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The *Drosophila* morphogenetic protein Bicoid binds DNA cooperatively

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SUMMARY

The *Drosophila* morphogenetic protein Bicoid, encoded by the maternal gene *bicoid*, is required for the development of the anterior structures in the embryo. Bicoid, a transcriptional activator containing a homeodomain, is distributed in an anterior-to-posterior gradient in the embryo. In response to this gradient, the zygotic gene *hunchback* is expressed uniformly in the anterior half of the embryo in a nearly all-or-none manner. In this report we demonstrate that a recombinant Bicoid protein binds cooperatively to its sites within a *hunchback* enhancer element. A less than 4-fold increase in Bicoid concentration is sufficient to achieve an unbound/bound transition in DNA binding. Using various biochemical and genetic methods we further demonstrate that Bicoid molecules can interact with each other. Our results are consistent with previous studies performed in the embryo, and they suggest that one mechanism to achieve a sharp on/off switch of gene expression in response to a morphogenetic gradient is cooperative DNA binding facilitated by protein-protein interaction.

Key words: *Drosophila*, Bicoid, cooperativity, DNA binding, *hunchback*, enhancer

INTRODUCTION

One of the central questions in developmental biology is how cells interpret morphogenetic gradients to establish qualitative, rather than quantitative, changes according to their relative positions in a developing embryo. One class of morphogenetic gradients is made of transcriptional activators, which can specify developmental fates of cells in different regions of the embryo by establishing discrete patterns of gene expression. An example is the *Drosophila* morphogenetic protein Bicoid (Bcd), which is encoded by the maternal gene *bicoid* (*bcd*). Bcd protein, synthesized from the anteriorly localized mRNA (Berleth et al., 1988), is distributed in an anterior-to-posterior gradient (Driever and Nüsslein-Volhard, 1988; Driever, 1992). It determines the polarity along the anterior-posterior axis of the embryo, and organizes the development of the anterior structures including head and thorax (Nusslein-Volhard and Roth, 1989; Nusslein-Volhard, 1991; Driever, 1992).

Bcd induces the anterior structures in a concentration-dependent manner. Transplantation experiments demonstrate that cytoplasm isolated from the anterior of a wild-type embryo, or *bcd* mRNA alone, can induce the development of the anterior structures near the injection site in another embryo (Frohnhöfer et al., 1986; Driever et al., 1989, 1990). These experiments further demonstrate that larger amounts of *bcd* activity (or *bcd* mRNA) are required to induce the head structures than the thoracic structures. In addition, experiments altering the *bcd* gene dosage in the female demonstrate that the blastoderm fate map can be shifted according to Bcd levels in the embryo (Frohnhöfer and Nüsslein-Volhard, 1986, 1987). For example, when the female carries additional copies of the *bcd* gene, and consequently the embryo has increased levels of Bcd protein, the headfold (a morphological marker between head and thorax) is shifted to a more posterior position. These experiments suggest that different anterior structures are induced by Bcd at discrete thresholds of concentration.

Bcd is a concentration-dependent transcriptional activator in the embryo (Struhl et al., 1989). One target gene of Bcd is the zygotic gap gene *hunchback* (*hb*; Tautz et al., 1987), which is required for the formation of the thoracic structures (Nüsslein-Volhard and Wieschaus, 1980). In response to the Bcd gradient, *hb* is expressed uniformly in the anterior half of the embryo with a sharp posterior border (Driever et al., 1989; Struhl et al., 1989). However, head-specific gap genes have been suggested to respond to higher levels of Bcd, and their expression is restricted to smaller domains at the anterior (Dalton et al., 1989; Finkelstein and Perrimon, 1990). The posterior borders of the expression domains of *hb* and the head-specific genes are determined by Bcd concentration (Dalton et al., 1989; Struhl et al., 1989; Finkelstein and Perrimon, 1990). Elevated levels of Bcd in the embryo, caused by a higher *bcd* gene dosage in the female, can shift each of these borders to a more posterior position respectively (Dalton et al., 1989; Struhl et al., 1989; Finkelstein and Perrimon, 1990).

The 489 amino acid Bcd protein (Berleth et al., 1988) contains a homeodomain (residues 92-151), a conserved

protein domain responsible for specific DNA binding (Qian et al., 1989; Kissinger et al., 1990; Wolberger et al., 1991; Klemm et al., 1994). Our previous experiments (Driever et al., 1989), as well as those by Struhl et al. (1989), suggest that Bcd contains two functional domains. The carboxy-terminal portion of the protein (residues 247-489), which includes the acidic and glutamine-rich regions, provides most of the transcriptional activating function. The amino-terminal portion of Bcd (residues 1-246), which includes the homeodomain, provides the DNA binding function. Our experiments further demonstrate that hybrid Bcd molecules containing this amino-terminal portion attached to heterologous activating sequences can functionally rescue the *bcd*$^{-}$ mutant phenotype of the embryo (Driever et al., 1989). These experiments strongly suggest that, except transcriptional activation, this amino-terminal portion of Bcd provides all the essential functions expected of a molecular morphogen.

In order to understand how Bcd works as a molecular morphogen in the embryo, we have begun to address the question of how the Bcd gradient directs *hb* gene expression in an all-or-none fashion. Several potential mechanisms have been proposed that could contribute to this sharp target response (see Discussion). The experiments described in this report are designed to address this question by studying the DNA binding properties of Bcd. Here we demonstrate that Bcd binds cooperatively to multiple sites in a *hb* enhancer element. A less than 4-fold increase in Bcd concentration is sufficient to achieve an unbound/bound transition in DNA binding. In addition, we demonstrate that Bcd molecules can interact with each other in various genetic and biochemical assays. We suggest that one mechanism of sharp target gene response to the Bcd gradient is cooperative DNA binding facilitated by protein-protein interaction.

MATERIALS AND METHODS

Bcd protein in Sf-9 cells

The recombinant Bcd protein was generated in insect Sf-9 cells. The *bcd* gene was isolated from the plasmid bcdTN3 (Driever et al., 1989) and inserted into the baculovirus vector pJV NheI (Vialard et al., 1990). Recombinant viruses were generated and purified in Sf-9 cells according to the method of Summers and Smith (1987). To produce Bcd protein, 50 ml Sf-9 culture cells were infected by the recombinant viruses, collected 3 days after infection, and washed once with 10 ml buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, and 1 μg/ml pepstatin). The cell pellets were resuspended in 5 ml of the above buffer containing 1% NP-40, and the insoluble fraction was resuspended (overnight in a cold room) in 5 ml denaturation buffer (50 mM Tris pH 7.4, 5 mM EDTA, 1 mM PMSF, and 6 M urea). The protein was renatured by a step-wise dialysis against buffers (50 mM Tris pH 7.4, 2 mM DTT, 5 mM EDTA, 100 mM NaCl) containing 4 M, 2 M and 0 M urea. As estimated by Coomassie Blue staining, Bcd constituted about 70-80% of the preparations. The concentration of active Bcd in the preparations, estimated by titration at excess amount of DNA probe (1×10^{-5} M; data not shown), was about 2.3×10^{-6} M. The protein preparations contained two major species of active Bcd protein, the ratio of which varied from preparation to preparation. These two species probably did not reflect a difference in phosphorylation because calf intestine phosphatase treatment shifted both species, suggesting that both of them were phosphorylated (data not shown).

