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The activity of the *Drosophila* morphogenetic protein Bicoid is inhibited by a domain located outside its homeodomain

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SUMMARY

The *Drosophila* morphogenetic protein Bicoid (Bcd) is a homeodomain-containing activator that stimulates the expression of target genes during early embryonic development. We demonstrate that a small domain of Bcd located immediately N-terminally of the homeodomain represses its own activity in *Drosophila* cells. This domain, referred to as a self-inhibitory domain, works as an independent module that does not rely on any other sequences of Bcd and can repress the activity of heterologous activators. We further show that this domain of Bcd does not affect its properties of DNA binding or subcellular distribution. A Bcd derivative with point mutations in the self-inhibitory domain severely affects pattern formation and target gene expression in *Drosophila* embryos. We also provide evidence to suggest that the action of the self-inhibitory domain requires a *Drosophila* co-factor(s), other than CtBP or dSAP18. Our results suggest that proper action of Bcd as a transcriptional activator and molecular morphogen during embryonic development is dependent on the downregulation of its own activity through an interaction with a novel co-repressor(s) or complex(es).

Key words: *Drosophila*, Bicoid, Transcription

INTRODUCTION

Bicoid (Bcd) is a *Drosophila* morphogenetic protein that is required for the formation of the anterior structures of the embryo (Berleth et al., 1988; Driever, 1992; Nüsslein-Volhard et al., 1987). An essential function of this homeodomain-containing protein is to activate transcription of specific target genes in a concentration-dependent manner (Arnosti et al., 1996; Driever and Nüsslein-Volhard, 1989; Gao and Finkelstein, 1998; La Rosee et al., 1997; Rivera-Pomar et al., 1995). Despite considerable efforts to understand the molecular action of Bcd (Arnosti et al., 1996; Burz et al., 1998; Chan and Struhl, 1997; Dave et al., 2000; Driever et al., 1989; Dubnau and Struhl, 1996; Hanes and Brent, 1989; Janody et al., 2000; Janody et al., 2001; Ma et al., 1999; Niessing et al., 1999; Niessing et al., 2000; Rivera-Pomar et al., 1996; Sauer et al., 1995a; Sauer et al., 1995b; Struhl et al., 1989; Yuan et al., 1996; Yuan et al., 1999), it is currently unclear how it works as a transcriptional activator. Our previous analysis suggests that this 489 amino acid protein contains two broadly defined domains (Driever et al., 1989). The N-terminal half of the protein (residues 1-246), which contains the homeodomain (residues 92-151), provides the DNA-binding function. This region of Bcd also plays an important role in cooperative DNA recognition, which is facilitated by a self-association function (Ma et al., 1996; Yuan et al., 1996; Zhao et al., 2000). The C-terminal half of the protein (residues 246-489) provides much of the activation function but can be replaced by heterologous activation domains for embryonic development (Driever et al., 1989). The C-terminal half of Bcd contains several sequences that are characteristic of activation domains, including an acidic region (residues 347-414), an alanine-rich region (residues 329-342; also see Discussion) and a glutamine-rich sequence (residues 256-289).

Biochemical studies by Sauer and Tjian have demonstrated that TAFs (TATA box-binding protein associated factors) can specifically interact with the putative activation domains located in the C-terminal region of Bcd (Sauer et al., 1995a; Sauer et al., 1995b). In particular, it was shown that TAF60 interacted with the alanine-rich region, while TAF110 recognized the glutamine-rich region. It was thus proposed that Bcd activated transcription by directly interacting with these TAFs (Sauer et al., 1995a; Sauer et al., 1995b; Sauer et al., 1996). However, the relevance of such interactions in Bcd function during development has been questioned recently (Schaeffer et al., 1999). Interestingly, it has been shown that a

Bcd derivative lacking the entire C-terminal half can rescue the *bcd*$^{-}$ phenotype when expressed at high levels (Schaeffer et al., 1999). These results further highlight the importance of the N-terminal region of Bcd, suggesting that this region may provide most or all the functions required for Bcd action *in vivo*. The importance of the N-terminal region of Bcd is also evidenced by the recent finding that this region of Bcd is evolutionarily conserved. The Bcd proteins from *Drosophila* and a primitive cyclorrhaphan fly, *Megaselia abdita*, share a highly conserved domain in their N-terminal portions while their C-terminal region diverge dramatically (Stauber et al., 1999).

In this report, we describe experiments that reveal an unexpected new function provided by the N-terminal region of Bcd. A self-inhibitory domain located immediately N-terminally of the homeodomain can repress Bcd activity in reporter activation assays conducted in *Drosophila* tissue culture cells. Mutations in this domain, or its removal by deletion, dramatically increase the activity of Bcd. Our experiments demonstrate that this domain operates as an independent module that does not require any other sequences of Bcd and can repress the activity of heterologous activators. We further show that a Bcd derivative with point mutations in the self-inhibitory domain causes severe defects in both embryonic patterning and target gene expression during development. The action of the self-inhibitory domain requires a *Drosophila* co-factor(s) absent in yeast cells, but our further studies suggest that neither CtBP nor dSAP18 directly target the self-inhibitory domain of Bcd. Our results suggest that proper action of Bcd as a transcriptional activator and molecular morphogen requires a novel co-repressor(s) or complex(es) interacting with its self-inhibitory domain.

MATERIALS AND METHODS

Plasmid construction

The plasmids used in this study are listed in Table 1. They were constructed according to standard procedures (Ausubel et al., 1994; Maniatis et al., 1982); more detailed information is available upon request. All effector plamids for S2 cells express hemagglutinin (HA)-tagged Bcd proteins from the *Drosophila actin 5C* promoter. The *bcd* cDNA gene and its derivatives all contain the *β-globin* leader sequence from the vector *bcd*TN3 (Driever et al., 1989). The effector plamids for yeast cells were based on AAH5 (Ammerer, 1983), whereas the plasmids for in vitro transcription/translation were based on pGEM3 (Promega). The P-element-mediated germline transformation constructs were based on pCasperBcd*Bgl*II, a vector kindly provided by Dr David Stein. HA-tagged wild type *bcd* and *bcd(A52-56)* cDNA genes (with the *β-globin* leader sequence) were isolated as *Bam*HI fragments and inserted into the *Bgl*II site of pCasperBcd*Bgl*II.

