Contributions to gene activation by multiple functions of Bicoid

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Bicoid is a *Drosophila* morphogenetic protein required for the development of anterior structures in the embryo. To gain a better understanding of how Bicoid works as a transcriptional activator, we systematically analysed various functions of Bicoid required for gene activation. We provide evidence suggesting that Bicoid is an intrinsically weak activator. First, our biochemical experiments demonstrate that the Bicoid–DNA complexes are very unstable, suggesting a weak DNA-binding function of Bicoid. This idea is further supported by our experiments demonstrating that the same number of LexA–Bicoid fusion molecules can activate transcription more effectively from LexA sites than from Bicoid sites. Secondly, we demonstrate that transcriptional activation by the weak activator Bicoid is readily influenced by the local enhancer environment. These influences are decreased when the Bicoid function is enforced by attaching to it either a known dimerization domain or the strong activation domain VP16. VP16 can also compensate for the loss of some Bicoid sites in an enhancer element. Our experiments demonstrate that the outcome of transcriptional activation by Bicoid is determined by multiple weak functions that are interconnected, a finding that can further help us to understand how this morphogenetic protein achieves its molecular functions.

Key words: GAL4, LexA, transcriptional activation, VP16.

INTRODUCTION

Gene transcription in eukaryotes is a complex but co-ordinated process involving many protein–protein and protein–DNA interactions [1–5]. These interactions, in particular those involving activator molecules, have an important role in modulating the level of transcription [3,4,6,7]. Therefore understanding the functions of an activator involved in these interactions is a key step towards understanding the molecular mechanisms of transcriptional activation in eukaryotes. The activator analysed in the current study is the *Drosophila* homeodomain protein Bicoid (Bcd), which has an essential role in embryonic pattern formation.

Bcd is a morphogenetic protein required for the development of anterior structures, including the head and thorax. in the embryo [8,9]. During early embryonic development, Bcd is distributed as an anterior-to-posterior gradient with the highest concentration at the anterior [10]. An essential molecular function of Bcd as a morphogenetic protein in the embryo is to act as a transcriptional activator. One of the earliest zygotic genes activated by Bcd is the gap gene *hunchback* (*hb*) [11–13], which is uniformly expressed in the anterior half of the embryo with a sharp posterior border [11,14]. Our previous studies have demonstrated that Bcd binds to a *hb* enhancer element co-operatively, providing one molecular mechanism for such on/off responses of its target genes [15]. We have further suggested that co-operative DNA binding is facilitated by an interaction between Bcd molecules [16]. Thus Bcd as a transcriptional activator possesses at least three important functions: DNA binding, transcriptional activation and interaction between Bcd molecules.

To understand more clearly how Bcd works as an activator, we systematically analysed these three functions of Bcd to access their contributions to gene activation. By taking advantage of the heterologous yeast system to avoid any potential dedicated partners of Bcd, we provide evidence suggesting that Bcd is an intrinsically weak activator, reflecting not only its dynamic interaction with DNA but also a weak interaction between Bcd molecules and a weak activation function. We demonstrate that the enforcement or alteration of any of these weak functions of Bcd can increase its activity. For example, the same number of LexA–Bcd fusion molecules can activate transcription from LexA sites more effectively than from Bcd sites. In addition, although transcriptional activation by the weak activator Bcd is readily influenced by the local enhancer environment, such influences are decreased by attaching to Bcd either a strong activation domain or a known dimerization domain. The strong activation domain VP16 can also compensate for the loss of some Bcd sites in a *hb* enhancer element. Our studies demonstrate that the level of transcriptional activation by Bcd is determined by multiple weak but interconnected functions. They also provide important insights into the question of how Bcd might function as a molecular morphogen for the proper control of target gene expression.

EXPERIMENTAL

DNA probes for biochemical assays

Table 1 lists the plasmids containing different numbers of Bcd sites derived from the *hb* enhancer element. Plasmids pMAX2′ and pMAX3′ were generated by cloning the *Hin*dIII–*Rsa*I and *Hin*dIII–*Fsp*I fragments respectively from p*hb*−298 [15] into the *Hin*dIII/*Sma*I sites of the Bluescript KS(−) vector, followed by a deletion of the *Hin*cII–*Hin*cII fragment. Mutation of A1 in *hb*(−298 to −50) was generated by a PCR-mediated mutagenesis

Abbreviations used: Bcd, Bicoid; *hb*, *hunchback*.

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Table 1 Plasmids used in the biochemical study

Bcd site	Plasmid	DNA fragment	Source
Six Bcd sites (A1X1X2X3A2A3)	pMAX1′	*hb* −298 to −50	[15]
Five Bcd sites (X1X2X3A2A3)	PMAX15	*hb* −298 to −50 with A1 site mutated	This study
Five Bcd sites (A1X1X2X3A2)	pMAX2′	*hb* −298 to −94	This study
Four Bcd sites (A1X1X2X3)	pMAX3′	*hb* −298 to −193	This study
One Bcd site (A1)	pMAX10	*hb* −298 to −267	[15]

Table 2 Plasmids used in the gene activation studies

Gene description	Plasmid names Yeast vector	Bacterial vector	Notes	Source Yeast vector	Bacterial vector
Reporter genes					
hb-WT	pMA630R		hb −298 to −50	[13]	
hb-Δ1	pMAX18	pMAX2′	hb −298 to −94	This study	This study
hb-Δ2	pMAX107		hb −298 to −119	This study	
hb-Δ3	pMAX106		hb −298 to −132	This study	
hb-Δ4	pMAX105		hb −298 to −147	This study	
hb-Δ5	pMAX104	pMAX108	hb −298 to −160	This study	This study
hb-Δ6	pMAX19	pMAX3′	hb −298 to −193	This study	This study
hb-Δ7	pMAX20	pMAX5	hb −267 to −193	This study	This study
hb-Δ8	pMAX21	pMAX9	hb −298 to −231	This study	[15]
hb-Δ9	pMAX22	pMAX10	hb −298 to −267	This study	[15]
mut-A1	pMAX114	pMAX109	Mutation at A1	This study	
mut-X2	pMAX115	pMAX110	Mutation at X2	This study	
mut-X3	pMAX116	pMAX111	Mutation at X3	This study	
mut-X1	pMAX117	pMAX112	Mutation at X1	This study	
mut-A2	pMAX118	pMAX113	Mutation at A2	This study	
two LexA sites	pJP167		Two LexA sites	J. Pearlberg	
Activator genes					
LexA–Bcd	pLexA–Bcd		*LEU2* marker	[19]	
Bcd	pMA625		*LEU2* marker	[13]	
Bcd–VP16	pMA1226		*LEU2* marker	This study	
LexA–Bcd–VP16	pMAX127		*LEU2* marker	This study	
Bcd	pTA3		*HIS3* marker	[15]	
GAL4–Bcd	pMA1220		*HIS3* marker	This study	

procedure with the template pMAX1′ [15]. pMAX15 was constructed by cloning the *Kpn*I–*Xba*I fragment of such a PCR product into the *Kpn*I/*Xba*I sites of the Bluescript KS(−) vector. To generate radioactively labelled DNA probes, the *Kpn*I–*Xba*I fragments containing different numbers of Bcd sites were isolated from the respective plasmids and filled in by Klenow enzyme in the presence of [α-^{32}P]dCTP.