DNA probes

Table 1 lists the plasmids containing various derivatives of the *hb* enhancer element. Briefly, these plasmids were constructed as follows. pMAX1 was constructed by inserting the *Mlu*I-*Hin*dIII fragment from the plasmid *hb-298* (with the *Mlu*I end filled-in by Klenow) at the *Sma*I/*Hin*dIII sites of pBluescript KS(−). The DNA fragment between *Hin*cII and *Hin*dIII of the polylinker of pBluescript KS(−), which contained a Bcd binding site (see Fig. 2) was subsequently deleted from pMAX1, resulting in pMAX1′. pMAX9 was constructed by inserting the *Hin*dIII-*Apa*I fragment of the *hb* enhancer element at the *Apa*I/*Sma*I sites of pBluescript KS(−). pMAX10 was constructed by inserting the *Bsr*FI-*Apa*I fragment (with the *Bsr*FI end filled-in by Klenow) at the *Apa*I/*Sma*I sites of pBluescript KS(−). pMAX7 was constructed by inserting the X1 site, isolated as the *Apa*I-*Hae*III fragment from a plasmid containing the sites X1, X2 and X3, at the ApaI/SmaI sites of pBluescript KS(−). The *Hin*cII-*Hin*dIII fragment from the polylinker was absent in pMAX7, 9 and 10. To generate

Table 1. Plasmids used in this study

Bicoid site or protein	Plasmid	Notes	Source
For in vitro DNA binding assays			
Intact *hb* enhancer	pMAX1′		This work
Intact *hb* enhancer	pMAX1	HincII-HindIII of polylinker present	This work
A1-X1	pMAX9		This work
A1	pMAX10		This work
X1	pMAX7		This work
For yeast experiments			
LexA(1-87)-Bcd(3-489)	Lex-bcd(wt)	*His3* marker	Hanes and Brent (1989)
LexA(1-87)-Bcd(3-489)(Q9)	Lex-bcd(Q9)	*His3* marker	Hanes and Brent (1989)
LexA(1-87)-B42	pMA457	*His3* marker	Ma and Ptashne (1987b)
Wild type Bcd (1-489)	pMA625	*Leu2* marker	Driever et al. (1989)
Bcd(1-396)-VP16	pMA650	*Leu2* marker	This work
Bcd(1-246)-VP16	pMA670	*Leu2* marker	Driever et al. (1989)
Bcd(1-396)-GAL4(768-881)	pMA671	*Leu2* marker	Driever et al. (1989)
Wild type Bcd (1-489)	pTA3	*His3* marker	This work
Bcd(1-479)(A9)-VP16	pMA1230	*Leu2* marker	This work
Bcd(1-479)(Q9)-VP16	pMA1231	*Leu2* marker	This work
For in vitro protein interaction experiments			
Wild type Bcd (1-489)	bcdTN3	Sp6 promoter; EcoRV to linearize	Driever et al. (1989)
Wild type LexA (1-202)	pMA1250	Sp6 promoter; XbaI to linearize	This work
LexA(1-87)-Bcd(3-489)	pMA1222	Sp6 promoter; XbaI to linearize	This work

radioactively labeled DNA probes, the Bcd binding sites were first isolated from the respective plasmids as XbaI-KpnI fragments, and then filled-in by reverse transcriptase in the presence of [α-^{32}P]dCTP.

Gel retardation

For gel retardation assays, each reaction was performed in 20 μl 1× BB buffer (20 mM Tris pH 7.5, 50 mM NaCl, 0.5 mM EDTA, 0.2 mM EGTA, and 1 mM DTT) containing 0.1 mg/ml bovine serum albumin (BSA) and 0.1 mg/ml poly dI::dC (Pharmacia) for 15 minutes at room temperature. The concentration of the ^{32}P-labeled probe (from pMAX1) was 5.7×10^{-10} M. After adding 3 μl 20% Ficoll, the reaction mixtures were loaded onto a native polyacrylamide gel in 0.5× TBE buffer. Quantitative analysis was carried out using the Molecular Dynamics PhosphorImager system.

DNase I footprint

Radioactively labeled probes containing either the 250 bp *hb* enhancer element or its derivatives (approx. 5×10^{-10} M) were incubated with various amounts of protein in 25 μl buffer (20 mM Tris pH 7.9, 110 mM KCl, 12 mM $MgCl_2$, 0.05 mM EDTA, 1 mM DTT, 17% glycerol, and 0.02% NP-40) containing 4.9 μg/ml poly dI::dC (Pharmacia) for 45 minutes on ice. DNase I digestion was carried out on ice for 5 minutes, and the DNA samples were purified and separated on an 8% sequencing gel. Quantitative analysis was carried out using the Molecular Dynamics PhosphorImager system.

Genetic assays in yeast

Two plasmids, one with the *Leu2* marker and the other with the *His3* marker, were transformed into yeast cells. The yeast strain JPY27 (α *his3Δ200 leu2Δ1 trp1Δ63 lys2Δ385 URA3*::JH199) containing a *GAL1-lacZ* reporter gene under the control of two LexA sites, kindly provided by J. Pearlberg, was used for the assay in Table 2. For the assay in Table 3, the yeast strain used was GGY1::MA630R (*α Δgal4 Δgal80 leu2 his3 URA3*::MA630R), which contained a *GAL1-lacZ* reporter gene with the intact *hb* enhancer element located upstream (Driever et al., 1989). In both assays, the control plasmids that did not encode any Bcd derivatives were pMA201 (Ma and Ptashne, 1987a), pMA200 (Ma and Ptashne, 1987a) or AAH5 (Ammerer, 1983), the former two of which carried a *His3* yeast marker and the third, *Leu2*. These control plasmids were included to ensure that all the yeast cells were grown in identical selection media (2% glucose, 0.67% yeast nitrogen base without amino acids, supplemented with amino acids lacking both leucine and histidine). The β-galactosidase activities were measured according to previously published procedures (Yocum et al., 1984; Ma and Ptashne, 1987a; Ma, 1992); at least 3-5 independent transformants were assayed. See Table 1 for the plasmids used in the yeast genetic assays. Briefly, pTA3 was constructed by inserting the *Bam*HI fragment from pMA625 (Driever et al., 1989) into the *Bam*HI site of pMA210 (Ma and Ptashne, 1987a). pMA1230 and 1231 were constructed in two steps. First, the *Pst*I-*Sac*II fragment of pMA1225, a plasmid bearing the wild-type *bcd-vp16* fusion gene, was replaced by the *Pst*I-*Sac*II fragments from the mutant *bcd* plasmids provided by Hanes and Brent (1989), resulting pMA1227 and 1228. pMA1230 and 1231 were then constructed by inserting the *Hin*dIII fragments from pMA1227 and 1228, respectively, containing the mutant *bcd-vp16* fusion genes, into the *Hin*dIII site of AAH5 (Ammerer, 1983). pMA650 was constructed in two step. First, the *Bgl*II-*Eco*RV fragment of bcdTN3 (Driever et al., 1989), was replaced by the *Bgl*II-*Hin*dIII fragment (with *Hin*dIII end filled-in with Klenow) from CRF-2 (Sadowski et al., 1988), a plasmid bearing the *vp16* gene and kindly provided by S. McKnight, resulting in pMA640. The *Hin*dIII fragment of pMA640 was then inserted at the *Hin*dIII site of AAH5, resulting pMA650.

Chemical cross linking

The proteins used for chemical cross linking and immunoprecipitation experiments were generated by in vitro transcription and translation according to the manufacturer (Promega). For the chemical cross linking experiments, the intact Bcd protein, encoded by the plasmid bcdTN3 (Table 1), was generated and analyzed. The chemical cross linking assays, using ethylene glycol bis succinimidyl succinate (EGS), was conducted in 20 μl buffer (plus 1 μl translation lysate) for 20 minutes on ice at various concentrations of EGS. Two different buffers were used with similar results: Buffer B used in the immunoprecipitation assays (see below) and a buffer (100 mM triethanolamine pH 8.0, 2 mM EDTA, and 5% glycerol) described by Sorger and Nelson (1989). The reactions were stopped by adding 0.92 μl 0.5 M lysine and 20 μl 2× protein loading buffer, and the solution was then loaded onto an SDS polyacrylamide gel.

Immunoprecipitation

The immunoprecipitation assays were performed as follows. The translation lysates were incubated in 20 μl Buffer B (10 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl and 0.1% NP-40) on ice for 3-4 hours. The total amount of translation lysate, adjusted with mock lysate, was kept constant for all the experiments (1.0 μl). Antibodies against LexA, kindly provided by M. Ptashne's laboratory, were added to the reactions at a final dilution of 1/25,000. After overnight incubation on ice, 2 μl *Staphylococcus aureus* cells (Calbiochem), which had been washed (3 times) and equilibrated in Buffer B, were added and the incubation continued for a further 1 hour, on ice. The reactions were then diluted with 150 μl Buffer B, precipitated by spinning for 3-4 minutes at 4°C, and gently washed three times with 150 μl Buffer B. The samples were then loaded onto an SDS polyacrylamide gel, which, after being dried, was exposed to X-ray films. For the experiments with ethidium bromide (EtdBr), the same concentration of EtdBr was present in Buffer B used for washing. See Table 1 for a list of plasmids used in the assays. Briefly, pMA1222 was constructed by inserting the *Mlu*I-*Eco*RV fragment from the Lex-bcd (wt) plasmid, provided by Hanes and Brent (1989), into the *Mlu*I/*Sal*I sites (with the *Sal*I end filled-in by Klenow) of pMA526, a plasmid expressing from the Sp6 promoter the LexA-GAL4 (848-881) fusion protein. pMA1250 was constructed by inserting the *Mlu*I-*Xba*I fragment from pMA910 (Ma, 1992) into the *Mlu*I/*Xba*I sites of pMA526.