Transient transfection experiments

Transient transfection assays in *Drosophila* S2 cells were performed as described previously (Dave et al., 2000; Zhao et al., 2000). All CAT activities shown are normalized to β-galactosidase activity, which was expressed from the control plasmid pDS47-*lacZ*. The amount of cell lysates used in western blots was also normalized to β-galactosidase

Table 1. Plasmids used in this study

Activator/Reporter	Plasmid	Notes	Source
Effector plasmids for S2 cells			
Bcd(WT)	pFY442	*Drosophila actin 5C* promoter	This study
Bcd(92-489)	pFY413	*Drosophila actin 5C* promoter	This study
Bcd(42-489)	pFY414	*Drosophila actin 5C* promoter	This study
Bcd(52-489)	pFY418	*Drosophila actin 5C* promoter	This study
Bcd(62-489)	pFY419	*Drosophila actin 5C* promoter	This study
Bcd(72-489)	pFY420	*Drosophila actin 5C* promoter	This study
Bcd(82-489)	pFY421	*Drosophila actin 5C* promoter	This study
Bcd(1-389)	pFY424	*Drosophila actin 5C* promoter	This study
Bcd(1-346)	pFY449	*Drosophila actin 5C* promoter	This study
Bcd(1-246)	pFY450	*Drosophila actin 5C* promoter	This study
BcdΔ(152-246)	pFY416	*Drosophila actin 5C* promoter	This study
Bcd(A52-56)	pFY436	*Drosophila actin 5C* promoter	This study
Bcd(1-389) (A52-56)	pFY450	*Drosophila actin 5C* promoter	This study
Bcd(1-346) (A52-56)	pFY452	*Drosophila actin 5C* promoter	This study
Bcd (1-246) (A52-56)	pFY451	*Drosophila actin 5C* promoter	This study
BcdΔ(152-246) (A52-56)	pFY498	*Drosophila actin 5C* promoter	This study
Bcd-GAL4(2-94)	pAY503	*Drosophila actin 5C* promoter	This study
Effector plasmids for yeast cells			
Bcd(WT)	pFY480	*LEU2*; yeast *ADH1* promoter	This study
Bcd (A52-56)	pFY481	*LEU2*; yeast *ADH1* promoter	This study
Plasmids for in vitro transcription/translation			
Bcd(WT)	pFY441	SP6 promoter	This study
Bcd(A52-56)	pFY432	SP6 promoter	This study
Bcd(1-246)	pFY443	SP6 promoter	This study
Bcd(92-489)	pFY7015	SP6 promoter	This study
Reporter plasmids			
hb-CAT	pCZ3005	*CAT* reporter plasmid	Zhao et al., 2000
GAL4-CAT	pG5-TATA-CAT	*CAT* reporter plasmid	Lillie and Green, 1989
hb-lacZ	pMA630R	Integrating yeast plasmid	Driever et al., 1989
Transgenic *bcd* constructs			
Wt Bcd	pAY802	*bcd* promoter/enhancer and 3′UTR	This study
Bcd(A52-56)	pAY804	*bcd* promoter/enhancer and 3′UTR	This study

activity. The cytoplasmic and nuclear fractions from S2 cells were prepared according a modified protocol based on that described by Gossett et al. (Gossett et al., 1989).

Gel shift assays

The DNA probe used in gel shift assays contains a consensus Bcd binding site A1. Gel shift assays and Scatchard analysis were carried out according to procedures described previously (Dave et al., 2000; Zhao et al., 2000); all DNA-binding reactions (total volume 30 μl) contained 2 μg poly (dI::dC).

Yeast strain and β-galactosidase liquid assays

The yeast strain used in this study is CY26::MA630R which contains an integrated *hb-lacZ* reporter gene in CY26 (Zhao et al., 2000). The effector plasmids were introduced into yeast using the lithium acetate method (Ito et al., 1983), and three independent colonies were assayed for β-galactosidase units (Yocum et al., 1984).

GST pull-down assay

Expression of GST and GST-dSAP18 fusion in bacteria was performed as previously described (Zhang et al., 2000). Equivalent amounts of GST and GST-dSAP18 were used to pull down in vivo translated and ^{35}S-labeled Bcd derivatives. For each Bcd derivative, a similar amount, as estimated by autoradiography, was used in the pull-down assay.

P-element-mediated germline transformation and phenotypic examination

P-element constructs containing either wild-type or mutant *bcd* genes were injected with transposase helper plasmid into *w* embryos, and transformant lines were mapped using standard procedures (Rubin and Spradling, 1982; Spradling, 1986). For cuticle examination, transgenic female flies were crossed with w^{118} males and allowed to lay on grape agar for 24 hours. The flies were then removed and the vials left at room temperature for 18 to 24 more hours. Cuticles were prepared according to the Hoyer's mountant method (Ashburner, 1989) and photographed by dark-field (whole cuticles) and Nomarski (head regions) microscopy.

Embryo staining and *Drosophila* germline clones

Drosophila embryos were collected and stained for *hb* or *otd* mRNA using digoxigenin-labeled antisense RNA probes as previously described (Jiang et al., 1991). *CtBP* germline clones were generated according to Nibu et al. (Nibu et al., 1998a).

RESULTS

The amino terminal region of Bcd represses its own activity

Fig. 1 shows our unexpected, initial observation that the N terminus of Bcd (residues 1-91) can inhibit dramatically its own activity in a transient transfection assay in *Drosophila* S2 cells. In this assay, the full-length protein [Bcd(1-489)] and a truncated derivative [Bcd(92-489)] were analyzed for their ability to activate a *hb-CAT* reporter gene. This reporter contains a Bcd-responsive enhancer element from *hunchback* (*hb*), which is directly activated by Bcd during development (Driever and Nüsslein-Volhard, 1989). As shown in Fig. 1A, Bcd(92-489) exhibits an activity 40 times greater than that of the full-length protein on the *hb-CAT* reporter. The activity difference between these two proteins is not explained by their protein levels in transfected cells (data not shown; also see the systematic titration analysis).

