the ability of multiple copies of the fragment to restore activity suggests that cooperative interactions between two or more molecules of *bcd* protein could substitute for cooperative binding or activating interactions which might normally occur between *bcd* and the other factor.

These results emphasize the possibility that cooperative interactions between several *bcd* molecules and perhaps other factors may be necessary for triggering transcription. For example, the activation domain of *bcd* protein may be relatively weak, as suggested by the yeast experiments. Hence, activation might only occur when sufficient numbers of *bcd* activation domains are brought together in the vicinity of the promoter. Such synergistic effects on transcriptional activation are commonly observed for eukaryotic promoters and indeed account for why enhancers usually require the action of multiple DNA binding proteins. Accordingly, the ability to activate transcription would depend on the square or higher powers of *bcd* protein concentration, thereby increasing the sensitivity of the response to limiting amounts of protein. It should also be noted that the number of bound molecules of *bcd* protein necessary to activate transcription could depend on the presence of other bound factors which might facilitate or interfere with activation.

General Implications for the Control of Body Pattern by the *bcd* Gradient

Our results establish that the *bcd* protein gradient can define several distinct concentration thresholds, each "read" with remarkable sensitivity by the all or none response of a different target gene. At a minimum, this result establishes that the *bcd* gradient has the instructive capacity to trigger several distinct responses along the body. Moreover, it suggests that the ability of each target gene to read a different threshold depends primarily on the particular combination of *cis*-acting regulatory elements that mediate its direct response to *bcd* protein. Although all of the target genes considered here, with the exception of the *hb* gene itself, are artificial, their response to the *bcd* gradient may reflect a general mechanism by which the gradient controls the patterns of expression of a variety of other developmental control genes. Thus, the molecular rules that govern how avidly *bcd* protein binds to its potential targets, as well as how effective these molecules are in activating transcription once bound, might be tantamount to the rules that govern how the gradient can specify distinct molecular, and ultimately cellular, outcomes along the body.

Experimental Procedures

Fly Strains

The mutations bcd^{085}, bcd^{111}, bcd^{2-13}, bcd^{E1}, bcd^{E3}, bcd^{E4}, bcd^{E5}, bcd^{E6}, and bcd^{GB}, as well as the deletions *Df(3R)Lin*, bcd^- and *Df(3R)PXT115*, hb^- were kindly provided to us by Hans Georg Frohnhofer and Christianne Nüsslein-Volhard (Frohnhöfer and Nüsslein-Volhard, 1986; Lehmann and Nüsslein-Volhard, 1987); as described below and in Figure 7, bcd^{E6} is a new mutant allele which arose during an attempt to generate a recombinant between the bcd^{E2} and osk^{301} mutations. Females containing extra copies of the *bcd* gene were derived from transformant lines carrying an 8 kb genomic fragment including the *bcd* gene (BBB; Macdonald and Struhl, 1988). Two inserts on the second chromosome, number 9 and number 16, each of which is capable of rescuing the fertility of bcd^{E1} females when present in a single copy, were placed in *cis* by recombination and then balanced in *trans* over a *CyO* chromosome. Balanced heterozygous females from the resulting stock contain four copies of the *bcd* gene; homozygous females, six copies. The *vas exu* mutant stock was kindly provided by Trudi Schüpbach (Schüpbach and Wieschaus, 1986). A homozygous ry^{506} stock was used as the host for all the transformation experiments and served as a wild-type (2× *bcd*) control.

Construction of *HB:Z* and *HB:HSZ* Hybrid Genes

The *hb:lacZ* (*HB:Z*) fusion genes *HB4.2*, *HB0.8*, and *HB0.3* (Figure 1) were constructed as follows. An 8 kb BamHI fragment containing the *hb* gene beginning 4.7 kb upstream of the transcriptional start site (Tautz et al., 1987) was cut at an NcoI site ~230 bp downstream of the translational start site and fused in-frame to the amino-terminal end of the *lacZ* coding sequence (this particular *lacZ* coding sequence is fused to 3′ terminating sequences from the α1 tubulin gene, as described in Lawrence et al., 1987). The *HB0.8* and *HB0.3* genes were derived from the resulting hybrid gene *HB4.2* by truncation at the HindIII and NheI sites shown in Figure 1. All three genes were then inserted into the *C20* transformation vector (Rubin and Spradling, 1982) just downstream from, and in the same orientation as, the ry^+ marker gene.