Order of addition assay

The binding reactions were carried out on ice in 1.0 ml 1× BB buffer containing 10 μl recombinant Bcd, 34.4 μg/ml poly dI:dC and 2×10^{-10} M radioactively labeled *hb* enhancer element (from pMAX1′). For one reaction the Bcd protein had been pre-diluted in 990 μl 1× BB buffer (without poly dI:dC and DNA probe) and incubated at room temperature for 5 minutes. For the other reaction, Bcd had not been pre-diluted. Aliquots (50 μl) were taken from the reactions at various time points for filter binding assays (Ausubel et al., 1994).

RESULTS

Cooperative DNA binding of Bcd to the *hb* enhancer element

To study the DNA binding properties of Bcd in vitro we produced a recombinant intact Bcd protein in Sf-9 insect cells (see Materials and Methods). A 250 bp enhancer element of the *hb* gene, which contains six previously identified Bcd binding sites (Driever and Nüsslein-Volhard, 1989; see Fig. 1A and its legend), was used in our studies. This enhancer element has been shown previously to be sufficient for conferring the spatially restricted *hb* expression pattern in response to the Bcd gradient in the embryo (Driever et al., 1989; Struhl et al., 1989). In this paper we designate this 250 bp DNA fragment as the intact *hb* enhancer element.

Fig. 1B shows the results of our initial DNA binding studies of the *hb* enhancer element in a gel retardation assay under equilibrium conditions. At low concentrations of Bcd, a small

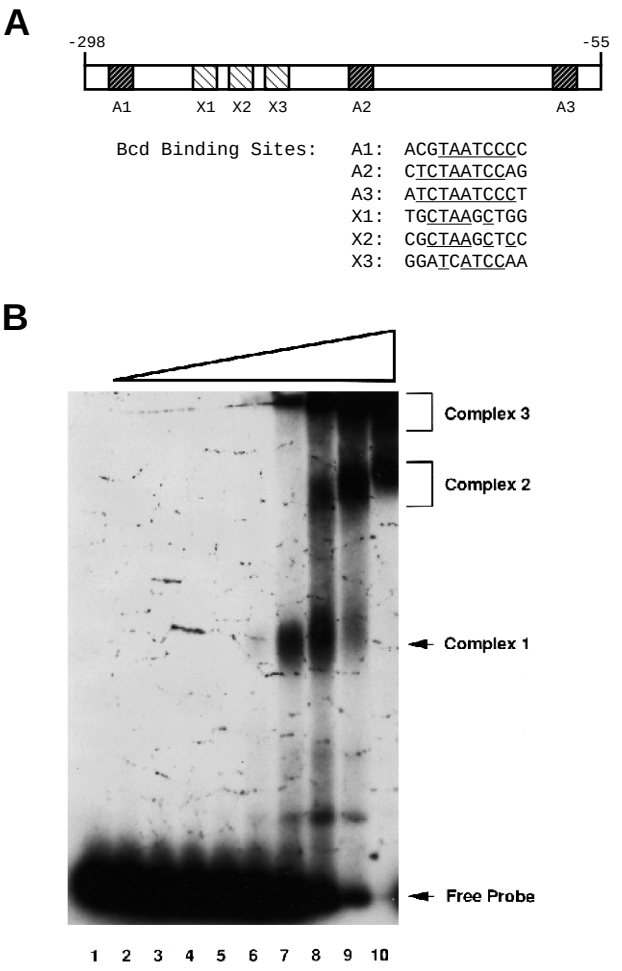


Fig. 1. Cooperative binding of Bcd to the *hb* enhancer element. (A) A schematic diagram of the 250 bp *hb* enhancer element and sequences of the Bcd binding sites. This DNA fragment is located between -298 and -55 upstream from the start site of the 2.9 kb *hb* zygotic transcript. The positions that match the putative consensus site TCTAATCCC generated by Driever and Nusslein-Volhard (1989) are underlined. In addition to these six previously identified Bcd binding sites, our footprint experiments (Fig. 2A) detected another less well protected site between A2 and A3. The protected region contains the sequence CCTCAATCCGCGAT with 5 bp matching the putative consensus. (B) Gel retardation assay using the 250 bp *hb* enhancer element. The amounts of protein added to the reactions are: 0 μl, 0.0156 μl, 0.031 μl, 0.065 μl, 0.125 μl, 0.25 μl, 0.5 μl, 1 μl, 2 μl, and 4 μl for lanes 1 through 10, respectively. Because of multiple bands that are shifted, the unbound free probe is measured for the quantitative analyses. The excess amount of non-specific DNA prevents non-specific DNA binding by Bcd (see Materials and Methods). Kinetic studies (Fig. 7) suggest that the Bcd binding assays are at equilibrium. We attempted but failed to show any heterodimer formation using two Bcd derivatives of different sizes in gel retardation assays. First, no heterodimer was detected when a single site was used in the assay (J. M. unpublished results), suggesting that a single Bcd site is bound by one Bcd molecule. Second, when two sites were used in the assay, a complex containing a single Bcd molecule, rather than two, was always detected first. These experiments suggest that the functional units for DNA binding are monomers rather than dimers. The relative position of Complex 1 in this gel suggests that it contains only one Bcd molecule. We do not know exactly how many Bcd molecules are present in the complexes migrating near the top of the gel (Complex 3) but we suspect that there are at least more than six of them. As discussed in the text, these complexes are not due to non-specific protein aggregation. It is possible that they represent the formation of super complexes of Bcd (see Discussion).

amount of DNA probe was shifted to a complex containing one Bcd molecule (Complex 1, lanes 6 and 7; see legend to Fig. 1b for further discussion). This complex did not reflect Bcd binding to any one particular site in the *hb* enhancer element as demonstrated by the DNase I footprint assays (see below). A slight increase (about 4 fold) in Bcd concentration shifted almost all the probe to much slower mobility complexes (Complexes 2 and 3, lanes 8-10). Our estimates suggest that at least three (up to six or more) Bcd molecules are present in Complex 2. The protein complexes migrating near the top of the gel (Complex 3), were not due to non-specific protein aggregation (see Discussion for a possibility of super complex formation). First, when probes of single sites were used in similar gel retardation assays, only complexes containing one Bcd molecule could be detected (X. M., D. Y., and J. M., unpublished data). In addition, all the shifted complexes, including Complex 3, were specifically competed away by unlabeled DNA probes containing Bcd sites (data not shown). To quantitatively analyze our gel retardation results, a Hill coefficient was estimated by measuring the unbound free probe at various Bcd concentrations. The estimated value of 2.5 strongly suggests that Bcd binds to the *hb* enhancer element cooperatively.

Concerted Bcd binding to multiple sites

To further demonstrate cooperative binding of Bcd, we performed a DNase I footprint analysis (Fig. 2A) using the *hb* enhancer element. A less than 4-fold increase in Bcd concentration was sufficient to achieve virtually complete protection of all the sites (except A3) from little or no protection (compare lane 5 and lane 3, left panel). Quantitative analyses revealed similar equilibrium binding curves (Fig. 2B) for each of the Bcd binding sites (except A3; see Discussion), suggesting that Bcd binds to these sites in a concerted manner. These binding curves exhibit strong sigmoid character, further supporting the idea that Bcd binds to the *hb* enhancer element cooperatively. A Hill coefficient of 3.6 for the *hb* enhancer element was estimated by averaging the coefficients for all the six sites (5, 4, 3, 3.7, 5, and 1.2 estimated for the sites A1, X1, X2, X3, A2, and A3, respectively). This value is higher than that estimated from the gel retardation data, suggesting that the binding of Bcd appears more cooperative in DNase I footprint assays than in gel retardation assays (see Discussion for further consideration of this issue).