To delineate the domain responsible for the observed difference between the full-length protein and Bcd(92-489), we

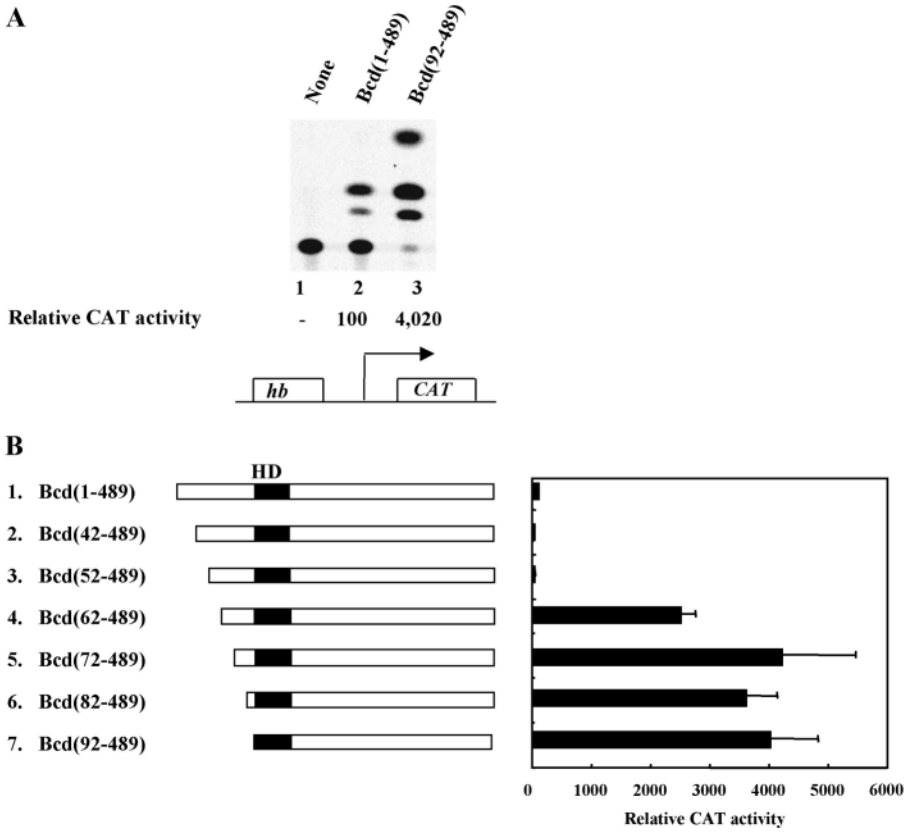


Fig. 1. The N-terminal domain of Bcd inhibits its own activity. (A) A truncated Bcd derivative [Bcd(92-489)] exhibits an activity higher than that of the full-length protein. CAT assay results from *Drosophila* S2 cells that were transfected with the *hb-CAT* reporter plasmid and effector plasmids that express Bcd(1-489) or Bcd(92-489). The CAT activity for wild-type Bcd (expressed from 1 μg transfected effector DNA) on this reporter is arbitrarily assigned as 100 throughout this report. The standard error (s.e.m.) for the activity of Bcd(92-489) on *hb-CAT* was 23%. While the representative CAT assay results shown here were obtained with the same length of enzymatic reaction time (30 minutes), accurate CAT activities (as measured numbers) were obtained with different lengths of reaction time to keep the assays in the linear range. (B) Delineating the self-inhibitory domain of Bcd. Shown are CAT activities for the N-terminal deletion derivatives of Bcd in transient transfection assays on the *hb-CAT* reporter. The homeodomain (HD) of Bcd (residues 92-151) is marked.

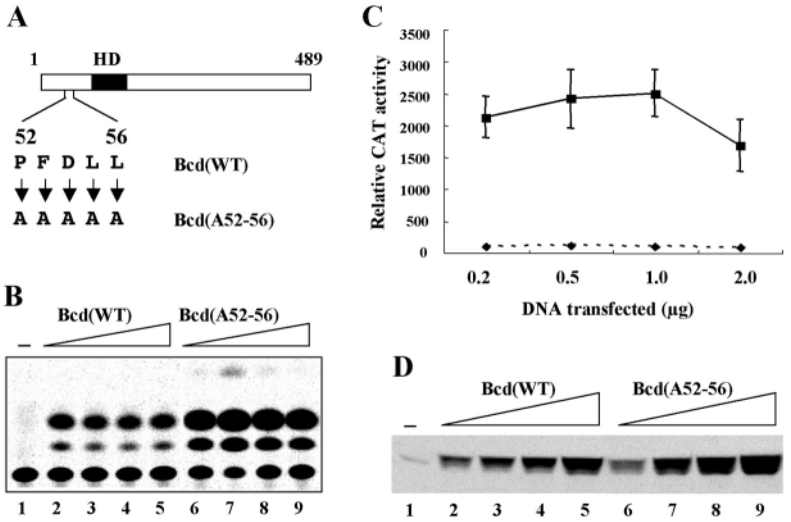


Fig. 2. Alanine mutations at residues 52-56 of full-length Bcd increase its activity. (A) Wild-type Bcd and Bcd(A52-56), which contains five alanines at positions 52-56 of full-length Bcd. HD, homeodomain. (B) CAT assay results from S2 cells that were transfected with increasing amounts of effector plasmids expressing wild-type Bcd or Bcd(A52-56): 0.2 μg (lanes 2, 6), 0.5 μg (lanes 3, 7), 1.0 μg (lanes 4, 8), 2.0 μg (lanes 5, 9). Lane 1 shows the result from cells transfected with an empty vector expressing no Bcd. All CAT reactions shown here were carried out in 30 minutes. (C) A plot of CAT activities against the amount of the transfected effector DNA. To measure accurately the activity difference between the proteins, a shorter length of the CAT reaction time was used for the mutant protein (also see legend to Fig. 1). (D) Western blot analysis showing the total amount of Bcd proteins in transfected cells. The amount of transfected DNA is the same as in B.

generated and analyzed Bcd derivatives with its N-terminal region progressively deleted. Our results shown in Fig. 1B demonstrate that the first 51 amino acids of Bcd can be removed without increasing its activity. By contrast, deletion derivatives lacking the first 61 amino acids or more dramatically increase the activity of Bcd. These results define a 40 amino acid domain of Bcd (residues 52-91) – possibly much smaller because its precise C-terminal border has not been defined – that can repress its own activity. We refer to this domain, which is located immediately adjacent to its homeodomain, as a self-inhibitory domain.

Our deletion analysis shown in Fig. 1B further suggests that residues 52-61 play a most crucial role in the self-inhibitory function. Five residues in this region (52-56) share significant homology with a consensus motif interacting with CtBP, a major co-repressor in *Drosophila* (see below). To determine the importance of residues 52-56 in regulating Bcd activity, we generated a full-length Bcd derivative, Bcd(A52-56), with these five amino acids changed to alanines (Fig. 2A). A systematic titration assay of this Bcd derivative and the wild-type protein was performed, using increasing amounts of effector DNA for transfection. Our results show that, under all protein concentrations analyzed, Bcd(A52-56) was 18-24 times more active than the wild-type protein (Fig. 2B,C). More importantly, this mutant protein was more active than the wild-type protein even when it was expressed at lower levels than the wild-type protein (Fig. 2C,D). As Bcd mutants with critical residues individually mutated also exhibited higher activity (not shown), it is unlikely that the five alanine residues in Bcd(A52-56) may have created fortuitously an alanine-rich activation domain responsible for the observed strong Bcd activity. Together, our experiments demonstrate an essential role for residues 52-56 of Bcd in repressing its own activity.

