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Enhancer Sequences Influence the Role of the Amino-Terminal Domain of Bicoid in Transcription

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Bicoid (Bcd) is a *Drosophila melanogaster* morphogenetic gradient that controls embryonic patterning by activating target gene expression in a concentration-dependent manner. In this study we describe experiments to determine how different enhancers respond to Bcd distinctively, focusing on two natural Bcd-responsive enhancer elements, *hunchback* (*hb*) and *knirps* (*kni*). Our results show that, on the *hb* enhancer element, the amino-terminal domain of Bcd (residues 1 to 91) plays primarily an inhibitory role, whereas on the *kni* enhancer element this same Bcd domain plays a positive role at low protein concentrations. We further demonstrate that while the amino-terminal domain is largely dispensable for cooperative binding to the *hb* enhancer element, it is preferentially required for cooperative binding to the *kni* enhancer element. Alteration of the arrangement of Bcd binding sites in the *kni* enhancer element reduces the role of the amino-terminal domain in cooperative DNA binding but increases the effectiveness of the self-inhibitory function. In addition, elimination of symmetric pairs of Bcd binding sites in the *kni* enhancer element reduces both DNA binding and activation by Bcd. We propose that the amino-terminal domain of Bcd is an enhancer-specific switch that contributes to the protein's ability to activate different target genes in distinct manners.

Bicoid (Bcd) is a *Drosophila melanogaster* morphogenetic protein required for anterior patterning during early embryogenesis (8, 24). Embryos lacking Bcd fail to develop anterior structures, including the head and thorax (13). Bcd protein, which is synthesized from the maternally contributed and anteriorly localized *bcd* mRNA (4), is distributed in early embryos as an anterior-to-posterior gradient (11). This 489-amino-acid protein is a homeodomain-containing transcriptional activator, with its homeodomain located at residues 92 to 151 (4). An essential function of Bcd during development is to activate gap genes in a concentration-dependent manner (8). For example, while Bcd activates the head-specific gap gene *orthodenticle* (*otd*) in the most anterior part of the embryo (14, 15), it activates the expression of *hunchback* (*hb*), a gap gene required for thoracic development (30), in the anterior half of the embryo (10, 28). Another gap target gene of Bcd, *knirps* (*kni*), contains a Bcd-responsive enhancer element that can sense even lower concentrations of Bcd in the embryo (6, 26). How Bcd activates different target genes at discrete concentrations remains poorly understood, and a previously proposed simple affinity threshold model has been challenged by recent findings (see Discussion for details).

Previous studies have shown that Bcd binds DNA in a highly cooperative manner (19). Such cooperativity is likely to play an important role in facilitating the sharp on/off switches of target genes, such as *hb*, in response to the Bcd gradient in embryos. Cooperative DNA binding by Bcd is achieved through interactions between Bcd molecules (19), primarily relying on pro-

tein sequences outside the homeodomain (35), including the amino-terminal domain (residues 1 to 91). In particular, the homeodomain alone, which fails to self associate efficiently in vitro, has little cooperative DNA binding activity (35, 37). Experiments that used an altered-specificity mutant of another homeodomain protein, Ftz(Q50K), further suggest that protein-protein interaction mediated by Bcd sequences outside its homeodomain represents an important mechanism for selecting specific target genes for activation (36, 37).

In addition to protein-protein interaction and cooperative DNA binding, the evolutionarily conserved amino-terminal domain of Bcd also provides a self-inhibitory function (38). In particular, Bcd derivatives lacking the amino-terminal domain exhibit a greatly increased ability to activate a *hb-CAT* reporter gene containing the Bcd-responsive *hb* enhancer element in *Drosophila* S2 cells. A mutant protein, Bcd(A52-56), which has the critical residues 52 to 56 mutated to alanines, exhibits a dominant effect in wild-type embryos, causing a posterior shift of the fatemap and patterning defects (38). The expression of Bcd target genes and other segmentation genes is also significantly altered in embryos containing Bcd(A52-56) (38; unpublished data). These results suggest that the self-inhibitory function provided by the amino-terminal domain of Bcd is essential to embryogenesis.

In this report we demonstrate that, depending on enhancer sequences, the amino-terminal domain of Bcd is preferentially utilized for one of two distinct functions: cooperativity or self inhibition. Specifically, while cooperative DNA binding to the *kni* enhancer element is highly dependent on the amino-terminal domain of Bcd, this domain is largely dispensable for cooperative binding to the *hb* enhancer element. In contrast, while activation from the *hb* enhancer element is highly sensitive to the self-inhibitory function, mutations affecting this function only cause limited effects on the *kni* enhancer ele-

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TABLE 1. Plasmids used in this study

Activator or reporter[a]	Plasmid	Note	Reference or source
Plasmids for in vitro transcription and translation			
Bcd(WT)	pFY441	SP6 promoter	38
Bed(92–489)	pFY7015	SP6 promoter	38
Bed(1–246)	pFY443	SP6 promoter	38
Bcd(92–246)	pFY7009	SP6 promoter	This study
Plasmids for transient transfection			
Bcd(WT)	pFY442	*Drosophila* actin 5C promoter	38
Bcd(92–489)	pFY413	*Drosophila* actin 5C promoter	38
Bcd(A52–56)	pFY436	*Drosophila* actin 5C promoter	38
Bcd(A57–61)	pFY465	*Drosophila* actin 5C promoter	38
Effector plasmids for yeast cells			
Bcd(WT)-VP16	pMA1226	*LEU2*; yeast *ADH1* promoter	20
Bcd(92–152)-VP16	pCZ74	*LEU2*; yeast *ADH1* promoter	This study
Bcd(92–165)-VP16	pCZ1007	*LEU2*; yeast *ADH1* promoter	This study
Bcd(92–205)-VP16	pCZ1008	*LEU2*; yeast *ADH1* promoter	This study
Bcd(92–246)-VP16	pCZ1010	*LEU2*; yeast *ADH1* promoter	This study
Bcd(1–246)-VP16	pCZ71	*LEU2*; yeast *ADH1* promoter	This study
Bcd(12–246)-VP16	pCZ1011	*LEU2*; yeast *ADH1* promoter	This study
Bcd(27–246)-VP16	pCZ1012	*LEU2*; yeast *ADH1* promoter	This study
Bcd(42–246)-VP16	pCZ93	*LEU2*; yeast *ADH1* promoter	This study
Bcd(1–151)-VP16	pCZ32	*LEU2*; yeast *ADH1* promoter	This study
Bcd(1–165)-VP16	pCZ69	*LEU2*; yeast *ADH1* promoter	This study
Bcd(1–205)-VP16	pCZ70	*LEU2*; yeast *ADH1* promoter	This study
Reporter plasmids			
hb-CAT	pCZ3005	*CAT* reporter plasmid	37
kni-CAT	pCZ3006	*CAT* reporter plasmid	37
kni(3R)-CAT	pDF520	*CAT* reporter plasmid	This study
3HH-CAT	pDF521	*CAT* reporter plasmid	This study
3TT-CAT	pDF522	*CAT* reporter plasmid	This study
hb-lacZ	pMA630R	Integrating yeast reporter plasmid	9
kni-lacZ	pTA123	Integrating yeast reporter plasmid	37

[a] WT, wild type.

ment. Our further analysis of *kni* enhancer derivatives suggests that enhancer structure plays a critical role in determining the characteristics in responding to Bcd. We propose that a coordinated execution of the two functions provided by the amino-terminal domain—cooperativity and self inhibition—helps define distinctive responses to the Bcd gradient.