Higher affinity of Bcd to double DNA sites than to single sites

One prediction of cooperative DNA binding is that Bcd would bind better to multiple sites than to single sites. To test this idea we generated various deletion derivatives of the *hb* enhancer element. One derivative contained A1 and X1 in their natural configuration (separated by 41 bp from center to center) and two others contained the individual binding sites separately. While individual X1 and A1 sites were poorly protected from DNase I digestion even at the highest concen-

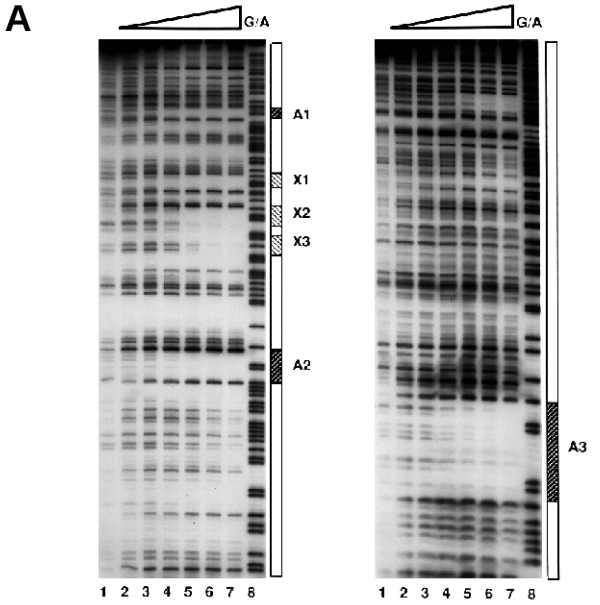
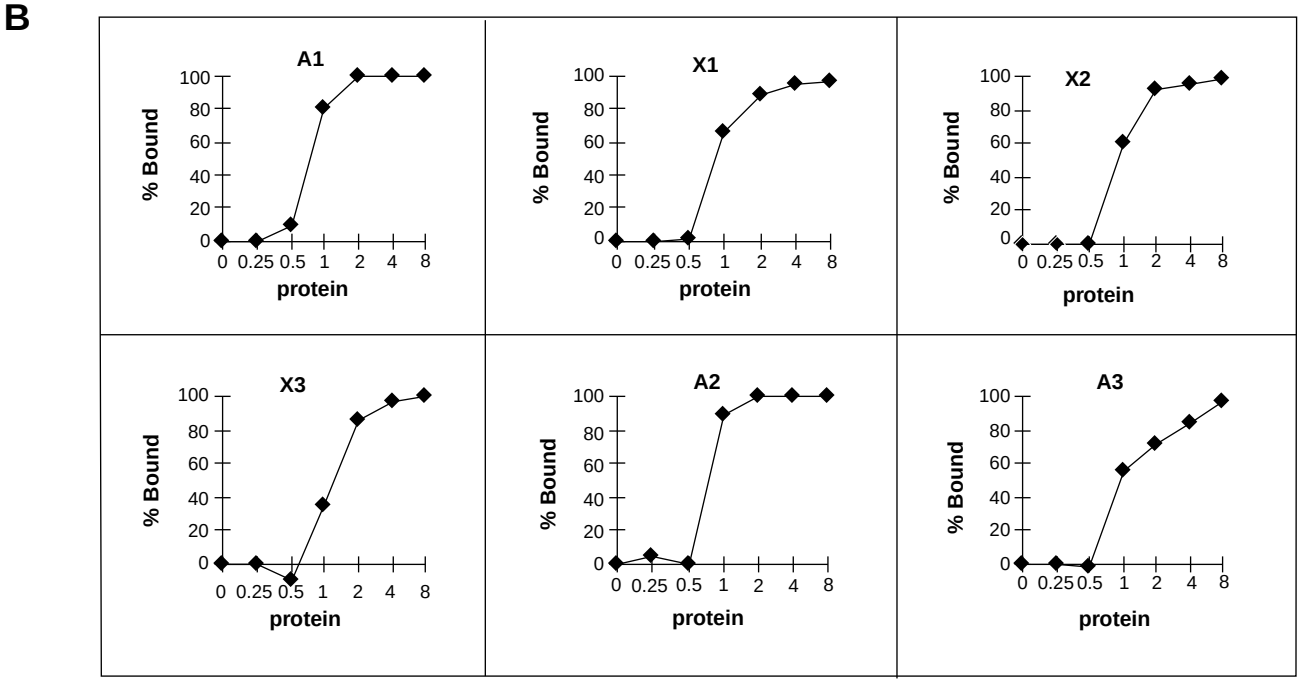


Fig. 2. Cooperative binding of Bcd in DNase I footprint assay. (A) DNase I footprint assay of the *hb* enhancer element. The two panels represent exactly the same footprint assay. To ensure a good resolution of all the binding sites, the same samples are loaded at two different times onto the same sequencing gel. On the right panel, only the site A3, which is well resolved, is marked. The amounts of protein are 0 μl, 0.125 μl, 0.25 μl, 0.5 μl, 1 μl, 2 μl, 4 μl, and 8 μl for lanes 1 through 7, respectively. Lane 8 represents a G+A DNA sequencing ladder. A previously unidentified Bcd binding site is detected between A2 and A3 (see legend to Fig. 1A for further discussion). A sequence in the polylinker of the pBluescript vector (between the *Hin*cII and *Hin*dIII sites) is also protected by Bcd (see the protected region above the A1 site). (B) Binding curves for each site in the *hb* enhancer element. The intensities of the protected bands were determined using the Molecular Dynamics PhosphorImager system and the percentage of binding (assuming % binding = % protection) is plotted against the amount of protein (μl).



tration of Bcd (Fig. 3A,B), the two sites together were much more efficiently protected (Fig. 3C). DNA binding curves resulting from quantitative analyses of the footprint data are presented in Fig. 3D. These analyses indicate that the affinity of Bcd to two sites, as estimated by protein concentrations required for half maximal protection, is almost an order of magnitude higher than that to single sites (estimated average K_d values: $5.5\pm0.4\times10^{-8}$ M and $4.6\pm2.1\times10^{-7}$ M for two sites and single sites, respectively). The quantitative analyses also show that Bcd binds to individual A1 and X1 sites with similar affinity (Fig. 3D; see legend for further discussion). Furthermore, the binding curves for the intact *hb* enhancer element (Fig. 2B) are much steeper than those for two sites (A1-X1; Fig. 3C) or three sites (X. M. and J. M., unpublished results), suggesting that Bcd binds to the intact *hb* enhancer element with the highest degree of cooperativity.

Two additional sets of experiments further support our conclusion that Bcd binds DNA cooperatively and suggest that a strict alignment (spacing and distance) between Bcd sites is not required for cooperative DNA binding. First, we specifically mutated either A1 or X1 by site-directed mutagenesis, generating two probes that contained one site mutated while keeping the other site intact in its natural configuration. These two probes, together with the probe containing both intact A1 and X1, were tested in DNase I footprint assays. Our results showed that, similar to the results in Fig. 3, Bcd bound to A1-X1 more efficiently than to either of the mutated probes (X. M. and J. M., unpublished results). To determine if there were strict require-

ments of spacing and orientation between A1 and X1 for cooperative DNA binding, we constructed two additional probes. One probe contained the A1 site in reverse orientation while keeping the distance to X1 the same. The other probe contained the A1 and X1 sites separated by 45 bp (center to center) while keeping their natural orientation. Our DNase I footprint assays demonstrated that, similar to the probe bearing the intact A1 and X1 in their natural configuration, Bcd bound to both of these probes more efficiently than to single sites (X. M. and J. M., unpublished results).

Yeast genetic assays demonstrating Bcd-Bcd interaction

One potential mechanism of cooperative DNA binding is an interaction between Bcd molecules. We have demonstrated by various genetic and biochemical assays that Bcd molecules can interact with each other. In this section we describe two yeast genetic assays. The first assay is based on the concept that the DNA binding domain and activation domain of an activator can be brought together by protein-protein interactions (Ma and Ptashne, 1988; Fields and Song, 1989; Dalton and Treisman, 1992; Gyuris et al., 1993). Two types of fusion proteins were used in this assay: one contained Bcd attached to the DNA binding domain of LexA (Hanes and Brent, 1989), and the other Bcd to the strong activating sequences of VP16 (Sadowski et al., 1988) or GAL4 (Ma and Ptashne, 1987a). These fusion proteins were assayed in yeast cells bearing a reporter gene (*GAL1-lacZ*) with two LexA binding sites located upstream. The LexA-Bcd fusion protein activated transcription poorly in this assay (line 1, Table 2). The Bcd-VP16 or Bcd-GAL4 fusion proteins in the

