The Gradient Morphogen bicoid Is a Concentration-Dependent Transcriptional Activator

Gary Struhl,* Kevin Struhl,† and Paul M. Macdonald*†
* Howard Hughes Medical Institute
        Center for Neurobiology and Behavior
        Columbia University College of Physicians and Surgeons
        New York, New York 10032
† Department of Biological Chemistry and Molecular Pharmacology
        Harvard Medical School
        Boston, Massachusetts 02115

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 (Frohnhiifer 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., Frohnhiifer and Nüsslein-Volhard, 1987; Tautz, 1988; reviewed in Ingham, 1988). 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; Frohnhiifer 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.
hb gene observed in vivo. Our approach has been to define $\alpha$-acting regulatory sequences in the hb gene that binds distinct target sites in the hb gene and that its affinity for these sites determines the minimum concentration of bed protein for these sites. Based on these results, we argue that the bed protein normally contains distinct regions capable of binding bed and hb DNA control the localized activation of the hb gene. We then show that these elements, which contain hb sequences between the Sall (S) and Xba (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, Sall, Ncol, 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 during early embryogenesis (see Experimental Procedures). The final 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 transcription 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 hbC7lacZ described by Schröder et al. (1988) is also shown; this construct, which contains hb sequences between the Sall (S) and Xba (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, Sall, Ncol, and FspI, respectively.

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 transcription 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 hbC7lacZ described by Schröder et al. (1988) is also shown; this construct, which contains hb sequences between the Sall (S) and Xba (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, Sall, Ncol, and FspI, respectively.

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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., 1989). 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 HSZ: lacZ 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 (HB477) 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...
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 and HbZ Genes

(B), (C), (D), (E), and (F) show the pattern of lacZ expression generated by the HbZ 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 HbZ 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 (Frühhörer 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 observed 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 an 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
Figure 4. Expression of HB:HSZ and hb Genes in vasa exuperantia Mutant Embryos

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

The experiments described above demonstrate that bed 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
bcd-Dependent Transcriptional Activation

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 1–67) 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 6, the bcd+ protein and bcd+ mutations are amber mutations that truncate the protein at amino acids 157, 183, and 263, respectively; the bcd+F and bcd+F mutations change amino acids 127 and 131, the bcd+F 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 I, 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 X7–263). Its sequence was used as a probe to isolate fragments streaming the LexA-GCN4-bcd+ DNA. The restriction map of the 263 bp fragment (Figure 6) was compared with that of the LexA-bcd+ fragment (Figure 5) and the genome adjacent to the core domain and the more severely truncated 82 bp core fragment fail to respond.

bcd coding sequence were placed under the control of the yeast ded7 promoter (Hope and Struhl, 1986). The resulting strains were assayed for his3 expression by their abilities to grow in medium containing amnitoriazole; the degree of amnitoriazole 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 amnitoriazole resistance indicates that the level of bcd protein 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
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; Nüsslein-Volhard, 1988a) by alternative expression mediated by the bcd regulatory sequences, though these proteins are capable of mediating expression via the bcd binding site (Table 1).

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 bcd 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 and lexA-GCN4-bcdR derivatives 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 bcd regulatory sequences in yeast.
Figure 7. Mapping bed Mutations

Labeled sense or antisense transcripts of mutant alleles were generated as described in Experimental Procedures, hybridized to labeled complementary RNA probes, the hybrids digested with RNAase to cleave mismatches, and the products displayed by electrophoresis and autoradiography. The bed 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 bcdE3, bcdE4, bcdE5, bcdG5, bcdG6, bcdG7, and bcdG8, 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 simultaneous 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.

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

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Levels of activation of the HB:HIS3 (Y283) and LEXA:LACZ genes mediated by the lexA-bed and lexA-GCN4-bed 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.

bcd Homeobox Mutations

All three lexA-GCN4-bcd hybrid proteins containing mutations in the homeobox domain (bcdG5, bcdG6, and bcdG7) 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 Truncation