MATERIALS AND METHODS

Plasmid construction. Table 1 lists the plasmids used in this study. The genes encoding truncated and point mutation derivatives of Bcd were generated by a PCR-mediated mutagenesis procedure with pFY441 (38) as the template, which is based on pGEM3 and contains wild-type *bcd* with the hemagglutinin (HA) tag-coding sequence immediately upstream of the unique *Nde*I site. To construct pFY7009, the resulting PCR product was cloned into the *Nde*I-*Xba*I sites of pFY441 to replace the full-length *bcd*. The plasmids pFY7015 and pFY443 were described previously (38). The plasmids expressing Bcd-VP16 derivatives in yeast cells were based on AAH5 (1) with the yeast *Leu2* marker. These deletion derivatives were generated by PCR. Effector plasmids expressing Bcd derivatives in S2 cells were based on pAc5.1/V5-HisC vector (Invitrogen). The *kni* enhancer derivatives *kni(3R)*, *3HH*, and *3TT* were generated by annealing oligonucleotides and filling in by reverse transcriptase. These DNA fragments were cloned into the *Eco*RI-*Not*I sites of pBluescript KS(−) vector (Stratagene) to generate pDF501, pDF510, and pDF511. The oligonucleotides were as follows: 5′-AAT TCGTACTGGCTTAGGCGATTTCGTTACGCGATTAGGGGATCAGCTTAC CAGCTTAGCAGATTATCCTAGC and 5′-GGCCGCTAGGATAATCTGCTA AGCTGGTAAGCTGATCCCCTAATCGCGTAACGAAATCGCCTAAGCCAG TACG for *kni(3R)*, 5′-CCGGAATTCGTAATCCAGGGATTACGCCATAGAC AACCGGTGGACAACGTAATCCAGGGATTACGGATCCAAGTGCGC

and 5′-TTTTCCTTTTGCGGCCGCGTAATCCGCGGATTACGAACGCT ATGCGCACTTGGATCCGTAATCC for *3HH*, and 5′-CCGGAATTCGGA TTACGTTACCTAATCCCCCCATAGACAACCGGTGGACAACCGGAT TACGTTATGTAATCCCGGATCCAAG and 5′-TTTTCCTTTTGCGGCC GCGGATTAGCCTACGTAATCCCGAACGCTATGCGCACTTGGATCC GGGATTACA for *3TT*. To construct pDF520, pDF521, and pDF522, the *Not*I (Klenow filled-in)-*Sal*I fragments from pDF501, pDF510, and pDF511 were inserted into the *Xho*I (Klenow filled-in)-*Sal*I sites of pCZ3005 (37) to replace the 250-bp natural *hb* enhancer.

Gel shift assays. To generate the radioactively labeled probes for gel shift experiments, the DNA fragments containing the natural or artificial enhancers were isolated from the respective plasmids and were filled in with Klenow in the presence of $[\alpha\text{-}^{32}P]dCTP$. In our experiments the *kni* probe was isolated as an *Xba*I-*Sac*I fragment from pCZ72, and the *hb* probe was isolated as an *Xho*I-*Xba*I fragment from pMAX1 (37). The *kni* enhancer derivatives *kni(3R)*, *3HH*, and *3TT* were isolated as *Eco*RI-*Not*I fragments from pDF501, pDF510, and pDF511, respectively. Probes for single pairs of head-to-head (HH) and tail-to-tail (TT) sites were isolated as *Not*I-*Bam*HI fragments from pDF510 and pDF511, respectively. The probes were diluted to a final concentration of 1.6×10^{-11} M. The experimental procedures and conditions for gel shift assays were described previously (37). Wild-type Bcd and its derivatives used in this assay were expressed in vitro by using TnT quick coupled transcription/translation systems (Promega). The active protein concentrations were estimated by using high concentrations (4×10^{-8} M) of a ^{32}P-labeled consensus Bcd binding site, A1 (7). The same amounts of active Bcd proteins were used in gel shift experiments. The Molecular Dynamics PhosphorImager ImageQuant program was used for quantitative analysis.

Yeast strain and β-galactosidase liquid assays. The yeast strain used in this study is CY26 containing the integrated *hb-lacZ* or *kni-lacZ* reporter gene (37). The Bcd-VP16 effector plasmids were introduced into yeast cells by using the
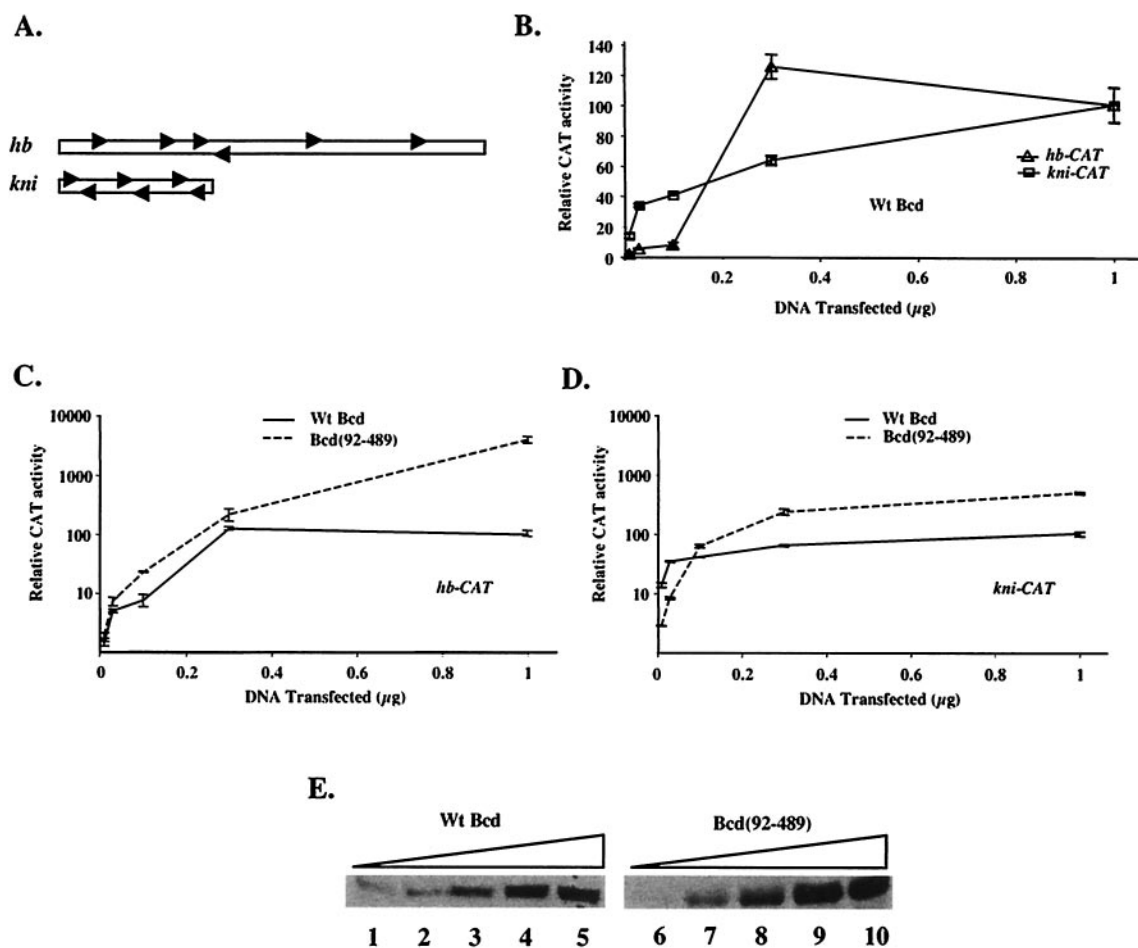
FIG. 1. The amino-terminal domain of Bcd plays different roles on *hb* and *kni* enhancer elements. (A) Schematic diagrams of the 250-bp *hb* enhancer element and the 64-bp *kni* enhancer element. The Bcd binding sites are represented by arrows. (B) CAT assay results of S2 cells transfected with *hb-CAT* and *kni-CAT* reporter plasmids (1 μg) and increasing amounts of an effector plasmid expressing Bcd. Activities obtained with 1 μg of transfected effector plasmid on each reporter were arbitrarily set to 100 (fold activation was 72 and 74 for *hb-CAT* and *kni-CAT*, respectively). (C and D) CAT assay activities (in logarithmic scale) for wild-type Bcd and Bcd(92-489) on *hb-CAT* (C) and *kni-CAT* (D) reporters at different concentrations. The activities of wild-type Bcd at 1 μg of transfected effector DNA on each reporters were set to 100. (E) Representative Western blot results detecting Bcd proteins in transfected cells. For the experiments shown in this figure, the amounts of the transfected effector plasmids were 0.01, 0.03, 0.1, 0.3, and 1 μg. Wt, wild type.