Off-rate

The conditions and procedures for the gel retardation assay have been described previously [15]. Recombinant Bcd used for biochemical studies was generated from the baculovirus expression system as described previously [15]. The estimated concentration of active Bcd in the final protein preparation was 2.3 μM. For off-rate measurements, binding reactions containing an estimated 0.115 μM active Bcd and probes with a single site (A1) or six sites (A1X1X2X3A2A3) were incubated in 100 μl of 1 × BB (20 mM Tris pH7.5, 50 mM NaCl, 0.5 mM EDTA, 0.2 mM EGTA, and 1 mM dithiothreitol) [15] at room temperature for 15 min, followed by the addition of a 100-fold excess of double-stranded oligonucleotides containing a Bcd-binding site. Aliquots (10 μl) of the reaction were loaded on a 4% (w/v) polyacrylamide gel at different time points after the addition of the unlabelled competitor.

Reporter and effector plasmids

Table 2 lists the plasmids used for gene activation studies in yeast cells. Reporter plasmids were derived from the yeast integrating plasmid LR1Δ1Δ2μ [17]. They contained different deletion and mutation derivatives of the *hb* enhancer element placed approx. 100 bp upstream of the *GAL1–lacZ* reporter gene. To construct reporter plasmids pMAX107, 106, 105 and 104, PCR products were first generated from pMAX2′ using the sense primer R to the Bluescript KS(−) vector sequence and anti-sense primers (with a *Sma*I site at the 5′ end) to the corresponding deletion positions of the *hb* element. These PCR products were then digested with *Xho*I/*Sma*I and inserted into the *Xho*I/*Sma*I sites of LR1Δ1Δ2μ. Plasmids pMAX18, 19, 20, 21, 22, 114, 115, 116, 117 and 118 were generated by cloning the *Xho*I–*Sma*I fragments from the corresponding bacterial plasmids (Table 2) into the *Xho*I/*Sma*I sites of LR1Δ1Δ2μ (for pMAX116, an *Xho*I–*Xba*I fragment with its *Xba*I site blunt-ended by Klenow fragments was used). pMAX5 was constructed by ligating the *Bsr*FI–*Xba*I fragment from pMAX3′ into the *Hin*cII/*Xba*I sites of the Bluescript KS(−) vector. pMAX108 was constructed by inserting

the *Xho*I–*Sma*I fragment from pMAX104 into the Bluescript KS(−) vector. pMAX108 was used as the template for a PCR-mediated mutagenesis procedure to generate mutations at individual Bcd sites in the region between −298 and −160 of the *hb* enhancer element. pMAX109, 110, 111, 112 and 113 were generated by inserting into the *Xho*I/*Sac*II sites of the Bluescript KS(−) vector *Xho*I–*Sac*II fragments of PCR products produced with the primer R, and primer F to the vector sequence and primers with mutations at respective Bcd sites. A1 was mutated from 5′-CG**TAA**TCCC-3′ to 5′-CG**AGC**TCCC-3′; X1 was mutated from 5′-GC**TA**AGCTG-3′ to 5′-GC**AG**AGCTG-3′. X2 was mutated from 5′-GCT**AAGC**TC-3′ to 5′-GCT**GACG**TC-3′. A2 was mutated from 5′-TC**T**AAT**C**CA-3′ to 5′-TC**G**AAT**T**CA-3′. X3, which contains two mutually exclusive subsites [18], was mutated from 5′-G**A**TC**AT**CCAAAT**C**-3′ to 5′-G**G**TC**GA**CC-AAAT**G**-3′, destroying both subsites. All these mutated sites were tested; it was shown that they could not be recognized by Bcd in gel-retardation assays (results not shown). Reporter plasmid pJP167, which was kindly provided by L. Gaudreau, contains two LexA sites upstream of the *GAL1–lacZ* reporter gene at the same location as other reporter genes bearing the *hb* enhancer elements.

Effector plasmids contain the 2μ replication origin and express the corresponding activator molecules under the yeast *ADH1* promoter. pMA1220 was generated by ligating the *Bam*HI–*Xba*I fragment from pLexA–Bcd [19] into a pMA424 [20]. pMAX127 was generated in three steps. First, the *Hin*dIII fragment from pMA1226 was ligated into the *Hin*dIII site of Bluescript KS(−), resulting in pMAX120. Secondly, the *Xba*I–*Bgl*II fragment from pMAX120 was cloned into the *Xba*I/*Bgl*II sites of pMA1222 [15], resulting in pMAX126. Thirdly, the *Hin*dIII–*Hin*dIII fragment from pMAX126 was ligated into the expression vector AAH5 [21] to yield pMAX127. pMA1226 was constructed in two steps. First, the *bcd–VP*16 fusion gene was constructed by inserting the *Hin*dIII–*Eco*RI fragment (with *Hin*dIII being filled in with Klenow enzyme) from pMA540 [22] into the *Eco*RI–*Eco*RV sites of pMA1224, a plasmid bearing the wild-type *bcd* gene. pMA1226 was then constructed by inserting the *Hin*dIII fragment from pMA1225 into the *Hin*dIII site of AAH5. AAH5 and pMA200 [23], which encode no activator protein but contain the yeast *LEU2* and *HIS3* markers respectively, were used as vector controls for gene activation assays.

Yeast strains and β-galactosidase liquid assays

Yeast strains were generated by integrating the reporter plasmids at the *URA3* locus of GGY1 (α Δgal4 Δgal80 leu2 his3 ura3) [24]. Only strains with single-copy integrants were used for further studies. To determine the copy number, genomic DNA was extracted as described previously [25] and subjected to PCR analysis. Two sets of primers, assay primers and control primers, were used in the PCR assay. The assay primers annealed to sequences outside the *URA3* gene and the product spanned the entire *URA3* gene. The length of this product was used to determine whether the strains had single or multiple copy integrations. Control primers were used in the same PCR reactions for an internal control because they annealed to the vector sequence, yielding an identical PCR product from all the integrants.

To assay for the activity of different proteins from various reporter genes, the corresponding effector plasmid was transformed into the yeast strains with integrated single-copy reporter genes. Five independent colonies from each transformation were cultured and assayed for β-galactosidase activity as described previously [15].