Derivatives of the three amber mutations (bcdG5, bcdG6, and bcdG7), 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 bcdG7 mutation is most revealing. As shown in Table 1, the lexA-bcdG7 protein fails to activate either the HB:HIS3 or LEXA:LACZ target genes, whereas the lexA-GCN4-bcdG8 protein activates both. Thus, the bcdG7 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-bcdG7 protein can be restored by adding the GCN4 activation domain, suggesting that the carboxy-terminal region of bcd deleted by the bcdG7 mutation is functionally analogous to an activation domain. The remaining amber mutations, which leave the homeobox domain intact but truncate the coding sequence 28 (bcdG6) and 108 (bcdG5) amino acids downstream, behave differently from the bcdG7 mutation in that they retain the ability to activate partially both the LEXA:LACZ and HB:HIS3 genes when fused only to the
The phenotypic properties of these mutant proteins in Drosophila (Frohnholzer 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 (bcd65 and bcd90) act as strong hypomorphic or amorphic alleles. Conversely, the bcdF2 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; Midzik and Gerhart, 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 recognition 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 \( \text{bcd} \) copy number cause roughly proportional changes in the local concentrations of \( \text{bcd} \) protein (Driever and Nüsslein-Volhard, 1988b), this result suggests that the concentration of \( \text{bcd} \) protein normally falls around 2-fold over intervals of \(~10\%\) EL. This interval is somewhat larger than the interval in which the expression of \( \text{hb} \) protein falls precipitously from high, uniform levels anteriorly to undetectable levels posteriorly (an interval of \(~5\%-8\%\) EL). Thus, a 2-fold difference in \( \text{bcd} \) protein concentration appears sufficient to distinguish between on or off states of \( \text{hb} \) gene expression. A similar relationship is also observed for the \( \text{HB}:\text{HSZ} \) genes (e.g., Figures 3B-3F): in each case, the shift in the posterior boundary of \( \beta\text{gal} \) expression associated with a 2-fold difference in \( \text{bed} \) 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 \( \text{bcd} \) protein are sufficient to trigger the activation of the \( \text{hb} \) and \( \text{HB}:\text{HSZ} \) genes in an all or none fashion. This degree of sensitivity resembles that of the \( \lambda \) gene of bacteriophage \( \lambda \) which responds sharply to few-fold differences in repressor concentration (Meyer et al., 1980).

### Setting the Concentration Threshold for Transcriptional Activation

The \( \text{HR}:\text{HSZ} \) genes we have generated have identical promoters and coding and flanking sequences, but differ in the size or number of copies of \( \sigma \)-acting regulatory regions derived from the \( \text{hb} \) 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 \( \text{bcd} \) 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 \( \sigma \)-acting regulatory elements mediating its response to \( \text{bcd} \) protein. Although we currently know of only one endogenous gene, \( \text{hb} \), which responds directly to the \( \text{bcd} \) 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 own and Driever and Nüsslein-Volhard’s (1989) evidence that the \( \text{bcd} \) protein is a transcriptional activator which directly binds \( \text{hb} \) 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 \( \text{HB}:\text{HSZ} \) genes supports this possibility. Although elements contained within a critical 123 bp fragment are essential for any response to occur (HB123), the minimum \( \text{bcd} \) concentration required to activate the \( \text{HB}:\text{HSZ} \) 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 \( \text{bcd} \) protein. It is notable that three of the five \( \text{bcd} \) 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 \( \text{hb} \) 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 \( \text{bcd} \) protein (HB263) but fails to activate anterior expression on its own (HB167). Note, however, that the correlation between binding sites defined in vitro and \( \sigma \)-acting regulatory sites defined in vivo is not simple, as indicated by the fact that none of these proximal binding sites defined in vitro appears to be able to function without auxiliary sequences (see also below).

Thus, the number and quality of \( \sigma \)-acting regulatory sites present in the \( \text{HR}:\text{HSZ} \) genes appears to determine the threshold concentration of \( \text{bcd} \) protein necessary to trigger transcription, suggesting that the net affinity of \( \text{bcd} \) protein for the collection of sites present in each gene dictates the spatial pattern of expression. The \( \text{bcd} \) 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 contribution to mediating the response to \( \text{bcd} \) protein remains uncertain.

### Cooperative Interactions

Although it is easy to see how the number and affinity of \( \text{bcd} \) 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 \( \text{bcd} \) protein binds cooperatively to its target sites, as is observed in the binding of \( \lambda \) repressor to its tripartite operator (Johnson et al., 1979; reviewed in Ptashne, 1986). Another possibility is that \( \text{bcd} \) 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 \( \text{bcd} \) protein in both flies and yeast. Yet, this fragment retains the \( \text{bcd} \) binding site defined in vitro (Driever and Nüsslein-Volhard, 1989). Moreover, it can bind \( \text{bcd} \) protein in early embryos, as multiple copies of this fragment (HB82X4) respond in a concentration-dependent fashion to the \( \text{bcd} \) gradient. One explanation of this result is that the 82 bp fragment may lack a cryptic \( \text{bcd} \) 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 \( \text{bcd} \) 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 bcdR20, hbrR111, bcdR12, bcdR2, bcdR4, bcdR5, bcdR8, and bcdR10, as well as the deletions D(3R)lun, bcd- and D(3R)PXT115, hb- were kindly provided to us by Hans Georg Frohnhefer and Christiane Nüsslein-Volhard. In the experiments described below and in Figure 7, bcdR2 is a new mutant allele which arose during an attempt to generate a recombinant between the bcdR2 and dmcX11 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 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 ryR20 stock was used as the host for all the transfection experiments and served as a wild-type (2x bcd) control.