lithium acetate method, and three independent transformants were assayed for β-galactosidase activities as described previously (37).

Transient transfection experiments. *Drosophila* S2 cells (Invitrogen) were grown at 25°C in Schneider's *Drosophila* medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO). The cells were seeded in 60-mm-diameter tissue culture plates at 4×10^6/plate 24 h prior to transfection. Transfection was performed by the calcium phosphate transfection system following the protocol from GIBCO. Each plate was transfected with 1 μg of reporter plasmid, 1 μg of *Copia-lacZ* internal control plasmid, and the indicated amount of effector plasmids. Salmon sperm carrier DNA (GIBCO) was used to bring the total amount of DNA to 10 μg for each transfection. The cells were harvested 48 h later, and whole-cell lysates were prepared by using the lysis buffer (50 mM HEPES [pH 7.5], 300 mM NaCl, 0.5% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) following the freeze-thaw procedure (3). The transfection efficiency was determined by monitoring the β-galactosidase activity, and the amounts of lysates used in chloramphenicol acetyltransferase (CAT) and Western blotting assays were normalized accordingly. CAT assays were performed as described previously by using three independently transfected samples for each experiment (3). For Western blotting, cell lysates were separated on 10% polyacrylamide sodium dodecyl sulfate gels and were transferred to nitrocellulose membrane (Bio-Rad), which was blotted with anti-HA monoclonal antibody (HA-11; 1:500 final dilution; Babco).

RESULTS

The amino-terminal domain of Bcd plays different roles on two enhancer elements. Both *hb* and *kni* are direct target genes that respond to the Bcd gradient during early embryonic development (25). The Bcd-responsive enhancer elements from these genes are sufficient to sense different Bcd concentrations in embryos (6, 10, 26, 28). Although the 250-bp *hb* enhancer element and the 64-bp *kni* enhancer element (referred to as the *hb* and *kni* enhancers hereafter) each contain six Bcd binding sites, these sites are arranged differently (Fig. 1A; also see below). We conducted a titration experiment with transiently transfected *Drosophila* S2 cells to determine whether such differential responses to Bcd can be recapitulated in vitro. In our experiments, the reporter genes *hb-CAT* and *kni-CAT* under the control of the respective *hb* and *kni* enhancers were cotransfected with increasing amounts of an effector plasmid expressing the wild-type Bcd protein. Our CAT assay experi-
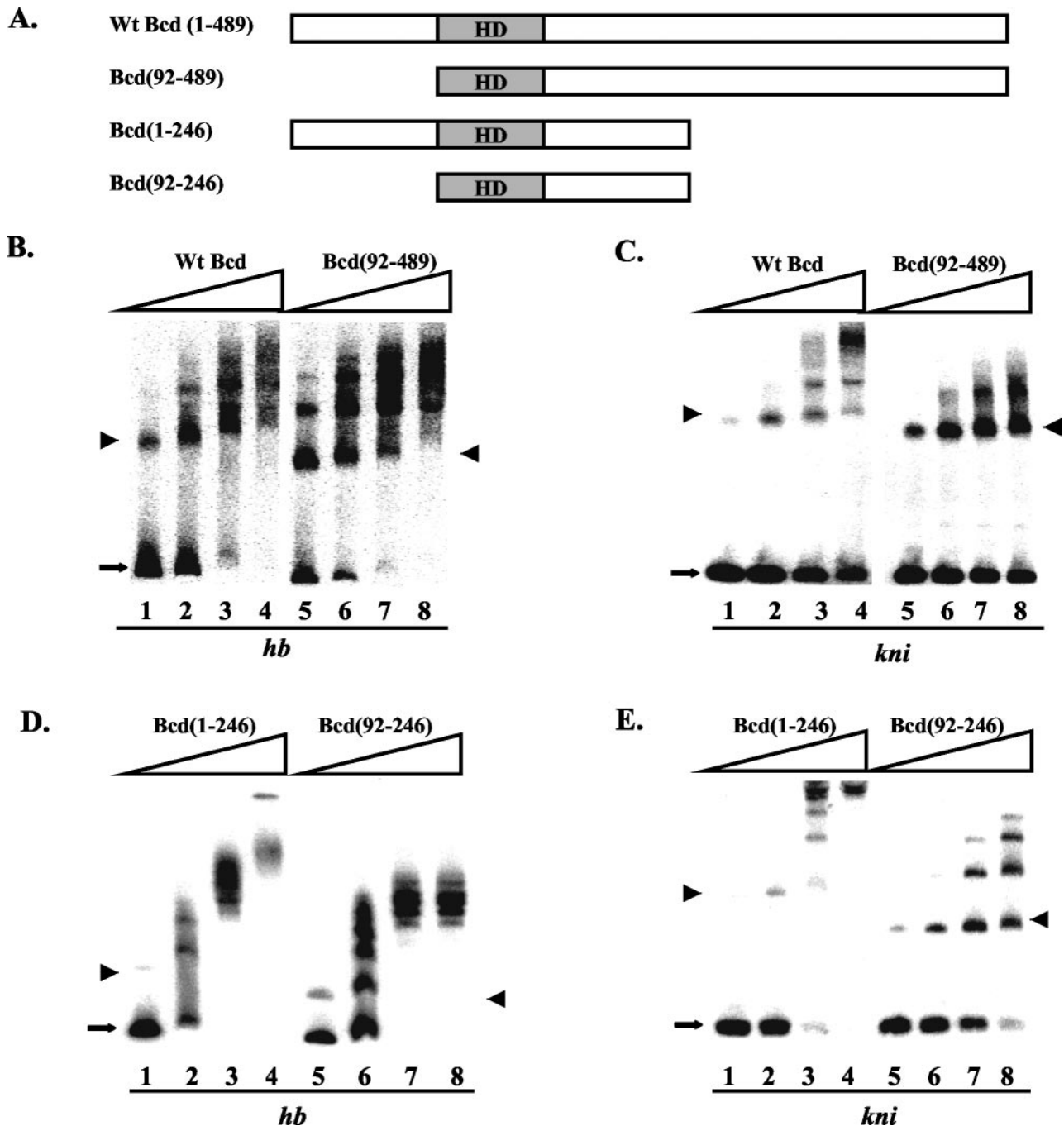
FIG. 2. The amino-terminal domain of Bcd is preferentially required for cooperative DNA binding to the *kni* enhancer element. (A) Schematic diagrams showing the Bcd proteins used in gel shift assays. HD, homeodomain. (B to E) Gel shift assay results on *hb* (B and D) and *kni* (C and E) probes with the indicated proteins. For these experiments, the DNA probe concentration was 1.6×10^{-11} M. The proteins were normalized according to their specific activity measured on a single consensus Bcd binding site. The estimated active protein concentrations were 8×10^{-11} M (lanes 1 and 5), 1.6×10^{-10} M (lanes 2 and 6), 3.2×10^{-10} M (lanes 3 and 7), and 6.4×10^{-10} M (lanes 4 and 8). The monomeric complexes are marked with arrowheads, and the free probes are marked with arrows. The data shown in panels B to E were used in a Scatchard analysis to estimate Hill coefficient values. The estimated values on *hb* and *kni* elements were, respectively, 1.7 and 2.4 for wild-type Bcd, 1.3 and 1.0 for Bcd(92-489), 3.1 and 3.7 for Bcd(1-246), and 2.3 and 1.8 for Bcd(92-246). Although poor resolution of protein-DNA complexes in some gels (due to protein sizes) offered only limited accuracy, these estimated Hill coefficient values show that the removal of the amino-terminal domain preferentially reduces cooperative binding to the *kni* enhancer element (over twofold reduction in Hill coefficient) compared to that with the *hb* enhancer element (less than 1.4-fold reduction). See Fig. 6C and D and the Fig. 6 legend for further details. Wt, wild type.