The *hb:hsp70:lacZ* (*HB:HSZ*) derivates *HB747*, *484*, *263*, *161*, *123*, and *82* were constructed by inserting the fragments indicated in Figure 1 into the "enhancer-trap" vector *HZ50PL* kindly provided by Yasushi Hiromi (Hiromi and Gehring, 1987). This vector is a *C20* derivative in which the unique SalI site positioned downstream from the ry^+ marker gene has been fused to an NruI site positioned 14 bp upstream of the TATA box and 47 bp upstream of the start site of the *hsp70* gene. The *hsp70* sequences extend from the NruI site to the first seven codons of the *hsp70* coding sequence where they are fused to the *lacZ* coding sequence followed by 3′ terminating sequences from the *hsp70* gene (Lis et al., 1983; Hiromi and Gehring, 1987). The polylinker immediately upstream of the SalI site includes XbaI, NotI, and KpnI sites. In general, the various fragments from the *hb* regulatory region shown in Figure 1 were cloned into *HZ50PL* vector by first inserting XbaI, NotI, or KpnI linkers at the chosen sites and then excising and inserting the desired fragments using the appropriate combination of enzymes. All fragments were cloned into the *HZ50PL* such that they retained their normal orientation relative to the TATA box. Insertions of multiple copies of the 82 and 123 bp fragments were generated by placing common linkers at both ends and ligating the fragments at high concentration into *HZ50PL* derivatives already having one or more copies of the same fragment.

Construction of *HB:HIS3* Hybrid Genes

All of the *hb* regulatory fragments shown in Figure 1 were cloned into the *Y0* derivative of the yeast plasmid *Sc3370* (Struhl and Hill, 1987) that contains a NotI linker inserted at the SacI site: this site is positioned just upstream of a truncated version of the *his3* gene in which all of the normal regulatory sequences have been deleted). The *Y263*, *Y161*, *Y123*, *Y102*, *Y85*, and *Y82* derivatives were all constructed by introducing NotI linkers at the appropriate restriction sites and then excising and inserting the resulting NotI fragment into the NotI site in the *Y0* plasmid. In all of these constructs, the cloned fragments retain their normal orientation relative to the TATA box.

Molecular Characterization of *bcd* Mutations

With the exception of bcd^{E2} and bcd^{E6}, all of the available *bcd* mutations (Frohnhöfer and Nüsslein-Volhard, 1986) were tested for sequence changes by a modification of the method of Myers et al. (1985) as outlined in Figure 7. Total RNA (5–10 μg) from 0–4 hr embryos obtained from mothers that were heterozygous, hemizygous, or homozygous for each mutation was hybridized to labeled antisense RNA probes (shown in Figure 7) and treated with RNAase as described previously (Macdonald et al., 1986). Sequence changes were detected for only three of the mutations. The use of probes in both orientations increases the probability of detecting mismatches (Myers et al., 1986); consequently, genomic clones of several alleles were isolated to allow