RESULTS

Dynamic interaction between Bcd and DNA

An activator–DNA complex is a prerequisite for gene activation; how an activator binds DNA might affect the extent of gene activation. To obtain a better understanding of the DNA-binding function of Bcd, we conducted kinetic studies on the formation of Bcd–DNA complexes. To measure the off-rate of

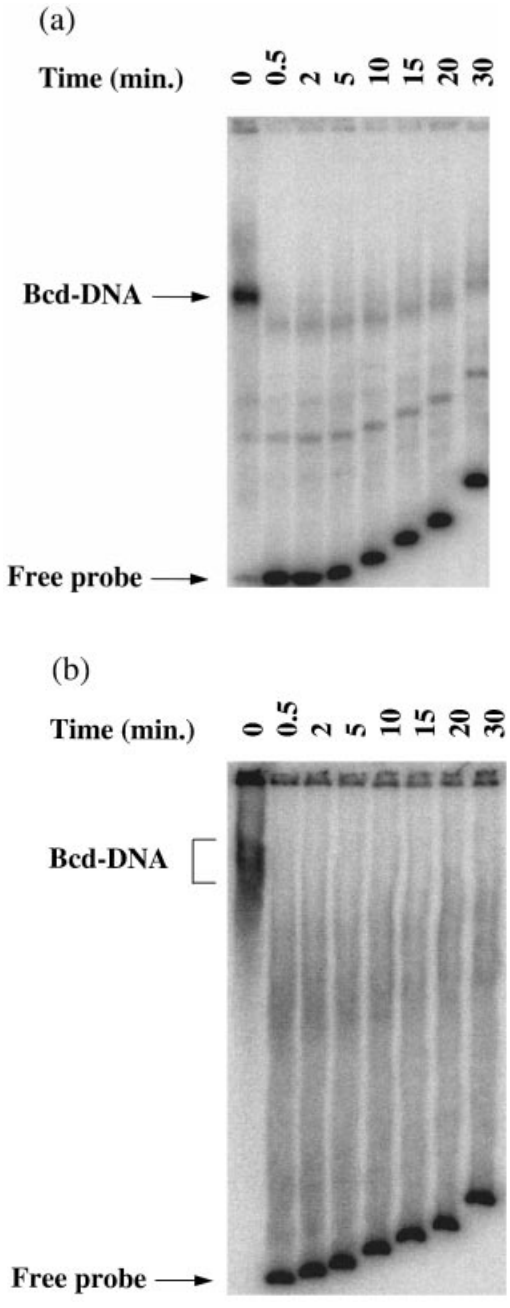

Figure 1 Bcd binds DNA with a fast off-rate

Off-rate measurement of Bcd binding to probes containing either a single site (**a**) or six sites (**b**). These probes are derived from the *hb* enhancer element (see the Experimental section for details). Binding reactions incubated at room temperature were loaded on a polyacrylamide gel at the indicated time points after the unlabelled competitor had been added. Non-specific bands visible in the gels are evidently not from Bcd binding.