Activator		hb-lacZ	kni-lacZ
1. Bcd(92-151)-VP16		<1	<1
2. Bcd(92-165)-VP16		1	2
3. Bcd(92-205)-VP16		11	<1
4. Bcd(92-246)-VP16		41	1
5. Bcd(1-246)-VP16		100	100
6. Bcd(12-246)-VP16		114	81
7. Bcd(27-246)-VP16		117	85
8. Bcd(42-246)-VP16		46	19
9. Bcd(1-151)-VP16		6	26
10. Bcd(1-165)-VP16		30	47
11. Bcd(1-205)-VP16		35	27
12. Bcd(1-489)-VP16		124	146
13. None		<1	<1

FIG. 3. Different domains of Bcd are required for activating transcription from *hb* and *kni* enhancers in yeast cells. Shown are relative activities of Bcd-VP16 fusion proteins on integrated *hb-lacZ* or *kni-lacZ* reporter genes in yeast cells. Only Bcd sequences are shown in schematic diagrams (not to scale), with the shaded region representing its homeodomain. The activities of Bcd(1-246)-VP16 were arbitrarily set to 100 on each reporter gene, with actual β-galactosidase activities of 576 and 91 on *hb-lacZ* and *kni-lacZ*, respectively.

ments (Fig. 1B) demonstrate that the *kni-CAT* reporter responded to Bcd at lower concentrations than the *hb-CAT* reporter did, suggesting that transfection assays can reveal enhancer-specific properties of Bcd.

To analyze the roles of the amino-terminal domain of Bcd on the *hb* and *kni* enhancers, we compared dose-response profiles for wild-type Bcd and a Bcd derivative lacking this domain [Bcd(92-489)] on both *hb-CAT* and *kni-CAT* reporter genes (Fig. 1C and D; see panel E for protein levels). Our results show that Bcd(92-489) was more active on *hb-CAT* than wild-type Bcd at all concentrations (Fig. 1C), confirming that the amino-terminal domain predominantly plays an inhibitory role on this reporter (38). In contrast, Bcd(92-489) had a lower activity on *kni-CAT* than wild-type Bcd at low concentrations (Fig. 1D), suggesting that the amino-terminal domain of Bcd plays a positive role on *kni-CAT* under these conditions. At high concentrations, Bcd(92-489) had a higher activity than wild-type Bcd on *kni-CAT*, but such an activity difference was much less significant (<4-fold) than that on *hb-CAT* (>40-fold). Together, these results demonstrate that the amino-terminal domain of Bcd plays distinctive roles on *hb* and *kni* enhancers.

The amino-terminal domain of Bcd is required for cooperative binding to the *kni* but not *hb* enhancer. The positive effect of the amino-terminal domain of Bcd on *kni-CAT* expression was observed predominantly at low Bcd concentrations (Fig. 1D), suggesting that this domain may play an important role in facilitating the protein to bind the *kni* enhancer. To test this possibility, we conducted gel shift assays with both full-length Bcd and Bcd(92-489) on *hb* and *kni* enhancers (Fig. 2B for *hb* and C for *kni*). Our results show that wild-type Bcd can bind to both enhancer elements cooperatively (Fig. 2B and C, lanes 1 to 4). In particular, as the protein concentration increased, the monomeric complex containing a single Bcd molecule (marked with an arrowhead) was depleted to form oligomeric com-

plexes. Interestingly, contrary to the simple affinity threshold model (see Discussion for further details), the *kni* enhancer did not exhibit a higher affinity for Bcd than the *hb* enhancer did (Fig. 2B and C, lanes 1 to 4).