The self-inhibitory domain of Bcd does not affect its properties of subcellular localization and DNA binding

As further outlined in the Discussion, protein domains with self-inhibitory properties have been identified on other transcription factors. These domains exert their inhibitory functions by different mechanisms, ranging from intramolecular interactions that conceal specific functions to cytoplasmic sequestration and recruitment of co-repressors. To understand the mode of action of the self-inhibitory domain of Bcd, we specifically determined the subcellular distribution of Bcd(A52-56) and wild type Bcd in transfected cells. Our results show that both proteins are predominantly localized to the nucleus in a similar manner (Fig. 3A), suggesting that the self-inhibitory domain does not function by sequestering the protein in the cytoplasmic region. It is interesting to note that Bcd is not strictly localized to the nucleus, a finding that is consistent with its previously demonstrated role of translation inhibition (Chan and Struhl, 1997; Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996).

To determine whether the self-inhibitory domain of Bcd works by affecting its DNA-binding function, we carried out gel shift studies in vitro (Fig. 3). Full-length Bcd proteins were either synthesized in vitro or expressed in transfected S2 cells. Our results show that, in gel shift assays, both wild-type Bcd and Bcd(A52-56) synthesized in vitro bound to a consensus Bcd site with a similar affinity (Fig. 3B). Dissociation constant (K_D) measurements (Fig. 3C,D) of these two proteins expressed in S2 cells further revealed a comparable DNA-binding affinity [estimated K_D values for wild-type Bcd and Bcd(A52-56) were 3.0±0.9 nM and 4.0±0.5 nM, respectively]. These results demonstrate that the self-inhibitory domain of Bcd does not inhibit its DNA binding function, thus making it highly unlikely that this domain physically masks its homeodomain (Amendt et al., 1999).

The self-inhibitory domain is an independent module that does not require any specific Bcd sequences and can repress the activity of heterologous activators

To further determine whether the self-inhibitory domain of Bcd specifically targets any other regions of the protein, we measured the activities of various deletion derivatives in transient transfection assays (Fig. 4A). For each deletion derivative, a pair was tested, one with residues 52-56 mutated to alanines [Bcd(A52-56)] and the other wild type. Our experiments demonstrate that the mutant proteins in each pair were always more active than their wild type counterparts (Fig. 4A). Specifically, these experiments show that neither residues 246-489 (lines 2-4) nor residues 152-246 (line 5) are required

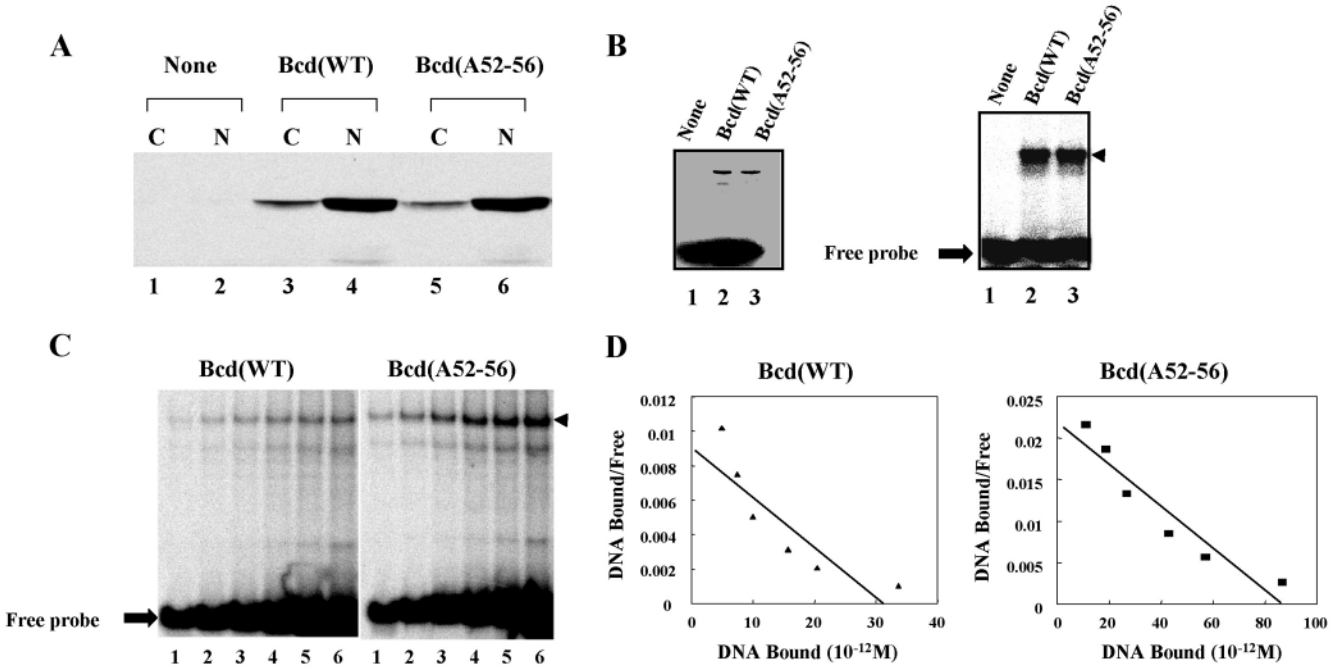
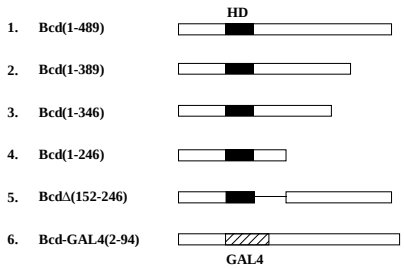