synthesis of antisense mutant RNAs and to allow sequencing of the mutations. DNA was prepared from hemizygous or homozygous mutant males, partially digested with Sau3A (bcd^{E3} and bcd^{GB}) or completely digested with EcoRI (bcd^{E1}, ^{E4}, ^{E5}, ^{E6}, 2–13, 085, 111), and gel-purified fragments of the appropriate sizes were used to construct phage libraries by standard means. Most of the *bcd* coding sequence falls within an XbaI–StuI fragment: this fragment was obtained from each library and subcloned into pGEM1 (Promega Biologicals) which allows both sense and antisense transcripts to be synthesized (Melton et al., 1984). Unlabeled sense and antisense transcripts were hybridized to the complementary wild-type probes shown in Figure 7, and digested with RNAase. With one exception (see below), this approach sufficed to identify single base changes (or in one case a small deletion) associated with each mutation. The appropriate regions were then sequenced to determine the mutations. The bcd^{E6} mutation was characterized differently. In an attempt to isolate a genomic clone of the bcd^{E2} mutation which Berleth et al. (1988) have shown to be due to a deletion of about 260 bp including the homeobox, flies homozygous for a bcd^{E2} osk^{301} chromosome were used as a source of DNA. Restriction mapping of a subclone of the appropriate region revealed a deletion of about 30 bp rather than 300 bp, which was confirmed by sequencing. In addition to differing in the size of the deletion, we found that mutant embryos make normal amounts of *bcd* antigen, in contrast to bcd^{E2} mutant embryos (Driever and Nüsslein-Volhard, 1988a). Thus, the mutation characterized here, which we refer to as bcd^{E6}, is clearly not bcd^{E2} (or any other known *bcd* mutation) and probably arose spontaneously during construction of the *bcd osk* recombinant chromosome. Each of the characterized mutations is listed below using the sequence numbering of Berleth et al. (1988): the homeobox is composed of amino acids 97–156; the opa repeat, of amino acids 262–304.

bcd^{E1} deletion of 2482–2650 (inclusive) with an insertion of TA; deletion alters the reading frame such that amino acids 156–494 are replaced by 55 out of frame amino acids.
bcd^{E3} 2406 C→T; amino acid 131 ser→leu
bcd^{E4} 2393 C→T; amino acid 127 leu→phe
bcd^{E5} 2804 C→T; amino acid 264 gln→amber
bcd^{E6} deletion of 2388–2420 (inclusive); in-frame deletion of amino acids 125–135 (inclusive)
bcd^{GB} 2486 C→T; amino acid 158 gln→amber
$bcd^{2\text{-}13}$ 3885 T→A; amino acid 453 leu→his
bcd^{085} 2564 C→T; amino acid 184 gln→amber
bcd^{111} 2798 C→T; amino acid 262 gln→amber

Construction of Hybrid Genes Expressing Wild-Type or Mutant *bcd* Proteins in Yeast

The coding sequence of wild-type *bcd* protein was derived from a cDNA clone obtained from a 0–4 hr embryo cDNA library (provided by Markus Noll), characterized by standard methods, and altered at the 5′ end so that it now reads GGA.TCC.TCT.GGG.AAA.ATG (the ATG being the initiator methionine codon; note that the first six nucleotides form a BamHI site). The entire coding sequence, which is contained on a 1580 bp BamHI–EcoRV fragment, was then cloned into *YCp88*, *YCp88-LexA*, and *YCp88-LexA-GCN4* vectors (Hope and Struhl, 1986) such that the constitutive promoter of the yeast *ded1* gene is positioned just upstream of the *bcd*, *LexA-bcd*, and *LexA-GCN4-bcd* coding sequences and the *LexA-bcd* and *GCN4-bcd* fusions are in-frame. These three *YCp88-bcd* derivatives were then used to generate additional constructs in which the appropriate portions of the wild-type *bcd* coding sequence were replaced by various mutant coding sequences. Finally, the *bcd* coding sequence was truncated at nucleotide 1572 (numbered according to Berleth et al., 1988) to eliminate the amino-terminal 53 amino acids (including the *PRD*-repeat) and the remaining coding sequence inserted into the *YCp88-LexA* and *YCp88-LexA-GCN4* vectors, as above.

Fly Transformants

The *HB:Z* and *HB:HSZ* hybrid genes were introduced into the Drosophila genome by P element–mediated transformation (Rubin and Spradling, 1982). In general, the resulting *P(ry+)* transformants were initially maintained in ry^{506} backgrounds, the moderate selective advantage of ry^{+} being sufficient to ensure stable propagation of the transformed gene. Between three and eight transformants were obtained for each of the genes shown in Figure 1. Roughly equal numbers of flies carrying each of the independent transformants of a given gene were then pooled and their embryos assayed for *lacZ* expression (see below). Stably balanced or homozygous stocks of two to four independent transformants were then generated for each gene and their embryos assayed independently for *lacZ* expression (see below). In all cases, the independent lines behaved like the pools, the only variation was the intensity of staining, which was less intense in occasional lines.