The N-terminally truncated derivative Bcd(92-489) exhibited contrasting behaviors on *hb* and *kni* enhancers in gel shift assays (Fig. 2B and C, lanes 5 to 8). This derivative bound cooperatively to the *hb* enhancer in a manner similar to that of the full-length protein (Fig. 2B, lanes 1 to 8), suggesting that the amino-terminal domain is largely dispensable for cooperative binding to the *hb* enhancer. However, Bcd(92-489) failed to bind to the *kni* enhancer in a highly cooperative manner (Fig. 2C, lanes 5 to 8). In particular, the monomeric complex (arrowhead) represented the major species of protein-DNA complexes at all protein concentrations. To further analyze the role of the amino-terminal domain of Bcd in cooperative DNA binding, we analyzed in gel shift assays two C-terminally truncated Bcd derivatives, Bcd(1-246) and Bcd(92-246), either containing or lacking the amino-terminal domain (Fig. 2A). Our gel shift results (Fig. 2D for *hb*, E for *kni*) with these proteins [lanes 1 to 4, Bcd(1-246); lanes 5 to 8, Bcd(92-246)] further confirm a preferential requirement of the amino-terminal domain for cooperative DNA binding to the *kni* enhancer. Hill coefficient estimates based on the data shown in Fig. 2 further suggest that removal of the amino-terminal domain of Bcd preferentially reduces cooperative binding to the *kni* enhancer (see the legend of Fig. 2 for details).

We also analyzed systematic Bcd deletion derivatives in yeast cells (Fig. 3). In this experiment, Bcd derivatives fused to the strong activation domain from VP16 (27) were compared for their abilities to activate integrated *hb-lacZ* and *kni-lacZ* reporter genes in yeast cells. Both full-length Bcd-VP16 and Bcd(1-246)-VP16 fusion proteins activated transcription from the *hb-lacZ* and *kni-lacZ* reporter genes effectively (lines 5 and 12). Therefore, all deletions were derived from the truncated

protein Bcd(1-246)-VP16, with its activities on each reporter set to 100. Figure 3 shows that a derivative lacking the amino-terminal domain, Bcd(92-246)-VP16, failed to activate transcription from the *kni-lacZ* reporter while remaining active on *hb-lacZ* (line 4). In contrast, Bcd(1-151)-VP16, which lacks the Bcd sequence on the C-terminal side of its homeodomain, had a more significant reduction in activity on *hb-lacZ* than on *kni-lacZ* (line 9). The data shown in Fig. 3 further suggest that protein sequences immediately flanking the homeodomain play positive roles on *kni-lacZ* (compare lines 4 and 8) and *hb-lacZ* (compare lines 9 and 10; also compare lines 2 through 4). Interestingly, Bcd(92-151)-VP16, which contains only the homeodomain of Bcd fused to VP16, failed to efficiently activate from both reporters (line 1). Since the self-inhibitory function conferred by the amino-terminal domain of Bcd is not operative in yeast cells (38 and unpublished data) and, moreover, since all the derivatives contain the strong activation domain VP16, these results are consistent with the suggestion that different Bcd sequences outside its homeodomain play important but differential roles in cooperative binding to *hb* and *kni* enhancers.

The self-inhibitory function of Bcd is implemented differently on *hb* and *kni* enhancers. The experiments described thus far relied on either full-length or truncated Bcd derivatives. Our recent systematic analysis of the self-inhibitory function has generated point mutations within the amino-terminal domain that either disrupt or strengthen the self-inhibitory function (38 and unpublished data). In particular, Bcd(A52-56), which has residues 52 to 56 of Bcd changed to alanines, disrupts the self-inhibitory function, and results in an increased ability to activate the *hb-CAT* reporter (38). In contrast, Bcd(A57-61), which contains the neighboring five residues converted to alanines, further strengthens the self-inhibitory function, resulting in a virtually inactive protein on the *hb-CAT* reporter.

To further determine whether the self-inhibitory function provided by the amino-terminal domain of Bcd is differentially implemented on different enhancers, we took advantage of these point mutation derivatives. We performed transient transfection assays by using high protein concentrations to specifically compare the effectiveness of the self-inhibitory function on the *hb-CAT* and *kni-CAT* reporters. Figure 4 shows that, while Bcd(A52-56) was 25 times more active than wild-type Bcd on *hb-CAT*, the difference was only 6.25-fold on *kni-CAT* (lane 3). In addition, while Bcd(A57-61) was virtually inactive on *hb-CAT*, its activity on *kni-CAT* was reduced only by 40% (lane 4). As shown in Fig. 1C and D, at high protein concentrations the activity difference between full-length Bcd and the N-terminally truncated derivative Bcd(92-489) was also much greater on *hb-CAT* (>40-fold) than on *kni-CAT* (<4-fold) (Fig. 4, lane 2). Together these results demonstrate that the self-inhibitory function is more robust on *hb-CAT* than on *kni-CAT*. Interestingly, Bcd(92-489) was more active on *hb-CAT* than Bcd(A52-56), but the opposite was true on *kni-CAT*; this difference is consistent with the finding that the amino-terminal domain is preferentially required for cooperative binding to the *kni* enhancer (Fig. 2).

Bcd binding site arrangements influence their dependence on the amino-terminal domain for cooperative DNA binding. Previous binding site selection experiments have isolated from
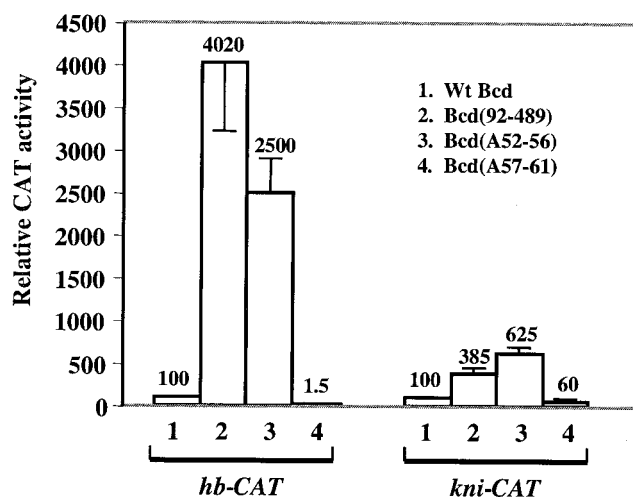

FIG. 4. The self-inhibitory function of Bcd is differentially implemented on *hb* and *kni* enhancers. (A) CAT assay results from S2 cells transfected with 1 μg of *hb-CAT* or *kni-CAT* reporter plasmid and 1 μg of effector plasmid expressing wild-type Bcd (lane 1), Bcd(92-489) (lane 2), Bcd(A52-56) (lane 3), or Bcd(A57-61) (lane 4). The activities of wild-type Bcd on each reporter were set to 100. The data for the *hb-CAT* reporter, except for that of Bcd(A57-61), are from Zhao et al. (38). Wt, wild type.

random DNA sequences symmetric head-to-head (HH) and tail-to-tail (TT) sites, suggesting that Bcd can preferentially recognize symmetric pairs of DNA sites that are separated by defined, short spacing (34). An examination of the *hb* and *kni* enhancers (Fig. 1A) reveals that, while all the Bcd binding sites in the 64-bp *kni* enhancer are arranged symmetrically similar to the selected patterns, those in the 250-bp *hb* enhancer are dispersed with only one centrally located head-to-head pair. Both enhancers have six Bcd binding sites. To determine whether the arrangements of Bcd binding sites can influence the dependence on the amino-terminal domain of Bcd for cooperative DNA binding, we generated three enhancer derivatives (Fig. 5A). In *kni(3R)*, the orientations of three of the Bcd binding sites were reversed, eliminating all symmetric pairs of sites while maintaining the same number and spacing of the sites. Two other enhancers, *3HH* and *3TT*, contain three closely spaced head-to-head and tail-to-tail pairs, respectively, that are separated by long spacing (25 bp).