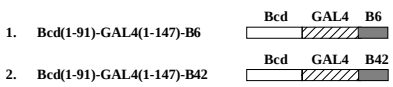
Fig. 3. Neither subcellular localization nor DNA binding is affected by mutations in the self-inhibitory domain of Bcd. (A) Western blot results for wild-type Bcd and Bcd(A52-56) in nuclear (N) and cytoplasmic (C) fractions of the transfected cells. Lanes 1 and 2 represent results from cells that were transfected with an empty effector plasmid expressing no Bcd. (B) DNA-binding assay using Bcd proteins synthesized in an in vitro transcription/translation system. The left panel shows the proteins (labeled with ^{35}S) and the right panel shows the gel shift results using a ^{32}P-labeled DNA probe containing a Bcd binding site. In both panels, lanes 1 to 3 represent no Bcd, wild-type Bcd and Bcd(A52-56), respectively. The Bcd-DNA complex is marked with a solid arrowhead. (C) Gel shift assays for a Scatchard analysis to determine the dissociation constants (K_D) for wild type Bcd (left panel) and Bcd(A52-56) (right panel) expressed in S2 cells. In this assay, the nuclear extracts generated from transfected S2 cells were used in gel shift assays with increasing concentrations of the radioactively labeled DNA probe: 5×10^{-10} M, 1×10^{-9} M, 2×10^{-9} M, 5×10^{-9} M, 1×10^{-8} M and 3.3×10^{-8} M for lanes 1 to 6, respectively. The solid arrowhead indicates the full-length Bcd-DNA complex, which was not formed using nuclear extracts made from non-transfected cells (not shown); smaller bands seen on the gel are presumably complexes containing breakdown products of Bcd. Quantitation was based on the amount of the full-length Bcd-DNA complex. (D) Scatchard plots for wild-type Bcd and Bcd(A52-56) expressed in S2 cells. Three independent assays yielded an estimated K_D value ($-1/K_D$=slope) of 3.0±0.9 nM and 4.0±0.5 nM for wild-type Bcd and Bcd(A52-56), respectively.

A



	Relative CAT activity		
	Bcd(WT)	Bcd(A52-56)	Fold Difference
1. Bcd(1-489)	100	2400 ± 372	24.0×
2. Bcd(1-389)	72.0 ± 17.6	1225 ± 218	17.0×
3. Bcd(1-346)	23.4 ± 5.9	576 ± 104	24.9×
4. Bcd(1-246)	23.0 ± 6.5	460 ± 48	20.0×
5. BcdΔ(152-246)	20.5 ± 2.5	595 ± 142	30×
6. Bcd-GAL4(2-94)	100*	610 ± 153	6.1×

B



	Relative CAT activity		
	Bcd(WT)	Bcd(A52-56)	-Bcd
1. Bcd(1-91)-GAL4(1-147)-B6	560 ± 24	9208 ± 1830	2230 ± 445
2. Bcd(1-91)-GAL4(1-147)-B42	1880 ± 126	9525 ± 1710	N.D.

Fig. 4. The self-inhibitory domain of Bcd works as an independent module. (A) Deletion derivatives of Bcd, either wild-type or Bcd(A52-56), were used in transient transfection assays. The derivative shown at the bottom (6) contains the DNA-binding domain of GAL4 (residues 2-94) in place of the homeodomain of Bcd. The activities, shown in the table, of the wild-type and mutant forms of this hybrid protein were obtained from the *GAL4-CAT* reporter gene, which contains five GAL4 sites upstream of the *CAT* gene. All other derivatives were assayed on the *hb-CAT* reporter gene. *The activity of the wild type Bcd-GAL4(2-94) hybrid protein on the *GAL4-CAT* reporter is assigned as 100, a standard for the relative CAT activity shown in B. (B) The self-inhibitory domain of Bcd can repress the activity of heterologous activators. Activities of hybrid activators that contain the DNA-binding domain of GAL4 (residues 1-147) fused to bacterially derived activation sequences (B6 and B42) are shown in the table. In addition, the first 91 amino acids of Bcd, either wild type or Bcd(A52-56), were attached to the N termini of these activators. The column designated –Bcd shows results of an activator lacking any Bcd sequence.

for the action of the self-inhibitory domain. As shown already in Fig. 1C, the first 51 N terminal amino acids are not required for the action of the self-inhibitory domain.

The homeodomain of Bcd was present in all of our deletion derivatives described in Fig. 4A (lines 1-5). As discussed above, our gel shift results (Fig. 3B-D) argue against the possibility that the self-inhibitory domain inhibits the DNA-binding function conferred by its homeodomain. To further analyze any possible role of the homeodomain in the action of the self-inhibitory domain of Bcd, we generated a hybrid Bcd protein (Fig. 4A, line 6) with its homeodomain replaced by the DNA-binding domain of GAL4 (residues 2-94). Again, a pair of these derivatives were analyzed, either wild type or Bcd(A52-56). For this assay, the CAT reporter gene contains GAL4-binding sites upstream. Our experiments show that the mutant Bcd(A52-56)-GAL4 hybrid protein was over six times more active than its wild-type counterpart (line 6), further demonstrating that the homeodomain of Bcd is not necessary for the action of the self-inhibitory domain.

We conducted experiments to determine further whether the self-inhibitory domain of Bcd is transferable, i.e. whether it can work on entirely heterologous activators. For this analysis, the N-terminal domain of Bcd (residues 1-91), either wild type or Bcd(A52-56), was attached to two different activators, GAL4-B6 and GAL4-B42. These two activators contain the DNA-binding domain of GAL4 (residues 1-147) fused to bacterially derived activation domains that have different activation potentials (Ma and Ptashne, 1987b). When assayed on the *CAT* reporter gene containing GAL4 binding sites, both activators with the Bcd(A52-56) N terminus exhibited higher activity than their wild-type counterparts (Fig. 4B), demonstrating that the self-inhibitory domain can function on entirely heterologous activators.

The function of the self-inhibitory domain requires a *Drosophila* factor(s) absent in yeast cells

Our studies described thus far demonstrate that the self-inhibitory domain of Bcd is an independent module that does not target any specific sequences from Bcd and can work on heterologous activators. One attractive model consistent with these findings is that this module provides a docking site for a *Drosophila* co-repressor(s) or complex(es) that can inhibit transcription. To test this hypothesis, we analyzed the activities of full-length Bcd proteins, either wild type or Bcd(A52-56), in yeast cells that contain an integrated *hb-lacZ* reporter gene. Our experiments show that, in striking contrast to its behavior in *Drosophila* S2 cells, Bcd(A52-56) did not have an increased activity (Table 2); it actually exhibited a moderately reduced activity when compared with the wild-type protein. These results suggest that the action of the self-inhibitory domain requires a co-factor(s) that is present in *Drosophila* S2 cells but missing in yeast cells.