Construction of HBZ and HBSZ Hybrid Genes

The hb: lacZ (HBSZ) fusion genes HB4.2, HBO.8, and HBO.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 (Teute et al., 1987) was cut at an NcoI site ~230 bp downstream of the transcriptional start site and fused in-frame to the amino-terminal end of the lacZ coding sequence (Figure 1). The HB4.2 and HBO.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 of, the ry marker gene.

The hb: hsp70: lacZ (HBSZ) derivatives HB747, 484, 263, 101, 123, and 62 were constructed by inserting the fragments indicated in Figure 1 into the enhancer-trap vector HZ250L, 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 downstream from 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 following 3' terminating sequences from the hsp70 gene (Lis et al., 1983; Hiromi and Gehring, 1987). The polylinker immediately upstream of the hb site includes XbaI, NotI, and KpnI sites. In general, the various fragments from the hb regulatory region shown in Figure 1 were cloned into HZ250L 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 HZ250L by a method described previously. The three constructs already have one or more copies of the same fragment.

Construction of HB3: HS3 Hybrid Genes

All of the hybrid regulatory fragments shown in Figure 1 were cloned into the YO 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 Y92 derivatives were all constructed by introducing NotI linkers at the appropriate restriction sites and then excising and inserting the NotI fragment into the NotI site in the YO plasmid. In all of these constructs, then cloned fragments retain their normal orientation relative to the TATA box.

Molecular Characterization of bcd Mutations

With the exception of bcdR2 and bcdR3, all of the available bcd mutations (Frohnhefer and Nüsslein-Volhard, 1986) were tested for sequence changes by a modification of the method of Myer 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 (Myer 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 (bcdE7 and bcdE9) or completely digested with EcoRI (bcdE1, E2, F1, F2, 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-Stul fragment: this fragment was obtained from each library and subcloned into pGEM1 (Promega Biotechnologies) 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 probe 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 bcdE10 mutation was characterized differently. In an attempt to isolate a genomic clone of the bcdE10 mutation which Berenst (1988) has shown to be due to a deletion of about 260 bp including the homeobox, flies homozygous for a bcdE2 osk707 chromosomes 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 bcdE10 embryos (Dreier and Nusslein-Volhard, 1988). Thus, the mutation characterized here, which we refer to as bcdE10, is clearly not bcdE2 (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 following the use of sequencing the number of Berenst et al. (1988); the homeobox is composed of amino acids 97–156; the oop repeat, of amino acids 262–304.

bcdE10 deletion of 2482–2650 (inclusive) with an insertion of 1A; deletion alters the reading frame such that amino acids 150–494 are replaced by 55 out of frame amino acids.

bcdE22 2406 C→T; amino acid 127 leu→phe

bcdE23 2583 G→T; amino acid 127 leu→phe

bcdE33 2904 C→T; amino acid 264 gin→amber

bcdE65 deletion of 2388–2420 (inclusive); in-frame deletion of amino acids 125–135 (inclusive)

bcdE25 2486 C→T; amino acid 158 gin→amber

bcdE26 2564 C→T; amino acid 184 gin→amber

bcdE27 2798 C→T; amino acid 262 gin→amber

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

The expression of a 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 remain unchanged in the resulting P(ry+) transformants). 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., 1990). 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 diaminobenzidine 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 negative showed 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 20–50-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:His3 genes in bcd and vasa mutant embryos.

Analysis of Endogenous bcd Protein in Early Embryos

bcd protein expression was assayed immunohistochemically using a rat anti-bcd antisera. To examine the expression of the HB263 gene in embryos lacking cytoplasmic bcd activity (Figure 3A), H263/+; Df(3R)PX7; hb+/+ parents were crossed and the resulting embryos double stained for lacZ and bcd antigens. Embryos lacking the bcd gene do not express bcd 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 bcd protein derived from maternal-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 bcd staining in that it is cytoplasmic, rather than nuclear. We infer that these embryos lack zygotic bcd activity but are nevertheless capable of expressing the H263 gene.

Yeast Transformants

DNAs containing the HB:His3 hybrid genes Y263, Y167, Y123, Y102, Y93, Y92, and Y0 were introduced into yeast strain KY250 such that they replaced the chromosomal his3 locus as described previously (Chen and Struhl, 1988). Those 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 amnomatrole, a competitive inhibitor of the his3 gene product (Hope and Struhl, 1986; Hill et al., 1986; Struhl, 1987, 1988). As the relative degrees of amnomatrole resistance are 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 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 KY1500 (Hope and Struhl, 1986; Struhl, 1986), which contains a plasmid in which a LexA operator is located upstream of the cyt TATA element and lacZ structural gene. The resulting strains were assayed for lacZ enzymatic activity as described previously (Hope and Struhl, 1986; Struhl, 1986).
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References


Dependent Transcriptional Activation