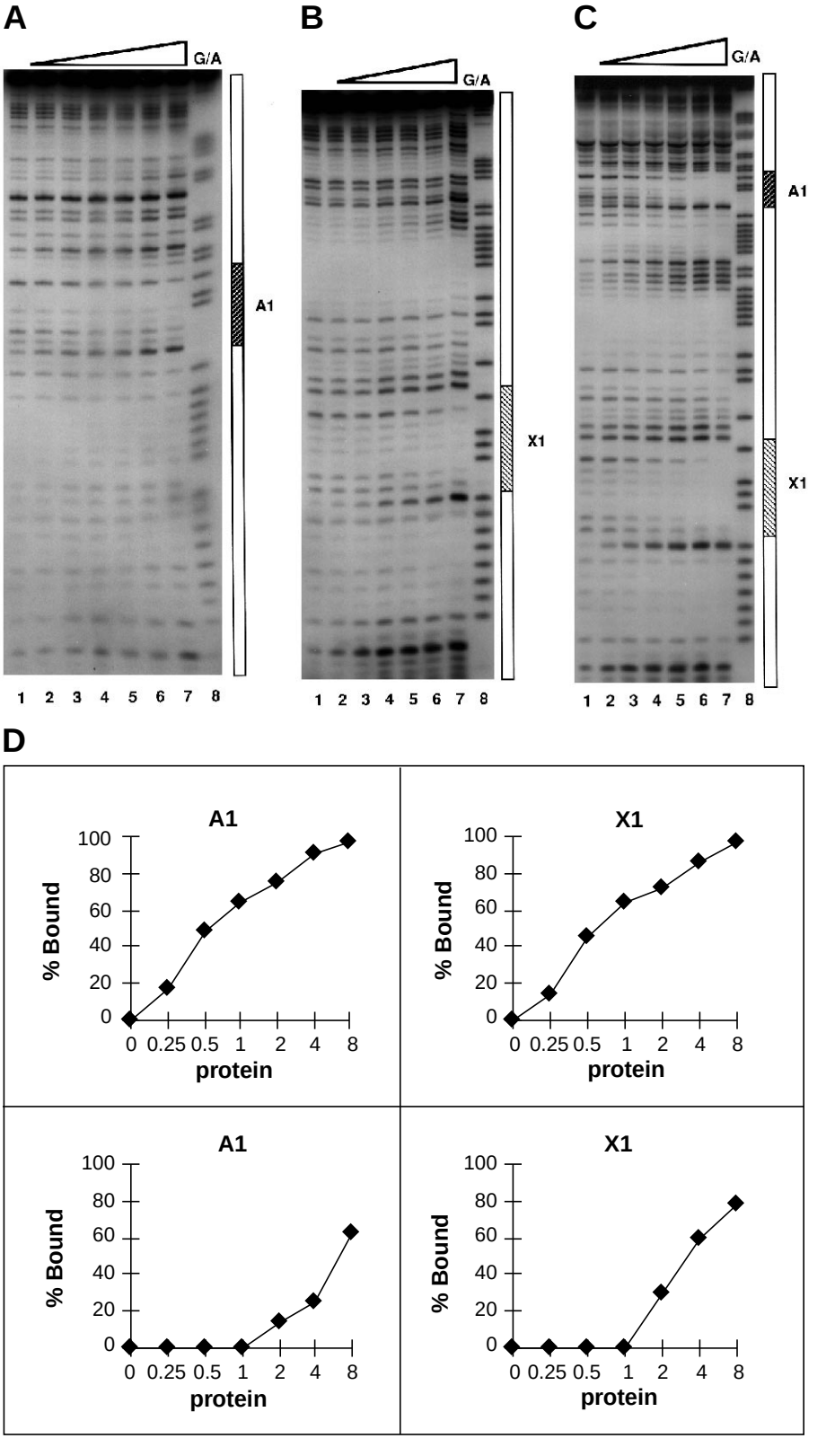


Fig. 3. More efficient binding of Bcd to multiple sites than to single sites. (A-C) DNase I footprint data of probes containing either A1 (A), X1 (B), or A1 and X1 together (C). These experiments were performed side by side to ensure an accurate measurement of Bcd binding. See Fig. 2A legend for a further description. (D) Binding curves for the sites A1 and X1 assayed either together (upper curves) or individually (lower curves). See Fig. 2B legend for a description of the curves. As opposed to previous suggestions that A-type sites may have higher affinity than X-type sites (Driever and Nüsslein-Volhard, 1989; Driever et al., 1989), our experiments did not detect any significant difference in Bcd affinity to A1 and X1 as individual sites. This could be attributed to many differences including protein sources and assaying methods and conditions. It should be noted that the concept of high- and low-affinity Bcd sites was originally proposed based on the observation that the X-type sites were less well protected than A-type sites in a DNase I footprint assay, rather than a detailed protein titration analysis as shown in this report.

Table 2. A yeast genetic assay demonstrating Bcd-Bcd interaction

	LexA derivative	Bcd derivative	β-galactosidase units
1	LexA-Bcd	None	7.9
2	LexA-Bcd	wt Bcd	9.4
3	LexA-Bcd	Bcd(1-396)-VP16	20.4
4	LexA-Bcd	Bcd(1-246)-VP16	16.0
5	LexA-Bcd	Bcd(1-246)-GAL(768-881)	15.5
6	LexA-Bcd(Q9)	None	5.7
7	LexA-Bcd(Q9)	Bcd(1-396)-VP16	24.1
8	LexA-Bcd(Q9)	Bcd(1-246)-VP16	27.6
9	LexA-B42	None	26.6
10	LexA-B42	Bcd(1-396)-VP16	25.8
11	LexA-B42	Bcd(1-246)-VP16	29.3
12	None	None	0
13	None	Bcd(1-396)-VP16	0
14	None	Bcd(1-246)-VP16	0

The experiments shown here demonstrate a specific interaction between Bcd molecules in yeast. In this assay, the strong activating sequences VP16 (Sadowski et al., 1988) or activating region II of GAL4 (residues 768-881) (Ma and Ptashne, 1987a), which are attached to either Bcd(1-246) or Bcd(1-396), are brought to DNA by interacting with the LexA-Bcd fusion proteins. See Table 1 for the plasmids used in this assay and see text for further details. The Bcd-VP16 molecules appear to increase the target gene activity more significantly from LexA-Bcd(Q9) (line 6-8) than from LexA-Bcd (lines 1, 4 and 5). We do not fully understand this difference, but it is possible that the mutated position (the ninth position of the third helix of the homeodomain) of Bcd may somehow influence, though not directly participating in, protein-protein interaction.

same cells increased the transcription levels (lines 3-5). Although this is only a modest increase, consistent with the idea that the interaction between Bcd molecules is relatively weak (see below), it reflects a specific interaction. The Bcd-VP16 fusion proteins neither activated transcription by themselves (line 12-14) nor increased the activity of an unrelated protein, LexA-B42 (Ma and Ptashne, 1987b) (lines 9-11). In a different set of experiments, the transcription levels induced by a GAL4-Bcd fusion protein, similar to those by LexA-Bcd, were also increased by the Bcd-VP16 fusion proteins (T. S. and J. M., unpublished results). The experiments in Table 1 also show that Bcd fusion molecules containing residues 1-246 of Bcd are sufficient to interact with LexA-Bcd (lines 4, 5 and 8), suggesting that this amino-terminal portion provides a protein-protein interaction domain. Furthermore, the wild-type Bcd-VP16 fusion proteins can interact with a mutant Bcd protein, Bcd(Q9) (lines 6-8). This mutant Bcd protein contains a mutation at the ninth position of the homeodomain's third helix, abolishing its ability to recognize Bcd sites (Hanes and Brent, 1989). These results suggest that the surface(s) required for protein-protein interaction may be different from that for DNA recognition (see additional results in Table 3).

A second yeast genetic assay (Table 3) further demonstrates that Bcd molecules can interact with each other. In this assay, the *hb* enhancer element was placed upstream of the yeast reporter gene *GAL1-lacZ*, and three Bcd proteins were tested. The first protein was the wild-type Bcd, which, as demonstrated previously (Driever et al., 1989; Struhl et al., 1989), activated transcription from the *hb* enhancer element in yeast (line 1). The other two proteins were Bcd-VP16 fusions [Bcd(Q9)-VP16 and Bcd(A9)-VP16] containing mutant but nearly intact Bcd proteins (residues 1-479). Similar to Bcd(Q9) (see above), Bcd(A9) also contains a mutation at the ninth position of the homeodomain's third helix and is thus unable to recognize Bcd sites (Hanes and Brent, 1989). Therefore, both of these two mutant Bcd-VP16 fusion proteins failed to activate transcription from the *hb* enhancer element in yeast (line 2 and 3). However, in the presence of both the mutant Bcd-VP16 fusion proteins and the wild-type Bcd, the reporter gene activity was increased to levels approximately ten times higher than those induced by the wild-type Bcd alone (compare lines 4 and 5 with line 1). These results suggest that the mutant Bcd-VP16 fusion proteins can functionally interact with the wild-type Bcd molecules bound to the *hb* enhancer element (see Discussion).