Analysis of *lacZ* Expression in Transformed Flies

lacZ expression was assayed by both standard immunohistochemical and enzymatic assays (Macdonald and Struhl, 1986; Glaser et al., 1986). *lacZ* antigen was detected using either a mouse monoclonal (Lawrence et al., 1987) or rabbit polyclonal antisera, followed by appropriate biotinylated secondary antibodies, ABC avidin:horse radish peroxidase conjugates (Vector Labs) and reaction with diamino-benzidine resulting in a tightly restricted, permanent brown precipitate. *lacZ* activity staining was employed in all cases in which no *lacZ* antigenicity could be detected immunohistochemically. All of the transformants giving a positive immunohistochemical response also showed a positive response in terms of *lacZ* activity within 30 min and often within 5 or 10 min (staining done at room temperature). However, none of the transformants that were antigen negative showed any evidence of *lacZ* enzymatic activity even when incubated for 2 or more days. Because the substrate remains stable during this period, we estimate conservatively that we could have detected levels of *lacZ* activity at least 20- if not 50-fold lower than that present in embryos showing a positive response. Note that both methods of assaying *lacZ* expression were invariably used for testing the expression of *HB:HSZ* genes in *bcd* and *vas exu* mutant embryos.

Analysis of Endogenous *hb* Protein in Early Embryos

hb protein expression was assayed immunohistochemically using a rat anti-*hb* antisera. To examine the expression of the *HB263* gene in embryos lacking zygotic *hb* activity (Figure 3A), *HB263/+*; *Df(3R)PXT, hb⁻/+* parents were crossed and the resulting embryos double stained for *lacZ* and *hb* antigens. Embryos lacking the *hb* gene do not express *hb* protein in the posterior half (in contrast to wild-type embryos which show a band of expression close to the posterior pole; e.g., Figure 3J). They do, however, express *hb* protein derived from maternally derived transcripts, though this protein dissipates toward the end of the cellular blastoderm stage. Among the embryos derived from this cross, we found a significant proportion of embryos at the cellular blastoderm stage that lack posterior staining but express high levels of anterior staining; this anterior staining is characteristic of *lacZ* but not *hb* staining in that it is cytoplasmic, rather than nuclear. We infer that these embryos lack zygotic *hb* activity but are nevertheless capable of expressing the *HB263* gene.

Yeast Transformants

DNAs containing the *HB:HIS3* hybrid genes *Y263*, *Y161*, *Y123*, *Y102*, *Y85*, *Y82*, and *Y0* were introduced into yeast strain *KY320* such that they replaced the chromosomal *his3* locus as described previously (Chen and Struhl, 1988). These strains were then transformed with the *YCp88* derivatives (Hope and Struhl, 1986) capable of expressing the various wild-type and mutant derivatives of *bcd* protein.

Analysis of *his3* and *lacZ* Expression in Transformed Yeast

Yeast strains were examined for their level of *his3* expression by the standard method of plating or streaking cells on minimal medium containing 20 mM aminotriazole, a competitive inhibitor of the *his3* gene product (Hope and Struhl, 1986; Hill et al., 1986; Struhl, 1987, 1988). As the relative degree of aminotriazole resistance is directly related to the level of *his3* mRNA (Hill et al., 1986), this test provides an excellent measure of *his3* expression in vivo. To analyze the ability of the *LexA-bcd* derivatives to activate transcription when bound to a *LexA* operator, the various *YCp88-LexA* and *LexA-GCN4* derivatives were transformed into yeast strain *KY330* (Hope and Struhl, 1986; Struhl, 1988), which contains a plasmid in which a *LexA* operator is located upstream of the *cyc1* TATA element and *lacZ* structural gene. The resulting strains were assayed for *lacZ* enzymatic activity as described previously (Hope and Struhl, 1986; Struhl, 1988).

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