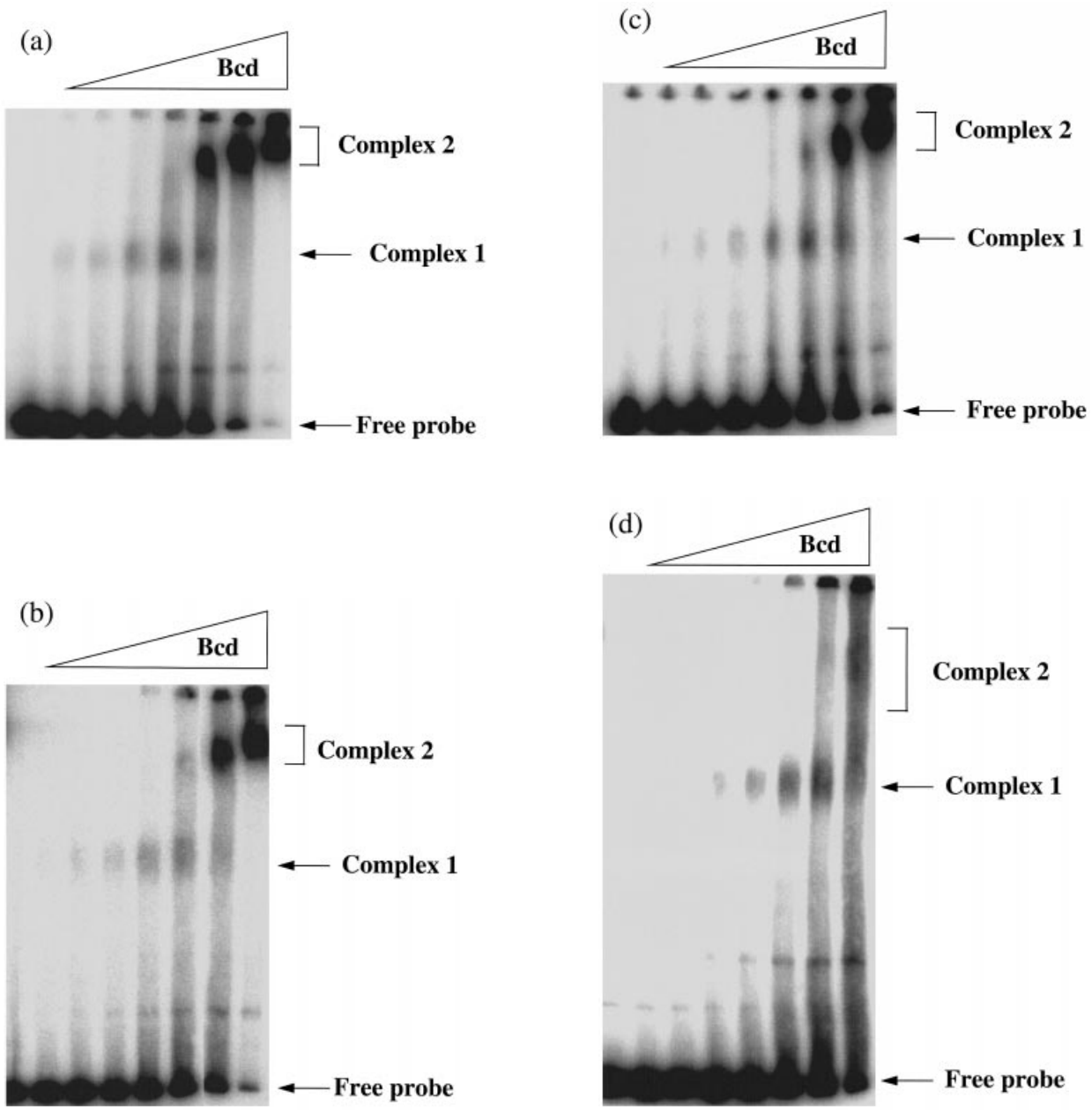

Figure 2 Bcd–DNA complexes are disrupted by gel electrophoresis

Binding reactions were performed with probes containing six (**a**), five (**b**, **c**) and four (**d**) Bcd sites. These probes are derived from the *hb* enhancer element (see the Experimental section for details). The probes with five sites contained sites A1X1X2X3A2 (**b**) and X1X2X3A2A3 (**c**). The estimated concentrations of active Bcd in the reactions are: 0, 0.007, 0.014, 0.029, 0.058, 0.115, 0.23 and 0.46 μM for lanes 1–8 respectively. Complexes 1 and 2 were Bcd–DNA complexes containing one and multiple Bcd molecules respectively. Consistently with our previous studies [15], no intermediate Bcd–DNA complexes were detected for probes containing five or six Bcd sites (**a**–**c**), reflecting a high degree of co-operativity of Bcd binding. In contrast, a smear consisting of intermediate complexes was observed for probes containing four Bcd sites (**d**) or fewer (X. Ma, D. Yuan and J. Ma, unpublished work), indicating that protein–DNA complexes containing four or fewer Bcd molecules are less stable and dissociable during electrophoresis. We have shown ([16]; C. Zhao, T. Scarborough and J. Ma, unpublished work) that Bcd has multiple self-associating domains. It is likely that one Bcd molecule can interact with at least two other Bcd molecules on DNA [18] and therefore more Bcd molecules can increase the stability of the protein–DNA complex. It should be noted that, because complex 1 is the smallest protein–DNA complex detectable in the gel retardation assay with one Bcd molecule, it does not appear as a smear even with probes containing four of fewer Bcd sites (**d**).

the protein–DNA complexes, binding reactions were loaded on a polyacrylamide gel at different time points after an excess (approx. 100-fold) of unlabelled competitor had been added to the reaction. Figure 1 shows that the protein–DNA complexes dissociated almost immediately (within 30 s) after the unlabelled competitor had been added, suggesting that the Bcd–DNA complexes are extremely unstable. We also measured the on-rate of Bcd–DNA interaction and observed that more than 50% binding was achieved within 10 s (results not shown). These kinetic studies demonstrate that the binding of Bcd to DNA is a dynamic process and that the Bcd–DNA complexes are very unstable.

Table 3 LexA–Bcd fusion proteins activate transcription more effectively from LexA sites than from Bcd sites

Fusion proteins LexA–Bcd and LexA–Bcd–VP16 were analysed for their ability to activate transcription from either four Bcd sites (hb-Δ6; see Figure 3a) or two LexA sites that were located approx. 100 bp upstream of the *GAL1–lacZ* reporter gene. Results are β-galactosidase units obtained from yeast cells containing the indicated activators and reporter genes (means ± S.D.).

		β-Galactosidase activity (units)	
DNA sites	Activator ...	LexA–Bcd	LexA–Bcd–VP16
Bcd sites		< 1	51.8 ± 5.4
LexA sites		21.5 ± 1.3	1185.6 ± 91.5

Although a similarly fast dissociation was observed with complexes on either a single Bcd site (Figure 1a) or multiple Bcd sites (Figure 1b), complexes containing different numbers of Bcd molecules exhibited different behaviours in gel-retardation assays (Figure 2). Specifically, the complex formed on a probe containing four Bcd sites seemed to be a smear (Figure 2d) rather than a discrete band as observed with probes containing five or six sites (Figures 2a–2c). It has been shown previously that weak protein–protein interactions can be disrupted by gel electrophoresis [26,27]. Our experiments support a previous suggestion that the interaction between Bcd molecules is weak [15], especially for complexes containing four or fewer Bcd molecules (also see Figure 2 legend). Taken together, our biochemical studies (Figures 1 and 2) suggest that the Bcd–DNA complexes are unstable, not only owing to the dynamic nature of the interaction between Bcd and DNA but also owing to a weak interaction between Bcd molecules.

LexA–Bcd and LexA–Bcd–VP16 activate transcription more effectively from LexA sites than from Bcd sites

We hypothesized that the dynamic Bcd–DNA interaction demonstrated by the above biochemical experiments might be one contributing factor for ineffective transcriptional activation by Bcd, in addition to its previously documented weak activation domain [28,29]. To test this idea directly, we took advantage of two Bcd fusion proteins, LexA–Bcd and LexA–Bcd–VP16, each containing an extra DNA-binding domain from the bacterial repressor LexA [19,30]. LexA–Bcd contains a nearly full-length Bcd (residues 3–489) attached to the DNA-binding domain of LexA (residues 1–87). LexA–Bcd–VP16 contains an additional activation domain from the herpes viral activator VP16 [22]. We determined the abilities of these two fusion proteins to activate transcription in yeast cells containing integrated single-copy *GAL1–lacZ* reporter genes bearing, at approx. 100 bp upstream, either two LexA-binding sites or four Bcd-binding sites. Because each LexA site is recognized by two LexA monomers, two LexA sites would accommodate the same number of activator molecules as four Bcd sites. The activities from the two different reporter genes would therefore reflect how well the fusion proteins bind to and activate from the LexA and Bcd sites *in vivo*.

The data shown in Table 3 reveal two results. First, the fusion protein LexA–Bcd activated transcription to a much lower level than LexA–Bcd–VP16, irrespective of Bcd or LexA sites. This result further confirms the previous findings that the activating function of Bcd is weak [28]. Secondly, and more importantly, both fusion activators resulted in much higher levels of gene expression from LexA sites than from Bcd sites. This finding further demonstrates the weak DNA-binding function of Bcd *in vivo*. We also note that levels of transcription activated by LexA–Bcd or LexA–Bcd–VP16 from two LexA sites were respectively higher than those activated by Bcd or Bcd–VP16 even from five Bcd sites (see Figures 3b and 3c), providing additional support for our conclusion that the DNA-binding function of Bcd is weak.

The LexA DNA-binding domain added to the molecules Bcd or Bcd–VP16 does not contain the dimerization function of LexA [31–33] and therefore does not affect the binding property of these molecules to Bcd sites. Specifically, we observed that Bcd or Bcd–VP16 activated transcription from *hb* enhancer derivatives to similar levels regardless of the presence or absence of the extra LexA DNA-binding domain (results not shown; see also Figures 3b and 3c). As noted previously [19], the efficient binding of the LexA–Bcd fusion proteins to LexA sites is presumably facilitated by an interaction, even though relatively weak, between Bcd molecules.