Table 3. Super activation assay in yeast

	Activators	β-galactosidase units	Fold increase
1	WT Bcd	4.8	-
2	Bcd(A9)-VP16	<1	-
3	Bcd(Q9)-VP16	<1	-
4	WT Bcd & Bcd(A9)-VP16	50.8	10.6×
5	WT Bcd & Bcd(Q9)-VP16	48.5	10.1×

These experiments demonstrate that the mutant Bcd-VP16 proteins, which are unable to activate transcription by themselves from the *hb* enhancer element in yeast (lines 2 and 3), can increase the level of gene activation by interacting with the DNA-bound wild type Bcd molecules (lines 4 and 5). Fold of increase is calculated by dividing the β-galactosidase activity from lines 4 and 5 by that from line 1. See Table 1 for plasmids used in this assay and see text for further details.

Biochemical assays demonstrating Bcd-Bcd interaction

The interaction between Bcd molecules was further demonstrated by chemical cross linking experiments. Radioactively labeled Bcd, generated by in vitro transcription and translation, was subjected to chemical cross linking by ethylene glycol bis succinimidyl succinate (EGS) (Sorger and Nelson, 1989). Protein complexes of twice the molecular mass of Bcd monomers were detected in an SDS polyacrylamide gel (Fig. 4). In addition to these dimeric complexes, higher molecular mass complexes were also evident though they could not be resolved in the gel. In this assay, the major fraction of Bcd is in its monomeric form, consistent with the idea that the interaction between Bcd molecules is relatively weak (see below).

Fig. 5 shows that the interaction between Bcd molecules can also be detected by an immunoprecipitation assay. In this assay, the wild-type Bcd and a LexA-Bcd fusion protein were generated and radioactively labeled by in vitro transcription and translation. These proteins were then incubated together and subsequently precipitated with antibodies against the LexA protein and *Staphylococcus aureus* cells (Lue et al., 1987). As shown in lanes 3 and 4, the wild-type Bcd was co-precipitated by the LexA-Bcd fusion protein. In the absence of LexA-Bcd fusion protein (lanes 5 and 6) or when the wild-type LexA protein was used in the same assay (lanes 13-16), no wild-type Bcd was co-precipitated above the background level. In a separate set of experiments, Bcd was similarly co-precipitated by a GAL4-Bcd fusion protein and antibodies against GAL4 (J. M., data not shown).

DNA-dependent interaction between Bcd molecules

Lai and Herr (1993) have demonstrated previously that ethidium bromide (EtdBr) can specifically inhibit DNA-

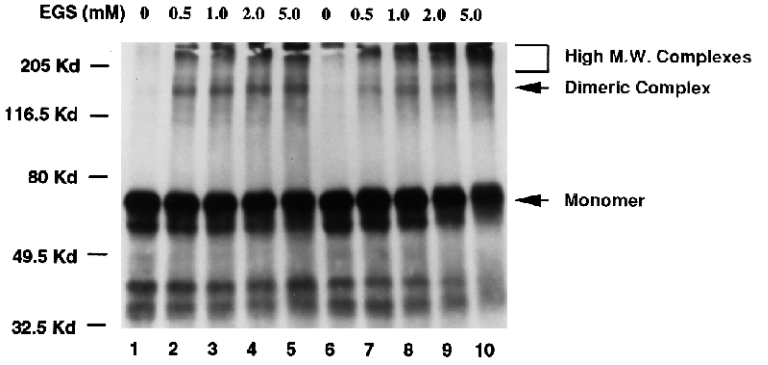


Fig. 4. Chemical cross linking assays. Protein complexes of roughly twice the molecular mass of Bcd protein are detected after chemical cross linking with EGS. Two different buffers were used with similar results: Buffer B was used for the assays shown in lanes 1-5 and a buffer described by Sorger and Nelson (1989; see Materials and Methods for further details) was used for those in lanes 6-10. In addition to dimeric complexes, higher molecular mass complexes were also detected. We do not know whether these complexes are caused by oligomeric interaction on DNA or super complex formation (see text), or both. We also cannot exclude the possibility that other proteins in the in vitro translation lysate may be cross-linked to the Bcd complexes.

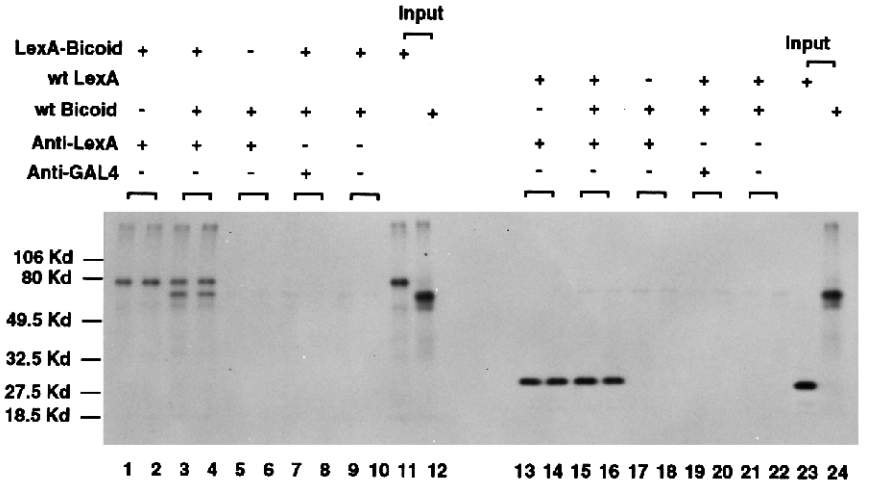


Fig. 5. Interaction between Bcd molecules demonstrated by immunoprecipitation assays. Two independent assays were performed for each experiment. The amounts of the translation lysate used for the experiments were: 0.2 μl for LexA-Bcd and the wild-type LexA, and 0.5 μl for the wild-type Bcd protein. The anti-GAL4 antibodies were used as a negative control (lanes 7 and 8), in addition to other necessary controls. 'Input' represents half the amount of the proteins used for the assays.

dependent, but not DNA-independent, protein-protein associations. To determine whether the interaction between Bcd molecules was DNA-dependent, we repeated the immunoprecipitation analysis in the presence of EtdBr. Fig. 6 shows that EtdBr greatly reduces the amount of wild-type Bcd co-precipitated by LexA-Bcd, suggesting that the interaction between Bcd molecules is DNA-dependent. The contaminating DNA could come from either the DNA templates used for synthesizing RNA or the in vitro translation lysate, or both. It is important to note that, consistent with the results of Lai and Herr (1993), EtdBr did not affect DNA-independent protein-protein interactions in our assay because LexA-Bcd was similarly precipitated with or without EtdBr (compare lanes 1 and 2 with lanes 5 and 6).

The following arguments rule out the objection that, without specific protein-protein interactions, the co-precipitation of wild-type Bcd was a result of non-specific tethering of the proteins to DNA (Lai and Herr, 1993). First, neither the DNA binding domain of GAL4 nor the wild-type LexA protein alone, both of which can bind DNA, co-precipitated the wild-type Bcd (Fig. 5 lanes 13-16; and data not shown). Second, co-precipitation of the wild-type Bcd was still detected when additional non-specific DNA (poly dI::dC) was added to the reactions at a molar concentration over 100 times higher than that of the proteins (data not shown). Under these conditions, it would be virtually impossible to have one piece of DNA bound by more than one protein molecule without specific protein-protein interactions. Third, experiments with varying amounts of the LexA-Bcd fusion protein ruled out the possibility of non-specific protein aggregation (J. M., unpublished results). Fourth, the homeodomain of Bcd alone, which is sufficient to bind DNA both in vivo and in vitro (Wilson et al.,

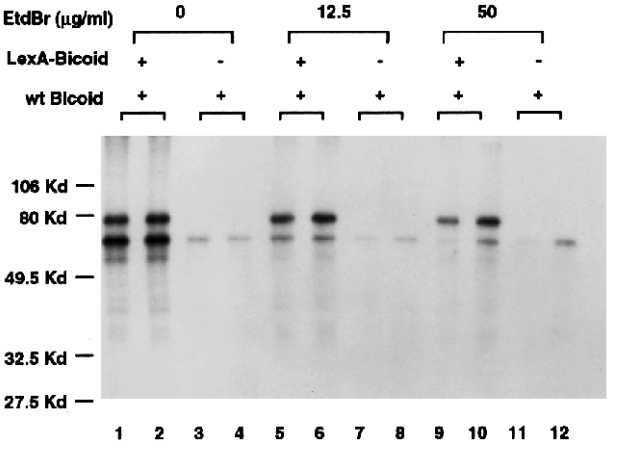


Fig. 6. DNA-dependent interaction between Bcd molecules. The assays are performed similarly to those of lanes 3-6 in Fig. 5, either in the absence (lanes 1-4) of presence (lanes 5-12) of ethidium bromide (EtdBr). The results show that EtdBr, which inhibits protein-DNA interaction, greatly reduces the amount of the wild-type Bcd co-precipitated by LexA-Bcd. The LexA-Bcd fusion protein is similarly immunoprecipitated in the presence of EtdBr demonstrating that the DNA-independent protein-protein interactions (between LexA and antibodies and between Protein A and antibodies) are insensitive to EtdBr.