Figure 5 shows our gel shift results on these three enhancer derivatives (and wild-type *kni* enhancer as a control), with full-length Bcd and Bcd(92-489). These results reveal the following findings. First, both full-length Bcd (lanes 1 to 4) and Bcd(92-489) (lanes 5 to 8) bound cooperatively to enhancer derivatives *3HH* (Fig. 5C) and *3TT* (Fig. 5D), suggesting that increased spacing between the symmetric pairs of Bcd binding sites reduces the dependence on the amino-terminal domain of Bcd for cooperative DNA binding (also see the legend to Fig. 5). Second, wild-type Bcd could no longer bind cooperatively to *kni(3R)* (Fig. 5E, lanes 1 to 4). Removing the amino-terminal domain did not further reduce DNA binding, suggesting that the amino-terminal domain plays little role in binding to *kni(3R)* (Fig. 5E, lanes 5 to 8). Together these results show that the role of the amino-terminal domain in cooperative binding to the *kni* enhancer is determined by the special arrangements
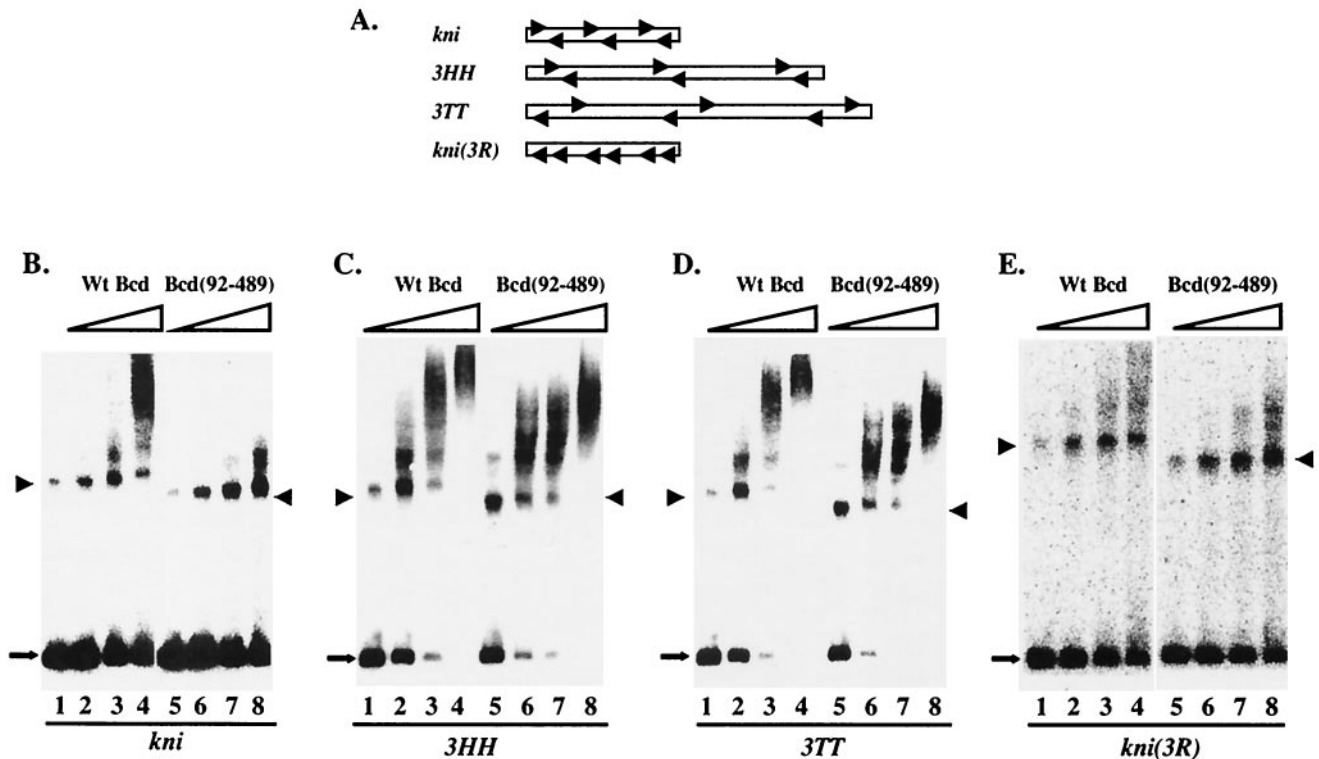
FIG. 5. Both spacing and orientation of Bcd binding sites affect the role of the amino-terminal domain in cooperative DNA binding. (A) Schematic diagrams of the enhancer derivatives. (B to E) Gel shift assay results on *kni* (B), *3HH* (C), *3TT* (D), and *kni(3R)* (E) with either wild-type Bcd or Bcd(92-489). The estimated active protein concentrations were 8×10^{-11} M (lanes 1 and 5), 1.6×10^{-10} M (lanes 2 and 6), 3.2×10^{-10} M (lanes 3 and 7), and 6.4×10^{-10} M (lanes 4 and 8). The DNA probe concentration was 1.5×10^{-11} M. The monomeric complexes are marked with arrowheads. Estimated Hill coefficient values on *3HH* and *3TT* were, respectively, 3.4 and 3.2 for wild-type Bcd and 2.7 and 1.7 for Bcd(92-489). See the legend to Fig. 2 for further details. Wt, wild type.

of the Bcd binding sites in this enhancer. They also suggest that, when the Bcd binding sites are tightly packed, such as in the *kni* enhancer, cooperative DNA binding by wild-type Bcd requires relatively rigid arrangements of these sites. This is in contrast to the Bcd binding sites that are separated by long spacing, where there is no such rigid requirement of orientation or spacing (34).

On enhancers that contain multiple Bcd binding sites, the Bcd-DNA complexes were poorly resolved in gel shift assays (Fig. 2 and 5). In contrast, the monomeric and dimeric Bcd complexes could be readily resolved on DNA probes containing only two Bcd binding sites (Fig. 6). This permitted a more accurate and quantitative analysis of the role of the amino-terminal domain in cooperative DNA binding. Specifically, we determined Hill coefficient values for full-length Bcd and Bcd(92-489) on single pairs of closely spaced HH (Fig. 6A and C) and TT (Fig. 6B and D) binding sites. Wild-type Bcd bound to both HH and TT pairs cooperatively, with an estimated Hill coefficient of 1.88 ± 0.28 and 1.48 ± 0.25, respectively. In contrast, Bcd(92-489) had a significantly reduced but measurable cooperativity on the HH pair, with an estimated Hill coefficient of 1.40 ± 0.30. This same Bcd derivative became completely noncooperative on the TT pair, with an estimated Hill coefficient of 0.66 ± 0.10, which is similar to the value (0.8) on a single Bcd binding site. These results further demonstrate

that the amino-terminal domain plays an important role on closely spaced symmetric pairs of Bcd binding sites, particularly the TT pair.