Transcriptional activation by Bcd is susceptible to influences by the local enhancer environment

Our analysis of the ability of Bcd to activate transcription from a series of deletion derivatives of a *hb* enhancer element (Figure 3a) reveals an important property of Bcd. We found that transcriptional activation by Bcd was readily influenced by Bcd-independent elements that acted either positively or negatively (Figure 3b). Because the analysis was conducted in the heterologous yeast system and therefore lacked any of the potential dedicated partners of Bcd, the Bcd property revealed here reflects an intrinsically weak nature of Bcd as an activator. For example, the level of reporter gene expression activated by Bcd was increased 6-fold when a negative element located between −94 and −50 was deleted (compare hb-WT and hb-Δ1). Further experiments indicated that the negative effect was not associated with the Bcd site (A3) in this region (results not shown). In addition, when a positive element located between −119 and −94 was removed, reporter gene activity was decreased to one-sixth (compare hb-Δ1 and hb-Δ2). This positive element contains no Bcd sites and, among all the reporter genes shown in Figure 3(a), hb-Δ1 had the highest background activity (4.3 β-galactosidase units; 1 unit = $1000 \times A_{420}/t \times v \times A_{600}$, where A_{420} is the reading of the reaction, t is reaction time, v is volume of yeast culture used in the reaction and A_{600} is the reading of the yeast culture density) in the absence of Bcd (also see Figure 3 legend).

The major relevant finding of this report is the demonstration of the inherently weak nature of Bcd and the further dissection of the functions contributing to such a weak nature (see below), rather than the identification of the elements themselves. These elements are presumably recognized by some yeast proteins that are normally unrelated to Bcd function, suggesting that the observed enhancer environment influences reflect the intrinsic property of Bcd. As shown below, Bcd molecules with enhanced functions are much less sensitive to these influences.

Stronger activation domain or a dimerization domain decreases the influences by the local enhancer environment

In addition to its protein–DNA interaction function, two other functions of Bcd, the transcriptional activation and the interaction between Bcd molecules, are also expected to be involved in transcriptional activation. Both of these functions have been shown to be weak [15,28,29], suggesting that they might also

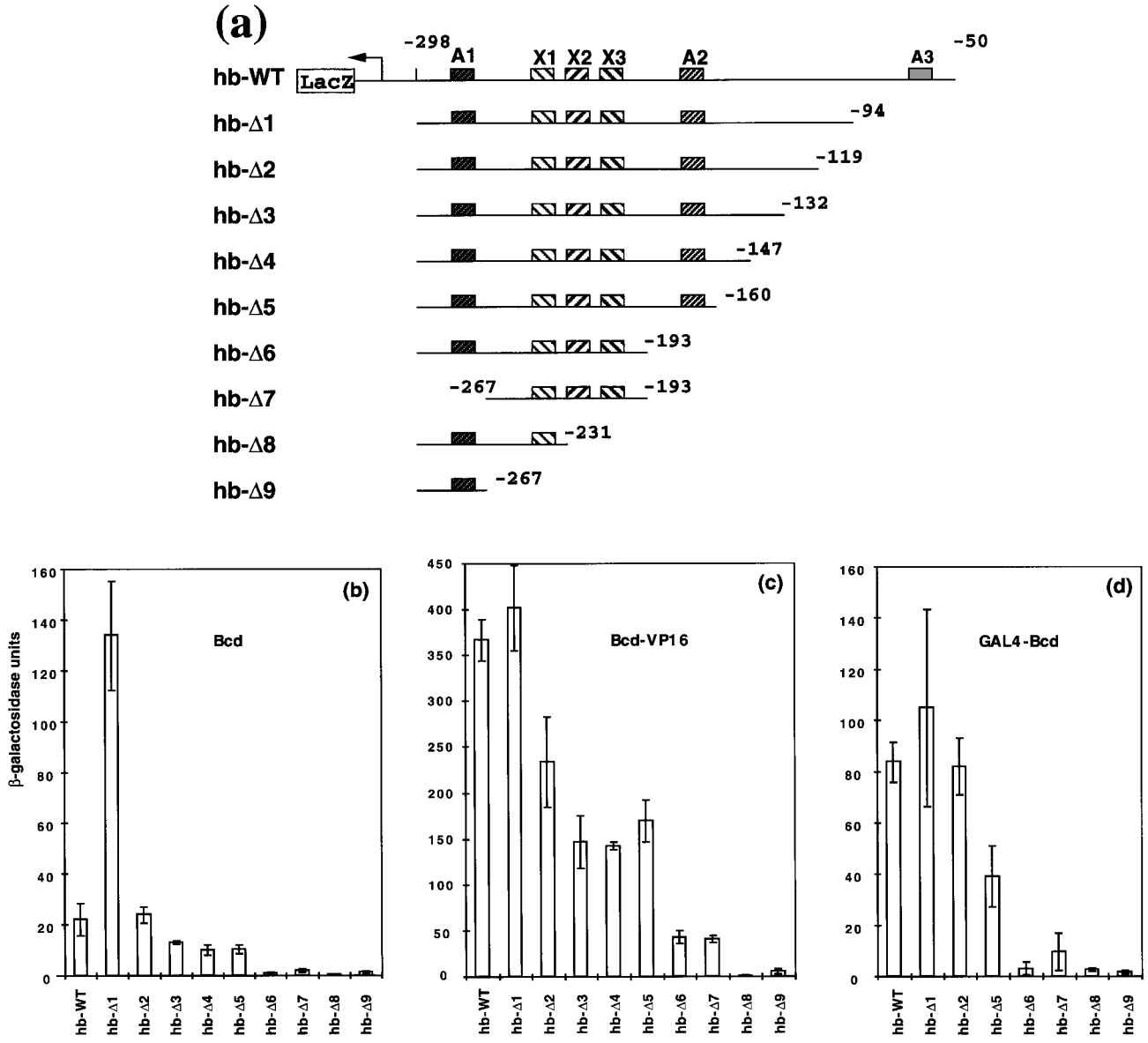

Figure 3 Stronger activation domain or a dimerization domain decreases the influences of the local enhancer environment on transcriptional activation

(**a**) Schematic diagrams of the *GAL1–lacZ* reporter genes bearing various deletion derivatives of the *hb* enhancer element. The wild-type *hb* enhancer element (hb-WT) is a 248 bp region from −50 to −298 upstream of the *hb* gene and contains six Bcd sites: A1, X1, X2, X3, A2 and A3. These reporter genes were integrated as single copies into the yeast genome for transcriptional activation assays (see the Experimental section for details). (**b**–**d**) Transcriptional activation by Bcd (**b**), Bcd–VP16 (**c**) and GAL4–Bcd (**d**) from different reporters in yeast cells. Background expression levels from the reporter genes in the absence of any introduced activator molecules were generally low, ranging from less than 1 to 4.3 (for hb-Δ1) β-galactosidase units. The background β-galactosidase activities have been subtracted from the values shown in the Figures.

contribute to the overall weak nature of Bcd, whose activation ability is influenced by the local enhancer environment. The following two sets of experiments demonstrated that such influences were decreased by enforcing the Bcd function with either a strong activation domain or an extra dimerization domain.