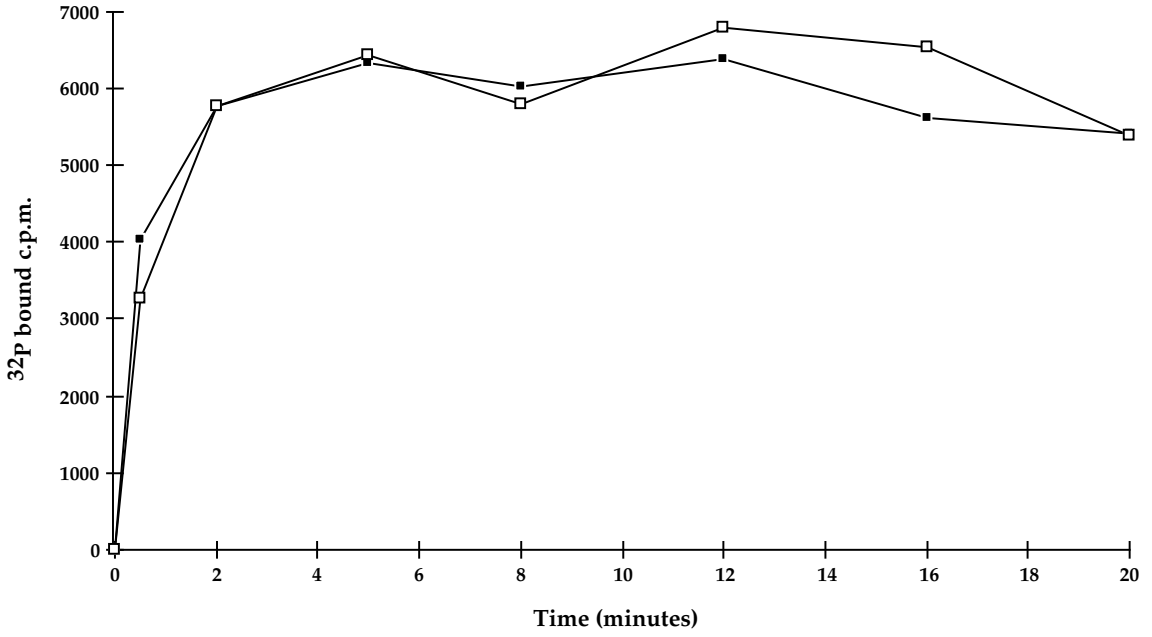


Fig. 7. Order of addition assay. Shown are two kinetic measurements of binding to the intact *hb* enhancer element (from pMAX1′) by Bcd that is either pre-diluted (open squares) or without pre-dilution (solid squares). These two kinetic curves are similar to each other, and in both cases equilibrium is achieved within 5 minutes. See Results and Materials and Methods for further details.

1993; N. Lehming, D. Y., and J. M., unpublished data), did not support protein-protein interaction (J. M., unpublished results). Finally, the interaction between Bcd molecules has been independently demonstrated in two functional assays in yeast (Tables 2 and 3). Taken together, these experiments suggest that the interaction between Bcd molecules is specific but relatively weak, and it is stabilized by DNA.

Order of addition assay

To determine whether Bcd molecules form complexes in solution or on DNA, we performed an order of addition assay (Pirrotta et al., 1970). This assay consists of two kinetic measurements of Bcd binding to the *hb* enhancer element, as determined by a filter binding analysis. Recombinant Bcd expressed in Sf-9 cells was either directly added to a reaction mixture containing the radioactively labeled DNA probe, or it was first diluted in a reaction mixture in the absence of the DNA probe and then incubated with the DNA probe. The purpose of the latter experiment was to dissociate protein complexes, if any, into monomers. It has been demonstrated previously that if a protein forms complexes in solution, and if the formation of protein-protein complexes is slower than protein-DNA interaction, the pre-diluted protein binds to DNA more slowly than without pre-dilution (Pirrotta et al., 1970). Our experiments demonstrate that the kinetic properties for these two measurements are similar (Fig. 7). Equilibrium is achieved within 5 minutes of incubation of Bcd and the DNA probe in both cases (Fig. 7). These results are consistent with the idea that Bcd forms complexes on DNA rather than in solution. Alternatively, the interaction between Bcd molecules could be much faster than the interaction between Bcd and DNA, and therefore, these kinetic assays reflect the rate-limiting step of protein-DNA association. We favor the former explanation because it is consistent with our immunoprecipitation experiments suggesting that the interaction between Bcd molecules is relatively weak and needs to be stabilized by DNA (see above).

DISCUSSION

As a first step to understanding how the Bcd gradient activates the *hb* gene expression in an all-or-none fashion in the embryo, we have initiated the current study to determine the biochemical properties of Bcd. Using both gel retardation and DNase I footprint analyses, we demonstrate that Bcd binds to the *hb* enhancer cooperatively. A sharp threshold of Bcd binding to the intact *hb* enhancer element is obtained in these assays. In addition, we demonstrate that Bcd binds to two sites with a much higher affinity than to single sites. Using two yeast genetic assays, a chemical cross linking assay and an immunoprecipitation assay, we demonstrate that Bcd molecules can interact with each other, an interaction that could facilitate cooperative binding to multiple sites. We also provide experimental evidence suggesting that the interaction between Bcd molecules is relatively weak and needs to be stabilized by DNA. Our experiments suggest that one molecular mechanism of achieving a sharp target gene response to the Bcd gradient is cooperative DNA binding facilitated by protein-protein interaction.

Our in vitro DNA binding results are consistent with previous studies in the embryo. First, our experiments estimate that a less than 4-fold change in Bcd concentration is sufficient for the bound/unbound transition in DNA binding. This estimate is very close to previous estimates of a 2-3 fold change in Bcd concentration needed for the on/off switch of *hb* gene expression in the embryo (Struhl et al., 1989; Driever, 1992). In addition, our in vitro experiments demonstrate that Bcd binds to the intact *hb* enhancer element not only with a high

affinity but also a high degree of cooperativity (Figs 2 and 3). Consistent with these biochemical results, previous in vivo studies have demonstrated that, as more Bcd binding sites are deleted from the *hb* enhancer, the posterior border of the *hb* expression domain is not only shifted toward the anterior but also becomes less sharp (Driever et al., 1989; Struhl et al., 1989). Our experiments suggest that cooperative DNA binding by Bcd is an important mechanism in achieving an on/off switch of *hb* expression in the embryo.

We propose that cooperative DNA binding is facilitated by the interaction between Bcd molecules. First, our experiments demonstrate that the amino-terminal portion of Bcd (residues 1-246) is sufficient for protein-protein interaction (Table 1, and unpublished data). Consistent with these results, our previous studies in the embryo (Driever et al., 1989) suggest that, except transcriptional activation, this amino-terminal portion provides all the essential biological functions expected of a molecular morphogen (see Introduction). Second, our immunoprecipitation assays demonstrate that, unlike the intact Bcd or its amino-terminal domain, the 60 amino acid homeodomain alone does not interact with other Bcd molecules (J. M., unpublished data). Consistent with this result, our preliminary DNA binding experiments show that the homeodomain of Bcd fails to bind DNA cooperatively (X. M. and J. M. unpublished data). In addition, while Wilson et al. (1993) were unable to detect any cooperative DNA binding by the homeodomain of Bcd in a DNA site selection assay, our preliminary experiments detect a possible cooperativity by the intact Bcd in a similar assay (D. Y. and J. M., unpublished data).