Bcd binding site arrangements influence the effectiveness of the self-inhibitory function of Bcd. Our analysis of Bcd activities on *hb* and *kni* enhancers suggests that the amino-terminal domain is preferentially used for self inhibition and cooperativity, respectively. This further suggests that cooperative DNA binding mediated by the amino-terminal domain may preclude (or hinder) this domain from being utilized effectively for self inhibition. To further test this possibility, we compared the effectiveness of the self-inhibitory function on the artificial enhancers described above. Since both *3TT* and *3HH* had a reduced dependence on the amino-terminal domain for cooperative DNA binding, a prediction of the preclusion model is that the self-inhibitory function would become more robust on these enhancers than on the *kni* enhancer. For these experiments, we analyzed the activities of wild-type Bcd, Bcd(92-489), Bcd(A52-56), and Bcd(A57-61) on these enhancers at high protein concentrations to specifically compare the effectiveness of the self-inhibitory function.

The results shown in Fig. 7 demonstrate that both *3HH-CAT* and *3TT-CAT* reporters became more sensitive to the self-inhibitory function of Bcd. In particular, Bcd(A57-61) had much lower activities on both *3TT-CAT* and *3HH-CAT* than on
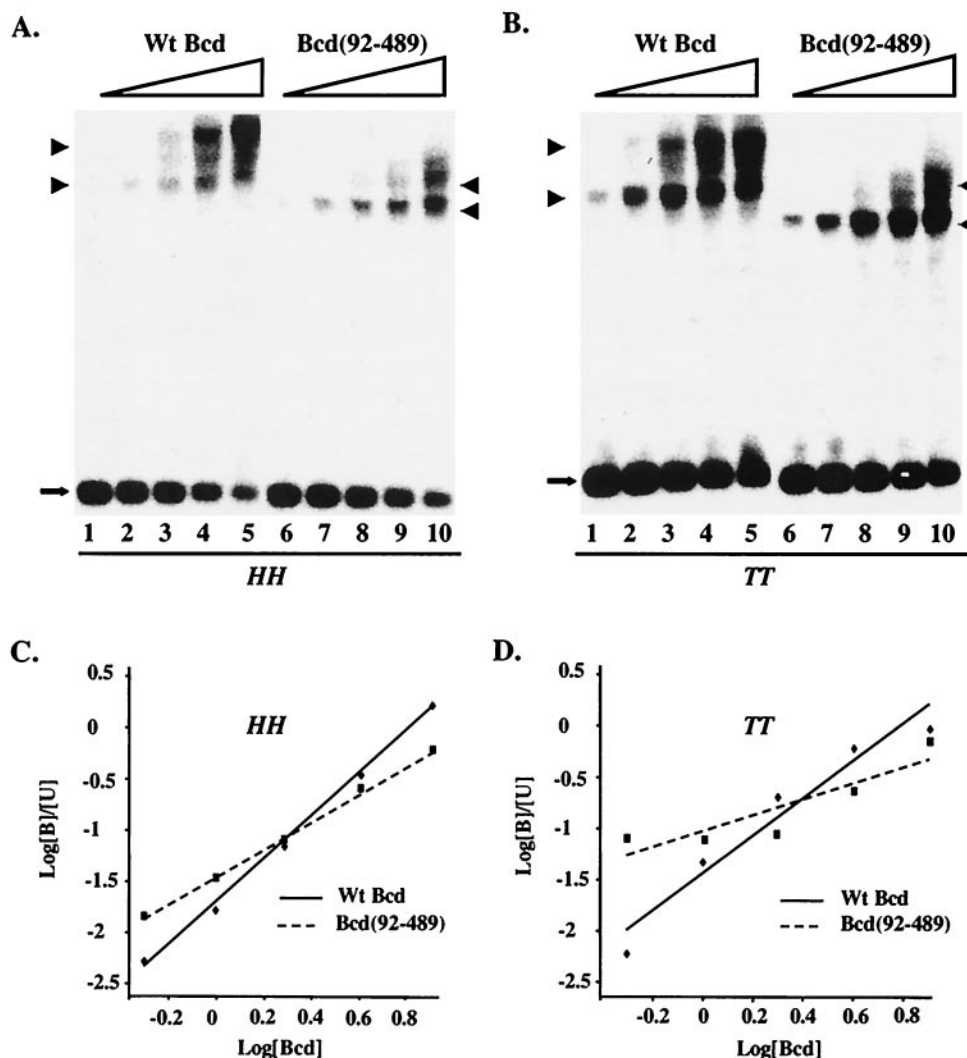
FIG. 6. The amino-terminal domain of Bcd plays an important role in cooperative binding to symmetric pairs of Bcd binding sites. (A and B) Gel shift experiments on single pairs of closely spaced HH (A) and TT (B) sites with wild-type Bcd (lanes 1 to 5) and Bcd(92-489) (lanes 6 to 10). The monomeric and dimeric complexes are marked with arrowheads, and the free probes are marked with arrows. (C and D) Hill coefficient measurements. The Scatchard plots shown here were based on the data shown in panels A and B. Hill coefficient values given in the text were averages of three independent gel shift assays. The estimated active proteins were 0.3×10^{-10}, 0.6×10^{-10}, 1.2×10^{-10}, 2.4×10^{-10}, and 4.8×10^{-10} M, expressed in arbitrary units of 0.5, 1, 2, 4, and 8, respectively. [B] and [U] represent bound and unbound probe concentrations as described previously (34). Wt, wild type.

kni-CAT (compare lane 4 of Fig. 7 with Fig. 4). In addition, both Bcd(A52-56) and Bcd(92-489) exhibited higher activities on *3TT-CAT* and *3HH-CAT* than on the *kni-CAT* reporter (Fig. 7, lanes 2 and 3). Our results also reveal a difference between *3HH-CAT* and *3TT-CAT* in their responses to the A52-56 mutation and removal of the entire amino-terminal domain (Fig. 7, lanes 2 and 3). This difference is consistent with the finding that cooperative binding to the TT pair is more dependent on the amino-terminal domain than to the HH pair (Fig. 6). Finally, although the responses to the mutations affecting the self-inhibitory function on the *kni(3R)-CAT* reporter were not dramatically altered compared to those of *kni-CAT*, all activities were significantly reduced on *kni(3R)-CAT* (27%) (see also reference 6). These results are consistent with the finding that Bcd fails to bind cooperatively to *kni(3R)*

(Fig. 5), demonstrating that reversion of the orientations of Bcd binding sites in the *kni* enhancer alters its characteristics in responding to Bcd.