In the first set of experiments, the strong activation domain VP16 was fused to Bcd and the ability of this fusion protein to activate transcription was assayed on the same reporter genes as described above. Figure 3(c) shows that Bcd–VP16 becomes much less sensitive than Bcd to the influences from the local enhancer environment. For example, the activity of Bcd–VP16 was virtually unaffected by the negative element (10% decrease; compare hb-WT and hb-Δ1) and was increased only slightly (60% as opposed to 6-fold) by the positive element (compare hb-Δ1 and hb-Δ2).

The experiments shown in Figures 3(b) and 3(c) also indicate that, as expected, Bcd–VP16 activated transcription to higher levels than Bcd alone for all the reporter genes (note the scale difference). In addition, whereas Bcd failed to activate transcription significantly from four or fewer sites (Figure 3b), Bcd–VP16 was able to activate transcription modestly from three or four sites (hb-Δ6 and hb-Δ7; Figure 3c), demonstrating that VP16 enables Bcd to activate transcription from fewer sites. These results are consistent with a previous study demonstrating that a stronger activation domain attached to Bcd also enables

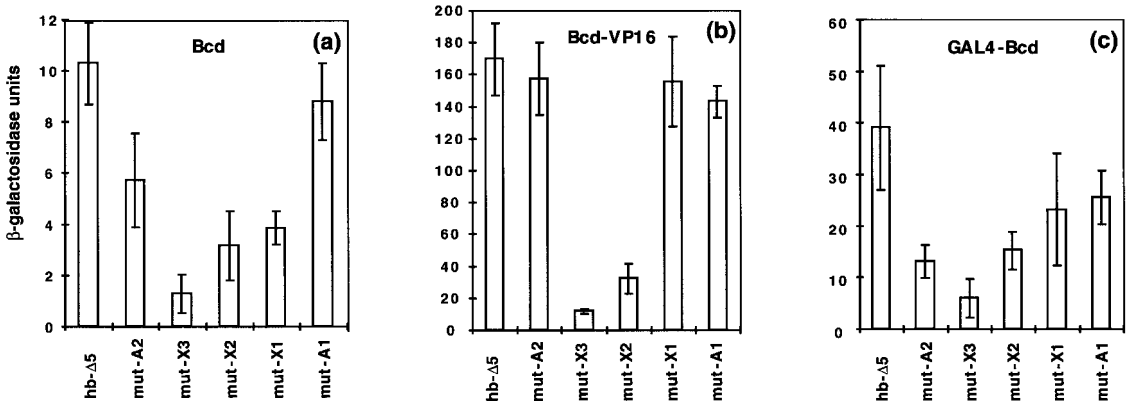

Figure 4 Bcd–VP16 is less sensitive to mutations of certain Bcd-binding sites *in vivo*

Transcriptional activation by Bcd (**a**), Bcd–VP16 (**b**) and GAL4–Bcd (**c**) from reporter genes bearing individual site mutations, mut-A1, mut-X1, mut-X2, mut-X3 and mut-A2, in yeast cells. These reporter genes are derived from hb-Δ5 and contain mutations at A1, X1, X2, X3 and A2 respectively. Sequences of the mutated sites are described in the Experimental section. The background activities have been subtracted from the β-galactosidase values shown in the Figures.

the protein to activate transcription from fewer sites in the embryo [34].

In the second set of experiments we attached a known dimerization domain to Bcd to enforce the interaction between Bcd molecules. Residues 1–147 of GAL4, which contain a dimerization domain [35], were fused to the nearly full-length Bcd (residues 3–489). This GAL4–Bcd fusion protein was then tested for its activity from the same *hb* reporter genes described above. Similarly to what was observed with the strong activation domain VP16, the additional dimerization domain from GAL4 markedly decreased the influences of the enhancer environment on transcriptional activation by Bcd. For example, the activity of GAL4–Bcd was decreased only slightly (25%) by the negative element (compare hb-WT and hb-Δ1) and increased (25%) by the positive element (compare hb-Δ1 and hb-Δ2). Figure 3(d) also shows that, although the protein sequence of GAL4 attached to Bcd does not contain any activation domain that can function in yeast [23], higher levels of reporter gene activity were detected from GAL4–Bcd than from Bcd for nearly all the *hb* reporter genes.

Bcd–VP16 is less sensitive to mutations at certain Bcd-binding sites *in vivo*

The experiments so far described demonstrate that the outcome of transcriptional activation by Bcd is determined not only by its DNA-binding property but also its activation strength and the interaction between Bcd molecules. With the use of the heterologous yeast system, we wished to determine more systematically the intrinsic contributions of individual Bcd-binding sites to transcriptional activation by Bcd. In addition we were interested in determining whether the fusion proteins Bcd–VP16 and GAL4–Bcd would behave differently from Bcd. We systematically mutated each of the Bcd sites individually in the reporter gene hb-Δ5 that contained sites A1, X1, X2, X3 and A2 from the *hb* enhancer element (see the Experimental section for details). This reporter gene contains the minimal enhancer fragment capable of supporting significant gene activation by Bcd (Figure 3). Gel retardation assays were performed on each of the mutated sites to make sure that they could no longer be recognized by Bcd (results not shown). Each reporter gene was integrated as a single copy into the yeast genome to assay for transcriptional activation by Bcd, GAL4–Bcd or Bcd–VP16.

Several conclusions can be drawn from the gene activation experiments shown in Figure 4. First, both GAL4–Bcd and Bcd–VP16 activated transcription to higher levels than Bcd for nearly all the reporter genes. Secondly, different Bcd sites contributed differently to transcriptional activation by Bcd, with a descending order of X3, X2, X1, A2 and A1 (Figure 4a). Thirdly, GAL4–Bcd responded in a similar manner to Bcd to mutations at individual Bcd sites (Figure 4c). Fourthly, the strong activation domain VP16 was able to compensate for the loss of the sites A2 and X1 (Figure 4b) but not for the defects associated with mutations at X2 and X3 (Figure 4b). The implications of these findings are addressed below.

DISCUSSION

An important function of Bcd as a molecular morphogen is to activate zygotic gene expression in a concentration-dependent manner. Our previous studies have demonstrated that Bcd can bind DNA co-operatively [15,16], providing a molecular mechanism for threshold responses of target gene expression to the Bcd gradient. The experiments described in this report systematically analysed various functions of Bcd as a transcriptional activator. Our experiments suggest that Bcd is an intrinsically weak activator, reflecting not only its dynamic DNA-binding property (Figure 1) but also its weak activation function and a weak interaction between Bcd molecules. Transcriptional activation by such an intrinsically weak activator can be enforced by strengthening or altering any of these weak functions (Table 3 and Figures 3 and 4), further demonstrating their contributions to the gene activation process. Our experiments also demonstrate a compensatory effect between some of these functions (Figures 3 and 4), suggesting that they are interconnected and collectively determine the outcome of transcriptional activation.