Table 2. The action of the self-inhibitory domain of Bcd requires a *Drosophila* co-factor(s)

	Relative activity	
	Drosophila cells	Yeast cells
Wild-type Bcd	100	100
Bcd(A52-56)	2400±372	50±7.2

Shown are activities of wild-type Bcd and Bcd(A52-56) either on the *hb-CAT* reporter gene in S2 cells or on an integrated *hb-lacZ* reporter gene in yeast cells. The activities of wild-type Bcd in each assay are arbitrarily assigned as 100. The exact β-galactosidase units for wild-type and Bcd(A52-56) in yeast cells are 47.4 and 23.7, respectively. The CAT activities are from Fig. 2 (1.0 μg transfected effector plasmid).

Bcd(A52-56) exhibits a dominant, gain-of-function effect, causing severe embryonic defects

To determine the biological role of the self-inhibitory function of Bcd during embryonic development, we generated transgenic flies expressing Bcd(A52-56). The P-element-mediated transformation vector used in our study contains both the native enhancer and 3′UTR of *bcd*. We reasoned that the strong activation function of Bcd(A52-56) may exhibit a dominant gain-of-function effect in *Drosophila* embryos that causes developmental defects. We systematically examined cuticle phenotypes of embryos from bcd^+ females carrying either one or two copies of the *bcd(A52-56)* transgene for nine independent lines that are viable homozygously, as well as a female sterile line, *18A* (Table 3). As a control, we also analyzed embryos from females carrying one or two copies of the wild-type *bcd* transgene for seven independent lines.

Table 3 summarizes our cuticle examination data and Fig. 5 shows the representative phenotypes. For all the independent lines examined, we observed defective embryos from *bcd(A52-56)* transgenic females. These defective embryos can be categorized into two classes according to the severity of their head defects, moderate (Fig. 5B,F) and severe (Fig. 5C-H). For both classes, the anteroposterior polarity is intact with detectable, but often reduced, filzkorper (a posterior marker) at

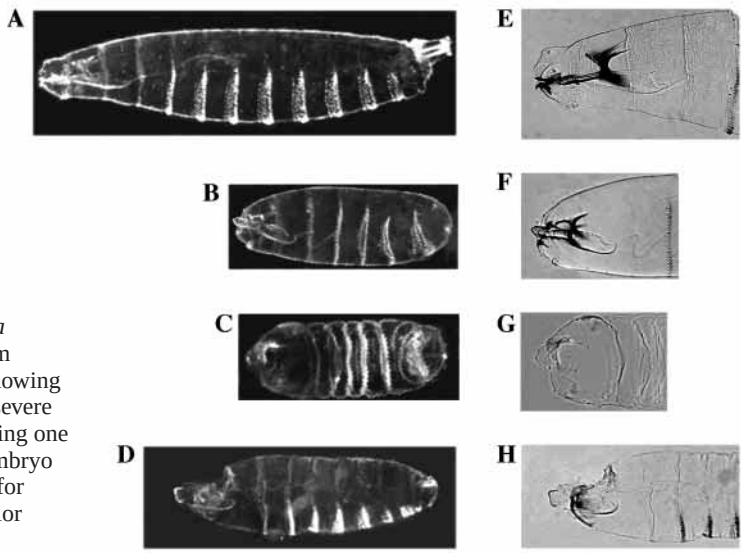


Fig. 5. Bcd(A52-56) causes severe patterning defects in *Drosophila* embryos. (A-H) Representative cuticle phenotypes of embryos from *bcd(A52-56)* transgenic females (A-D) and higher magnification showing their corresponding head regions (E-H). Both moderate (B,F) and severe (C-H) phenotypes are shown for embryos from bcd^+ females carrying one copy of $bcd(A52\text{-}56)^{18A}$. A and E represent a completely normal embryo from bcd^+ transgenic females carrying two copies of *bcd(A52-56)* for transgenic line *3-4* (see Table 3). Cuticles are orientated with anterior towards the left and (except C,G) dorsal upwards.

Table 3. Phenotypic analysis of embryos from *bcd(A52-56)* transgenic females

Line	Copy number	*n*	Moderate (%)	Severe (%)	Total (%)
3-2	1	46	4	7	11
	2	38	21	18	39
3-3	1	12	0	0	0
	2	39	5	3	8
3-4	1	87	5	0	5
	2	33	3	12	15
3-5	1	45	0	9	9
	2	22	9	14	23
3-6	1	37	0	3	3
	2	25	12	8	20
3-8	1	70	4	7	11
	2	48	8	0	8
3-10	1	40	7.5	2.5	10
	2	94	33	11	44
2-4	1	70	7	3	10
	2	16	9	25	34
18A	1	230	28	72	100
Wild-type *bcd* (*2-1*)	1	48	2*	0	2
	2	52	4*	0	4
Wild-type *bcd* (*2-6*)	1	55	0	1	2
	2	88	3*	0	3

Embryos from bcd^+ transgenic females carrying either one or two copies of the *bcd(A52-56)* transgene were scored according to their cuticle phenotypes. For line $bcd(A52\text{-}56)^{18A}$, only embryos from females carrying one copy of the transgene were examined because the penetrance of defective embryos was already 100%. See text for description of the two phenotypic classes (moderate and severe). Embryos from females carrying one or two copies of wild type *bcd* transgene were also scored for seven independent transgenic lines, with the results of two representative lines shown.

*The phenotype of these embryos does not exhibit the typical head defects characteristic of the moderate class embryos from females carrying *bcd(A52-56)* transgene (i.e. the head region is normal for these embryos). The defects in these embryos are generally restricted to the abdominal region, most frequently with fused or deformed A4 and/or A5. For one of the wild-type *bcd* transgenic lines (*2-2*) examined, 22% and 89% of the embryos from females carrying one or two copies of the transgene, respectively, were defective. However, the majority of these defective embryos (100% and 61% from females carrying one or two copies of the transgene, respectively) exhibited no head defects that are typical of the embryos from *bcd(A52-56)* transgenic females. Copy number, the number of transgenes in females; *n*, number of cuticles examined (naked cuticles lacking recognizable anterior structures not scored).

the posterior. However, the denticle bands are often fused or distorted for both classes, frequently lacking the entire A4-A7 region (Fig. 5B) or its various sections, thus causing the embryos to be much smaller. For the moderate class, the cuticles generally have a recognizable, but significantly deformed head (the cuticle shown in B has the mildest head defect in this class). The mouth parts are formed but positioned improperly (F), sometimes detached from the rest of the head skeleton. The dorsal bridge and posterior wall of the pharynx are often not formed, and the Lateralgraten are reduced (F) or completely non-existent. For the severe class, head deformation is more dramatic, often with only scrambled remnant head skeleton and mouth parts (G); sometimes the entire anterior region is missing or the anterior has a hole (H).