DISCUSSION

The experiments described in this report reveal that the amino-terminal domain of Bcd executes its cooperative DNA binding and self-inhibitory functions in a coordinated manner. On the *hb* enhancer this domain plays little role in cooperative DNA binding (Fig. 2B and D) but exhibits a potent self-inhibitory function (Fig. 4). In contrast, this domain is preferentially required for cooperative binding to the *kni* enhancer (Fig. 2C and E) but has a muted self-inhibitory function (Fig. 4). Our experiments further suggest that differential utilization of these
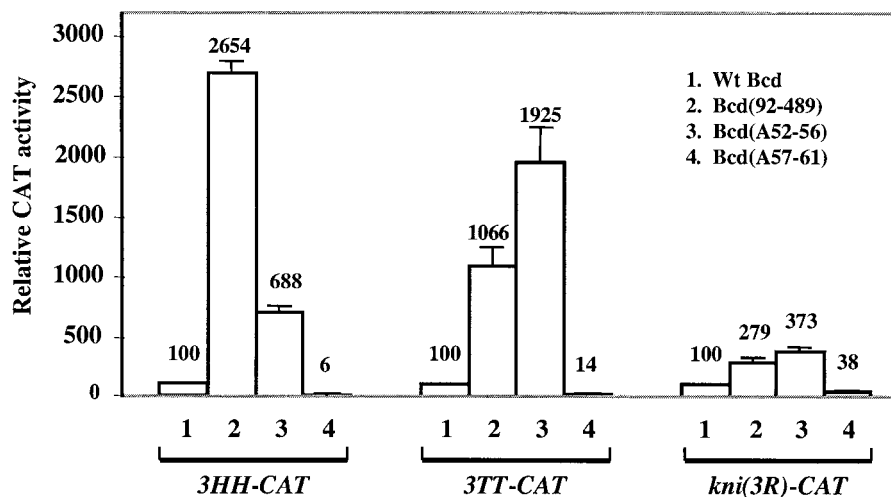
FIG. 7. Bcd binding site arrangements influence the effectiveness of the self-inhibitory function. Shown are CAT assay results on *3TT-CAT*, *3HH-CAT*, and *kni(3R)-CAT* by using Bcd derivatives at high concentrations (1 μg of transfected effector plasmid). The Bcd proteins used in these experiments were wild-type Bcd (lane 1), Bcd(92-489) (lane 2), Bcd(A52-56) (lane 3), and Bcd(A57-61) (lane 4).

two functions is reflective of the structural differences between the *hb* and *kni* enhancers. Although both enhancers have a comparable number of Bcd binding sites, they are arranged differently (Fig. 1A). The Bcd binding sites in the *kni* enhancer form tightly spaced symmetric pairs, whereas those in the *hb* enhancer are dispersed and predominantly arranged in tandem with only one closely spaced symmetric pair. Alternations of the arrangement of the Bcd binding sites in the *kni* enhancer dramatically changed the role of the amino-terminal domain in transcription control (Fig. 5C and D and 7). In addition, elimination of symmetric arrangements of Bcd sites significantly reduced both DNA binding (Fig. 5E) and activation by Bcd. These results demonstrate that enhancers are not merely docking sites for transcription factors; rather, their structure can dramatically influence the behaviors of activators.

Both cooperative DNA binding and self inhibition involve protein-protein interactions (19, 35, 38), suggesting that the amino-terminal domain of Bcd is engaged in distinct protein-protein interaction events on different enhancers. Our results suggest that the engagement of the amino-terminal domain in cooperative DNA binding on the *kni* enhancer likely hinders its availability for self inhibition. Tight spacing between the Bcd binding sites may also reduce the effectiveness of the self-inhibitory function. We presently do not know precisely how the amino-terminal domain of Bcd facilitates cooperative DNA binding. While this domain is strictly required for a single pair of closely spaced TT sites, its role on a single pair of closely spaced HH sites, though important, is somewhat less critical (Fig. 6). It is possible that the amino-terminal domains of two Bcd molecules are engaged in a direct homodimeric interaction. It is also possible that this domain of one Bcd molecule interacts with a different domain of another Bcd molecule on DNA. Our previous protein-protein interaction experiments suggest that the amino-terminal domain alone does not self associate efficiently without the homeodomain (35). In this context it is relevant that cooperative DNA binding can be achieved on the *hb* enhancer even without the

amino-terminal domain. Bcd contains multiple self-association domains (35) which play differential roles on distinct enhancers (Fig. 3). Regardless, our findings suggest that Bcd can accommodate and coordinate multiple interaction events that involve different parts of the protein, particularly its amino-terminal domain. Interestingly, the amino-terminal domain of Bcd also contains a motif that can interact with the translation initiation factor eIF4E and participates in translation repression of *caudal* (*cad*) mRNA (23), further underscoring the importance of this evolutionarily conserved domain.

One of the fundamental biological questions concerning the action of Bcd is how different target genes respond to distinct thresholds of the Bcd gradient in embryos. It was previously proposed that the affinity of Bcd binding sites within an enhancer dictates the concentration of Bcd required for activation in embryos (12, 14). However, this simple affinity threshold model has been challenged recently (36). In particular, the assumption of this model—the consensus A-type sites have higher Bcd affinity than the nonconsensus X-type sites—has been proven incorrect. Biochemical studies have shown that Bcd binds to a consensus A1 site and a nonconsensus X1 site with comparable affinities (7, 19). In addition, the *kni* enhancer, despite its ability to respond to lower Bcd concentrations, does not exhibit any higher Bcd affinity than the *hb* enhancer (Fig. 2). A more recent study also suggests that the concentration of Bcd in embryos, though important, is unlikely to be the only determinant for defining the target gene responses to the Bcd gradient (17). Our characterizations of the self-inhibitory function in embryos suggest that the activation potential of Bcd may play an important role in controlling target gene expression. In particular, Bcd(A52-56), which has a stronger activation potential, can exert a dominant effect in wild-type embryos, causing a posterior shift of the target genes (38). These results suggest that a stronger Bcd can activate transcription at a lower concentration (or with fewer DNA-bound molecules) in embryos, a concept that has been proposed previously for various activators (2, 5, 18, 20, 29). We

propose that the muted self-inhibitory function of Bcd on the *kni* enhancer contributes to the protein's ability to activate transcription at low concentrations.

Many homeodomain proteins have been shown to cooperate specifically with their DNA binding partners (16, 21, 22, 31–33). Although no such partner proteins have been identified to specifically help Bcd to select its target genes, this possibility cannot be formally ruled out at this time. If such proteins do exist, they could potentially play important roles in facilitating Bcd to act distinctively on different enhancers. In this context, it is interesting that the Bcd-responsive *kni* enhancer element used in this study contains binding sites for the Caudal (Cad) protein, although shorter *kni* enhancer fragments without these Cad binding sites can similarly respond to low concentrations of Bcd in embryos (26). The fact that the experiments described in this report use only Bcd-responsive enhancer elements in *Drosophila* cells indicates that Bcd may have an intrinsic ability to activate different target genes distinctively. These studies demonstrate that even relatively simple enhancers can have profound effects in influencing activator behaviors. Understanding precisely how complex enhancers orchestrate in vivo the individual actions of different transcription factors represents a rewarding challenge for molecular biologists.

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