Several recent studies also suggest that some of the interactions involved in gene transcription might influence each other. For example, the potency of the activation domain has been shown to influence the DNA-binding property of an activator *in vivo* [36,37]. In addition, a reciprocal compensatory effect is observed

between the strengths of the upstream activator-binding sites and the core promoter on transcriptional activation [38]. An emerging hypothesis from these and other studies [34,39–42] is that the level of gene transcription reflects the quality of an overall transcription complex that contains not only all the basal transcription factors and DNA but also activator molecules. Our experiments support this hypothesis and suggest that the concerted formation of such an overall complex might provide another mechanism, in addition to co-operative DNA binding, for the threshold responses of target gene expression to the Bcd gradient in the embryo.

DNA binding

The experiments described in this report demonstrate that the Bcd–DNA complexes are very unstable and can dissociate in less than 30 s under our experimental conditions (Figure 1). This is in sharp contrast with protein–DNA complexes formed by other homeodomain proteins; for example, the half-lives of complexes containing Ubx and Antp are 15–30 min and 90 min respectively [43,44]. Our finding that LexA–Bcd fusion proteins can activate transcription more effectively from LexA sites than from Bcd sites provides direct support for the idea that the weak DNA-binding property of Bcd is one contributing factor to its inefficient transcriptional activation by Bcd. It is possible that, unlike the efficient binding to LexA sites, these fusion proteins are unable to occupy Bcd sites at all *in vivo*. Alternatively, these proteins might occupy Bcd sites *in vivo* but the protein–DNA complexes formed on Bcd sites, unlike those formed on LexA sites, are too unstable to recruit or interact with the transcription machinery effectively, thus failing to activate transcription. Our current experiments cannot differentiate between these two possibilities.

Our experiments demonstrate that Bcd–DNA complexes formed on four or fewer Bcd sites are readily disrupted by gel electrophoresis (Figure 2, and results not shown), a finding that might help to explain why Bcd fails to activate transcription significantly from four or fewer Bcd sites in yeast (Figure 3b; hbΔ6–Δ9). It has been demonstrated previously that multiple Bcd sites are also crucial for transcriptional activation in the embryo [11,13,29,34,45]. Our experiments provide biochemical evidence suggesting the importance of multiple sites in the formation of Bcd–DNA complexes required for transcriptional activation (see also Figure 2 legend).

Other unstable protein–DNA complexes have been reported previously [46,47]. Similarly to our experiments, these studies also suggest that the quality of protein–DNA complexes can affect the outcome of transcriptional activation *in vivo*. It has been demonstrated that the half-life of a protein–DNA complex containing transcription factors Ets-1 and PEBP2α is less than 5 s [46]. In another case, the half-life of the *Drosophila* heat shock factors binding to a heat shock element can be less than 30 s [47]. In both cases, effective transcriptional activation can occur only when DNA binding is enforced either by other factors, such as LEF-1 and activating transcription factor/cAMP response element-binding protein (ATF/CREB) family molecules in the case of Ets-1 and PEBP2α [46], or by increasing the number of binding sites in the case of heat shock factors [47–49].

Activation domain

Our experiments confirm previous studies [28,29] and further demonstrate that Bcd has a weak activation function (Table 3). By attaching the strong activation domain VP16 to Bcd, we observed the following three effects, in addition to the expected increase in levels of gene transcription. First, the influences of the local enhancer environment on transcriptional activation by Bcd are greatly decreased (Figure 3c). Secondly, Bcd–VP16 can activate transcription from three or four Bcd sites, which are insufficient to support transcriptional activation by Bcd alone (Figures 3b and 3c). Thirdly, the strong activation domain VP16 can compensate for the loss of certain Bcd sites (Figure 4b). These effects are presumably a result of a stronger interaction between the activator molecules and the basal transcription machinery. In turn, such an enforced interaction might also increase the weak DNA-binding function of Bcd.

Dimerization domain

The outcome of gene transcription was affected in two ways when an extra dimerization domain was attached to Bcd: increased expression levels for nearly all the reporter genes and decreased influences from the local enhancer environment. Our previous studies have shown that co-operative Bcd binding requires interaction between Bcd molecules [16]. It is therefore possible that the dimerization domain of GAL4 enhances such an interaction, thus facilitating DNA binding. Alternatively the dimerization domain of GAL4 might help to bring additional Bcd molecules to the promoter region, thus providing additional contacts with the basal transcription machinery. Regardless of the detailed mechanisms, our experiments demonstrate that an enhanced interaction can have a positive effect on the outcome of transcription, presumably by promoting the formation of the overall transcription complex containing the activator molecules, DNA and the basal transcription machinery.

Although both the dimerization domain of GAL4 and the strong activation domain VP16 can enforce Bcd function, the following two observations suggest that they might do so by different mechanisms. First, unlike what was observed with Bcd–VP16, GAL4–Bcd did not increase the expression level of the reporter gene hb-Δ1 (Figure 3d). Secondly, unlike VP16, the dimerization domain of GAL4 cannot compensate for the loss of Bcd sites (Figure 4c).

Bcd-binding sites

Our studies suggest an exceptionally important role of sites X2 and X3 in mediating transcriptional activation. First, these sites contribute more to transcriptional activation than other sites in our mutagenesis studies (Figure 4a). Secondly, unlike other sites, mutations at X2 and X3 cannot be compensated for by the strong activation domain VP16 (Figure 4b), indicating a qualitatively different defect. One possible explanation might be related to DNA-binding affinity, but our experiments do not support this idea: Bcd appears to bind to both X and A types of sites with a similar affinity in DNase I footprint assays [15]. In addition, previous experiments with artificial enhancer elements and multimerized Bcd sites have suggested that A sites could actually respond to lower concentrations of Bcd than X sites in the embryo [14]. Alternatively, the contribution by individual Bcd sites might be determined by their spatial relationships with the neighbouring sites. Our recent binding-site selection studies *in vitro* have shown that the distance and alignment of adjacent Bcd sites are important parameters to determine co-operative DNA recognition by Bcd [18]. Further studies suggest that the specific alignment between X2 and an element in X3 might have a key role in supporting transcriptional activation by Bcd [18].