The analysis shown in Table 3 enables us to draw the following conclusions. First, as the same phenotypes were observed for all the independent transgenic lines examined, these phenotypes reflect the biological properties of Bcd(A52-56), rather than P-element insertion locations of the transgene. Second, the frequency of defective cuticles, in general, is significantly higher in embryos from females carrying two copies of the transgene than from females carrying one. This is consistent with the observation that the total amount of Bcd(A52-56) in 0-4 hour embryos from transgenic *18A* females was among the highest in several selected transgenic lines analyzed (not shown), but the difference is no more than a few fold at the most. Third, the frequency (penetrance) of defective embryos does not dictate the distribution among the two classes of phenotypes. This finding contrasts with the observation that an excessive amount of wild-type Bcd enhances both the total frequency of all cuticles that are defective and the percentage of the severe ones (Namba et al., 1997). It further suggests that the phenotypes caused by Bcd(A52-56) reflect its distinct properties, as opposed to a mere increase in protein levels. Fourth, the examination of embryos from bcd^+ females carrying one or two copies of the wild-type *bcd* transgene directly argues against the possibility that the observed phenotypes conferred by Bcd(A52-56) were caused by a mere increase in protein levels (Table 3). In this case, defective embryos were observed only at very low frequencies for all but one line examined and, more importantly, the embryonic defects were generally restricted to the abdominal region without the head malformations characteristic of embryos from *bcd(A52-56)* transgenic females (see legend to Table 3 for further details).

Bcd(A52-56) causes severe alterations in target gene expression during embryonic development

To determine how Bcd(A52-56) affects the expression of Bcd target genes, we conducted in situ hybridization assays for *hb* and *orthodenticle* (*otd*) in embryos from females that carry one copy of $bcd(A52\text{-}56)^{18A}$. These embryos were chosen because 100% of them exhibited developmental defects (Table 3). Our data shown in Fig. 6 reveal the following results. First, the Bcd-dependent expression domains of both *hb* and *otd* in the anterior are dramatically expanded towards the posterior at different developmental stages (e.g. compare C with D and I with J), demonstrating that Bcd(A52-56) can activate these target genes much more effectively in embryos. The posterior shift of the expression domains of these target genes is consistent with both a posterior shift of segmentation gene expression stripes (not shown) and our observed cuticle phenotypes resulting from a failed or incomplete head involution (Fig. 5). In embryos containing Bcd(A52-56), the parasegment 4 (PS4) domain of *hb* at a later stage is dramatically shifted toward the posterior (Fig. 5F), further illustrating a posterior shift of the fate map of these embryos.

Second, the Bcd-dependent anterior expression domain of *otd* (and to a lesser extent *hb*) retracts from the anterior tip upon cellularization in both wild-type embryos and those containing Bcd(A52-56), suggesting that the self-inhibitory domain is unlikely to be solely responsible for mediating the Tor-repression (Janody et al., 2000; Ronchi et al., 1993). As Bcd(A52-56) is a much stronger activator than the wild type protein, our finding that anterior retraction can proceed in embryos containing Bcd(A52-56) indicates that Tor-mediated repression cannot be overcome by a strong Bcd activator.

Third, the expression domain of *hb* at the posterior is greatly reduced (Fig. 5D,F) or missing. Both this expression domain

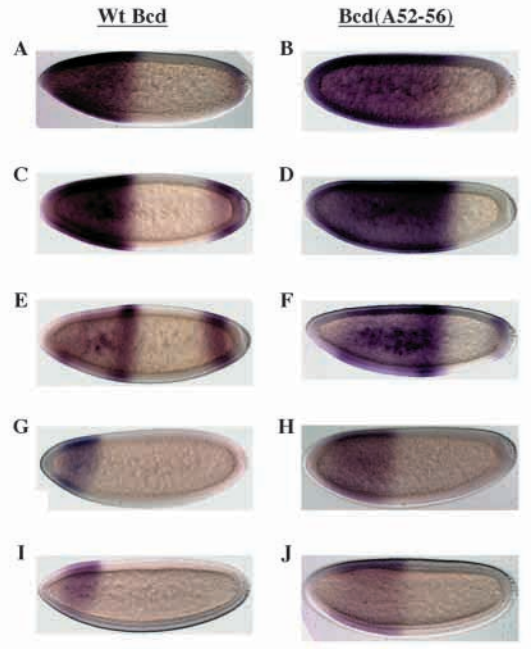


Fig. 6. Bcd(A52-56) severely affects the expression patterns of target genes in *Drosophila* embryos. Shown are embryos from bcd^{+} females carrying either no (A,C,E,G,I) or one copy (B,D,F,H,J) of $bcd(A52\text{-}56)^{18A}$, hybridized with digoxigenin-labeled *hb* (A-F) or *otd* (G-J) antisense RNA probes. Different developmental stages are shown: pre-cellular (A,B), cellularizing (C,D,G,H) and cellularized embryos (E,F,I,J). Embryos are oriented with anterior towards the left and dorsal upwards.

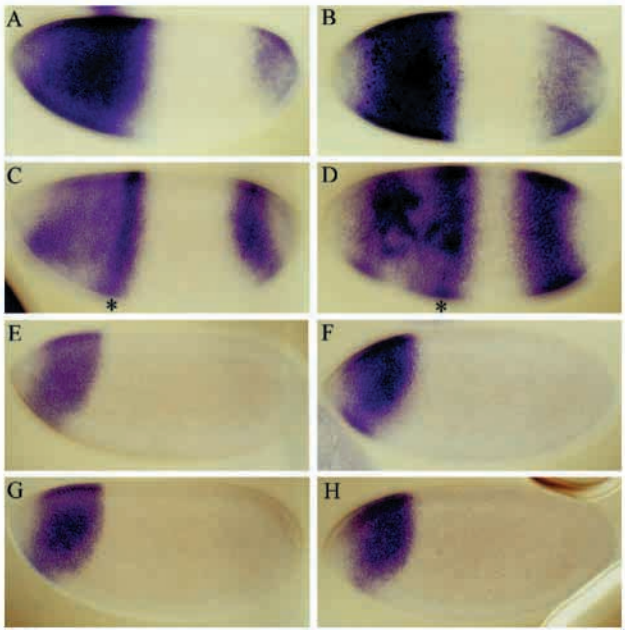


Fig. 7. Bcd-dependent activation of *hb* and *otd* is unaffected in embryos from P1590 GLCs. Cellularizing (A,B,E,F) and cellularized (C,D,G,H) embryos were hybridized with digoxigenin-labeled *hb* (A-D) or *otd* (E-H) antisense RNA probes. Embryos are oriented with anterior towards the left and dorsal upwards. Wild-type embryos (left column) are compared with embryos from P1590 GLCs (right column), which have greatly reduced maternal CtBP function. No change is detected in the expression pattern of either gene with the exception of the later *hb* pattern (D), which shows a posterior expansion of the PS4 stripe (*), and an anterior expansion of the posterior *hb* domain. This perturbation is only detected in a small percentage (~10%) of embryos from P1590 GLCs. See text for further details.