Our immunoprecipitation experiments show that the interaction between Bcd molecules is DNA-dependent, suggesting that this interaction is relatively weak and needs to be stabilized by DNA (Fig. 6). These results are consistent with the order of addition experiments suggesting that Bcd molecules may form complexes on DNA rather than in solution (Fig. 7). It is interesting to note that Bcd binds to the *hb* enhancer element more cooperatively in DNase I footprint assays than in the gel retardation assays. Previous experiments by others have suggested that gel electrophoresis can abolish weak protein-protein interactions (Grueneberg et al., 1992; Wagner and Green, 1993). It is therefore possible that the weak interaction between Bcd molecules may be partially disrupted during electrophoresis, leading to an underestimation of the extent of cooperativity. Consistent with this idea is our observation that Bcd can bind cooperatively to two Bcd sites in DNase I footprint assays (Fig. 3) but not in gel retardation assays (see legend to Fig. 1B).

Our experiments show that Bcd can bind cooperatively to A1 and X1 sites that have an altered spacing or orientation (data not shown). We have not systematically determined how far these sites can be separated from each other without abolishing the ability of Bcd to bind to them cooperatively. However, our DNase I footprint results using the intact *hb* enhancer element (Fig. 2) may provide some important clues. The Bcd binding sites in the *hb* enhancer element are separated by distances ranging from 12 bp to 104 bp (center to center). While the other five sites are relatively close to each other, A3 is separated from its nearest neighboring site (A2) by 104 bp (Fig. 1A). Our DNase I footprint results show that Bcd binds to A3 less cooperatively than to the other five sites (Fig. 2), suggesting that the ability of Bcd to bind DNA cooperatively starts to weaken as the distance between two sites approaches 104 bp. This weakened ability of cooperative DNA binding may be a result of the relatively weak interaction between Bcd molecules. Taken together, our experiments suggest that, within a certain distance between its binding sites, Bcd can bind DNA cooperatively with a relatively high degree of flexibility.

Based on the experiments presented in this report, we propose a model for how Bcd binds DNA cooperatively. In this model, the functional units of Bcd for DNA binding are monomers. Upon binding to DNA, Bcd molecules would interact with each other, thus facilitating cooperative DNA binding. In other words, the interaction between Bcd molecules, which facilitates cooperative DNA binding, in turn needs DNA. (Apparently, the interaction between Bcd molecules could also occur on non-specific DNA.) This model is consistent with the following observations. First, two Bcd derivatives of different sizes fail to form heterodimers on a single Bcd site in gel retardation assays (see legend to Fig. 1B for further details), suggesting that a single Bcd binding site is bound by one Bcd molecule. The A-type Bcd binding sites and the putative consensus (see legend to Fig. 1A) do not contain any obvious palindromic sequences or direct repeats, further suggesting that one Bcd site is bound by one Bcd molecule. (The X-type sites, on the other hand, may contain short palindromic sequences (Driever and Nüsslein-Volhard, 1989), but their significance, if any, is currently unclear.) Second, the order of addition and immunoprecipitation experiments suggest that Bcd molecules form protein complexes in a DNA-dependent fashion (Figs 6 and 7). Finally, Bcd can bind cooperatively not only to different numbers of sites (e.g., two or three sites) but also to sites that are separated by different spacing and orientation, suggesting an adaptability of Bcd to form appropriate complexes on DNA. This type of adaptability is not common to stable dimers or heterodimers, which may only bind to two properly aligned half-sites (Schwabe and Rhodes, 1991; Umesono et al., 1991). Although we currently favor the idea that Bcd molecules directly interact with each other and bind DNA cooperatively, our experiments do not rule out the possibility that Bcd may also interact with other regulatory proteins in the embryo. In this regard it is interesting to note that several homeodomain proteins have been suggested to form heterodimers (Passmore et al., 1989; Mendel et al., 1991; Grueneberg et al., 1992; Xue et al., 1993; Chan et al., 1994; Goutte and Johnson, 1994; van Dijk and Murre, 1994; Chang et al., 1995; Johnson et al., 1995).

Results from our second yeast genetic assay (Table 3) demonstrate that the mutant Bcd-VP16 fusion proteins, which are unable to recognize the Bcd sites (Hanes and Brent, 1989), can interact with the DNA-bound wild-type Bcd molecules. We do not know if these mutant Bcd-VP16 fusion proteins can nevertheless interact with DNA in the presence of the wild-type Bcd. If all the Bcd sites in the *hb* enhancer element are occupied by the wild-type Bcd molecules, then the mutant Bcd-VP16 fusion proteins must increase transcription activity by forming super complexes. The formation of such super complexes would bring to DNA additional, and stronger, activator molecules (the mutant Bcd-VP16 proteins), leading to super activation. (In the first yeast genetic assay (Table 2), DNA binding is mediated by LexA rather than Bcd and, therefore, super activation, if any, is irrelevant to this discussion.) Such super activation function has been previously

demonstrated for the mammalian transcription factor Sp1 (Pascal and Tjian, 1991). Like Sp1, Bcd can also form higher molecular mass complexes in chemical cross linking experiments (Fig. 4). In our gel retardation assays, the slow mobility complexes (Complex 3, Fig. 1B) presumably contain more than six Bcd molecules, and they could potentially represent possible super complexes. A super activation by Bcd would be important for its in vivo transcriptional activation so that a relatively weak activator could induce high levels of target gene expression in the embryo. In this regard, it is interesting to note that we have previously demonstrated that hybrid Bcd molecules bearing very strong activating sequences are toxic to the embryo (Driever et al., 1989).

In addition to the *hb* gene, Bcd also activates other zygotic genes in the embryo. For example, Bcd has been suggested to activate the expression of the gap genes *Kruppel* (Hoch et al., 1990) and *knirps* (Rivera-Pomar et al., 1995). The more anteriorly expressed head-specific gap genes *orthodenticle* (Finkelstein and Perrimon, 1990), *buttonhead* (Cohen and Jürgens, 1990; Wimmer et al., 1993), and *empty spiracles* (Dalton et al., 1989) have also been suggested to respond to the morphogenetic gradient of Bcd. In addition, one of the well characterized pair-rule genes, *even-skipped*, has been shown to be activated by Bcd (Small et al., 1991). It remains to be determined whether cooperative DNA binding by Bcd is a means by which the expression of these genes is controlled. It should be pointed out that the experiments reported here are not intended to address another important question: How do different enhancer elements sense the Bcd gradient differently? For example, if the head-specific genes are direct targets of Bcd, why is their expression restricted to smaller domains at the anterior? Although several potential models could be proposed, one possibility that remains to be tested is that the enhancers controlling the expression of these head-specific genes may have a lower affinity to Bcd than the *hb* enhancer element (Driever, 1992).

In addition to cooperative DNA binding, other mechanisms may also play a role in the formation of a sharp posterior border of the *hb* expression domain in the embryo. It is possible that Bcd molecules, once bound to DNA, may activate transcription synergistically, i.e., two molecules, for example, may activate transcription much better (more than additive) than one (Lin et al., 1988; Carey et al., 1990; Driever, 1992). It is also possible that neighboring gap gene products may exert inhibitory effects on *hb* gene expression (Jackle et al., 1986). In addition, cooperative DNA binding by Bcd molecules could be further facilitated in vivo by nucleosomes that are absent in our experiments (Taylor et al., 1991; Vettese-Dadey et al., 1994). Finally, Simpson-Brose et al. (1994) recently suggested that the *hb* gene product, Hb protein, could positively control its own gene expression. However, even in the absence of any functional Hb protein in the embryo, the *hb* expression border, though shifted toward the anterior, remains sharp (Simpson-Brose et al., 1994). This observation strongly suggests that autoregulation by Hb protein, though important, is not the only mechanism operating in the embryo. Although a Hb binding site is found in the *hb* enhancer element (Treisman and Desplan, 1989), it is currently unclear whether the cooperation between Bcd and Hb on *hb* gene expression is due to cooperative DNA binding or synergistic activation (see above), or both. To fully understand the mechanisms by which Bcd acts as a molecular morphogen in controlling the anterior development, these important issues remain to be addressed in the future. Nevertheless, the experiments reported here clearly demonstrate that Bcd can bind DNA cooperatively and that Bcd molecules can interact with each other, thus providing a molecular mechanism for achieving sharp target gene responses to the Bcd gradient. This could be a common mechanism used by other transcriptional activator gradients in controlling gene expression and embryonic pattern formation in animals.

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