Implications of the weak nature of Bcd

As a molecular morphogen, Bcd needs to co-ordinate with other factors to control gene transcription properly and instruct pattern formation in the embryo. It has been demonstrated that Bcd requires the presence of the Hunchback protein to activate transcription fully for virtually all of the Bcd target genes [42]. A stronger activator, Bcd-GCN4, can bypass the requirement for Hunchback and also activate from fewer Bcd sites for the *even-skipped* gene (*eve*) stripe 2 expression in the embryo [34]. It has been suggested that Bcd and Hunchback might contact different TATA-box-binding-protein-associated factors to achieve synergistic activation [41,50]. Negative factors have also been shown to affect transcriptional activation by Bcd in the embryo. For example, the Giant protein (Gt) represses the activity of Bcd and Hunchback in the *eve* stripe 2 enhancer [29,34]. One nearby Gt molecule is evidently sufficient to abolish transcriptional activation by five Bcd molecules and one Hunchback molecule [34]. Our present experiments demonstrate that the intrinsically weak activator Bcd can readily receive and integrate both positive and negative regulatory influences owing to its multiple weak functions that collectively contribute to transcriptional activation.

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REFERENCES

1 Triezenberg, S. J. (1995) Curr. Opin. Genet. Dev. **5**, 190–196
2 Hori, R. and Carey, M. (1994) Curr. Opin. Genet. Dev. **4**, 236–244
3 Zawel, L. and Reinberg, D. (1995) Annu. Rev. Biochem. **64**, 533–561
4 Tjian, R. and Maniatis, T. (1994) Cell **77**, 5–8
5 Ptashne, M. and Gann, A. (1997) Nature (London) **386**, 569–577
6 Chi, T., Lieberman, P., Ellwood, K. and Carey, M. (1995) Nature (London) **377**, 254–257
7 Choy, B. and Green, M. R. (1993) Nature (London) **366**, 531–536
8 Nusslein-Volhard, C., Frohnhoffer, H. G. and Lehman, R. (1987) Science **238**, 1675–1681
9 Frohnhofer, H. G. and Nusslein-Volhard, C. (1986) Nature (London) **324**, 120–125
10 Driever, W. and Nusslein-Volhard, C. (1988) Cell **54**, 83–93
11 Struhl, G., Struhl, K. and Macdonald, P. M. (1989) Cell **57**, 1259–1273
12 Tautz, D., Lehmann, R., Schnurch, H., Schuh, R., Seifert, E., Kienlin, A., Jones, K. and Jackle, H. (1987) Nature (London) **327**, 383–389
13 Driever, W. and Nusslein-Volhard, C. (1989) Nature (London) **337**, 138–143
14 Driever, W., Thoma, G. and Nusslein-Volhard, C. (1989) Nature (London) **340**, 363–367
15 Ma, X., Yuan, D., Diepold, K., Scarborough, T. and Ma, J. (1996) Development **122**, 1195–1206
16 Yuan, D., Ma, X. and Ma, J. (1996) J. Biol. Chem. **271**, 21660–21665
17 West, R. W. J., Yocum, R. R. and Ptashne, M. (1984) Mol. Cell. Biol. **4**, 2467–78
18 Yuan, D., Ma, X. and Ma, J. (1999) J. Biochem. (Tokyo), in the press
19 Hanes, S. and Brent, R. (1989) Cell **57**, 1275–1283
20 Ma, J. and Ptashne, M. (1987) Cell **51**, 113–119
21 Ammerer, G. (1983) Methods. Enzymol. **101**, 192–201
22 Sadowski, I., Ma, J., Triezenberg, S. and Ptashne, M. (1988) Nature (London) **335**, 563–564
23 Ma, J. and Ptashne, M. (1987) Cell **48**, 847–853
24 Gill, G. and Ptashne, M. (1987) Cell **51**, 121–126
25 Hoffman, C. S. and Winston, F. (1987) Gene **57**, 267–272
26 Wagner, S. and Green, M. R. (1993) Science **262**, 395–399
27 Grueneberg, D. A., Natesan, S., Alexandre, C. and Gilman, M. Z. (1992) Science **257**, 1089–1095
28 Small, S., Kraut, R., Hoey, T., Warrior, R. and Levine, M. (1991) Genes Dev. **5**, 827–839
29 Small, S., Blair, A. and Levine, M. (1992) EMBO J. **11**, 4047–4057
30 Brent, R. and Ptashne, M. (1985) Cell **43**, 729–736
31 Little, J. and Mount, D. (1982) Cell **29**, 11–22
32 Schnarr, M., Granger-Schnarr, M., Hurstel, S. and Pouyet, J. (1988) FEBS Lett. **234**, 56–60
33 Kim, B. and Little, J. W. (1992) Science **255**, 203–206
34 Arnosti, D. N., Barolo, S., Levine, M. and Small, S. (1996) Development **122**, 205–214
35 Carey, M., Kakidani, H., Leatherwood, J., Mostashari, F. and Ptashne, M. (1989) J. Mol. Biol. **209**, 423–432
36 Hyunsung, P., Ko, S., Okino, T., Ma, Q. and Whitlock, Jr., J. P. (1997) Mol. Cell. Biol. **17**, 3497–3507
37 Tanaka, M. (1996) Proc. Natl. Acad. Sci. U.S.A. **93**, 4311–4315
38 Lehman, A. M., Ellwood, K. B., Middleton, B. E. and Carey, M. (1998) J. Biol. Chem. **273**, 932–939
39 Lin, Y.-S., Carey, M., Ptashne, M. and Green, M. R. (1990) Nature (London) **345**, 359–361
40 Carey, M., Lin, Y.-S., Green, M. R. and Ptashne, M. (1990) Nature (London) **345**, 361–364
41 Sauer, F., Hansen, S. and Tjian, R. (1995) Science **270**, 1783–1788
42 Simpson-Brose, M., Treisman, J. and Desplan, C. (1994) Cell **78**, 855–865
43 Beachy, P. A., Varkey, J., Young, K. E., Kessler, D. P. V., Sun, B. I. and Ekker, S. C. (1993) Mol. Cell. Biol. **13**, 6941–6956
44 Affolter, M., Percival-Smith, A., Muller, M., Leupin, W. and Gehring, W. J. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 4093–4097
45 Rivera-Pomar, R., Lu, X., Perrimon, N., Taubert, H. and Jackle, H. (1995) Nature (London) **376**, 253–256
46 Giese, D., Kingsley, C., Kirshner, J. R. and Grosschedl, R. (1995) Genes Dev. **9**, 995–1008
47 Xiao, H., Perisic, O. and Lis, J. T. (1991) Cell **64**, 585–593
48 Dudler, R. and Travers, A. A. (1984) Cell **38**, 391–398
49 Amin, J., Mestril, R., Lawson, R., Klapper, H. and Voellmy, R. (1985) Mol. Cell. Biol. **5**, 197–203
50 Sauer, F., Wassarman, D. A., Rubin, G. M. and Tjian, R. (1996) Cell **87**, 1271–1284

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