and the PS4 domain of *hb* are thought to be regulated in a Bcd-independent manner (Margolis et al., 1995; Schroder et al., 1988; Tautz, 1988). In particular, previous studies have shown that the posterior expression domain of *hb* is repressed by the Hunchback protein (Hb) itself (Margolis et al., 1995; Struhl et al., 1989). It is possible that the dramatic posterior expansion of the Bcd-dependent anterior *hb* expression domain may contribute to the reduction or elimination of the posterior domain. Taken together, our phenotypic and staining analysis of embryos containing Bcd(A52-56) demonstrate that the self-inhibitory domain of Bcd is required for proper embryonic patterning and target gene activation during development.

The self-inhibitory domain of Bcd is targeted by a novel co-factor(s), rather than CtBP or dSAP18

As discussed above, residues 52-56 of Bcd, PFDLL, share similarity with the consensus motif for CtBP interaction, PLDLS, where the underlined residues are invariable (Postigo and Dean, 1999). CtBP is a major co-repressor that mediates the activity of a variety of transcriptional repressors in *Drosophila* and other organisms (Criqui-Filipe et al., 1999; Deconinck et al., 2000; Meloni et al., 1999; Nibu et al., 1998a; Nibu et al., 1998b; Poortinga et al., 1998; Postigo and Dean, 1999; Schaeper et al., 1995; Sollerbrant et al., 1996; Turner and Crossley, 1998). To test whether *Drosophila* CtBP is involved in modulating Bcd function, we analyzed the expression of *hb* and *otd* in embryos containing disruptions in *CtBP* activity. *CtBP* is expressed maternally and zygotically, and northern blots show a complex expression pattern in early *Drosophila* embryos (Poortinga et al., 1998). To disrupt maternal *CtBP* activity, we generated germline clones (GLCs) that are homozygous for the P-element insertion (P1590) using the *FRT-ovoD* technique (Chou et al., 1993). Previous experiments have shown that this technique disrupts the early functions of those repressors known to interact with CtBP, including Hairy, Kruppel (Kr), Knirps (Kni) and Snail (Morel et al., 2001; Nibu et al., 1998a; Nibu et al., 1998b; Poortinga et al., 1998).

Fig. 7 shows the in situ staining results for *hb* and *otd* in wild type or embryos from P1590 GLCs. There is no detectable change in the early expression patterns of these two genes, as judged by both their posterior borders and relative expression levels (Fig. 6B and 6F, compare with 6A and 6E, respectively). In addition, both genes appear to be downregulated at the anterior tip, indicating that CtBP does not mediate the repression of Bcd activity in this region. These results are consistent with the previous finding that the disruption of maternal *CtBP* does not grossly affect gap gene expression in *Drosophila* embryos (Nibu et al., 1998b; Poortinga et al., 1998). Furthermore, most embryos from P1590 GLCs exhibited normal *hb* expression in both the PS4 domain and the posterior domain at a later stage (not shown). However, in a small percentage (~10%) of embryos from P1590 GLCs, the PS4 stripe (marked *) expands posteriorly, and the posterior domain is expanded toward the anterior (Fig. 7D). These

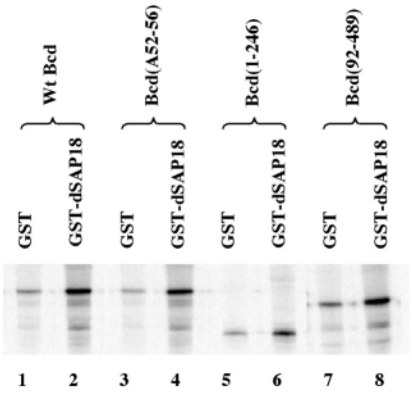


Fig. 8. Interaction between Bcd and dSAP18. Shown are results of GST pull-down experiments in which bacterially expressed GST-dSAP18 (lanes 2, 4, 6 and 8) or GST alone (lanes 1, 3, 5 and 7) were used to pull down in vitro translated and radioactive labeled Bcd proteins. Note that the Bcd derivatives are pulled down by GST-dSAP18 above the background levels for GST alone.

alterations may be due to disruptions of the functions of the gap proteins Kr and Kni, which are expressed in the region between these two *hb* domains. Consistent with this hypothesis, previous experiments have shown that Kni may act as a repressor of the PS4 stripe (Kosman and Small, 1997; Wimmer et al., 2000).

These experiments suggest that disrupting maternal CtBP function does not change Bcd activation of *hb* or *otd*. However, as the P1590 insertion does not completely remove maternal function (M. Levine, personal communication), and does not affect the zygotic paternal contribution, it is possible that low levels are sufficient for inhibiting Bcd activity. As mutants that completely lack maternal CtBP are not available, we analyzed in S2 cells the activity of a full-length Bcd mutant with residues 52-56 (PFDLL) converted to the consensus CtBP-interacting motif PLDLS. If CtBP is involved in the self-inhibitory function of Bcd, this mutant Bcd should further reduce its activity. By contrast, this mutant Bcd was about 17 times more active than the wild-type protein in S2 cells (not shown). Together, these experiments argue against CtBP as an essential co-factor involved in the self-inhibitory function of Bcd.

Amino acids 52-56 of Bcd, PFDLL, are not similar to the previously defined motifs for interaction with Groucho, another major co-repressor present in the early embryo (Chen and Courey, 2000; Tolkunova et al., 1998; Zhang and Levine, 1999; Zhang et al., 2001). However, a *Drosophila* protein called Bin1 was recently isolated as a Bcd-interacting protein in a custom-design yeast two-hybrid system (Zhu and Hanes, 2000). This protein shares homology with the SAP18 component of a mammalian histone deacetylase complex (Zhang et al., 1997). Histone deacetylase complexes represent another major mechanism of transcription repression (for a review, see Ahringer, 2000). Interestingly, dSAP18 (Bin1) has also been shown to interact with another *Drosophila* transcription factor GAGA both biochemically and genetically (Espinas et al., 2000). To determine whether dSAP18 directly targets our delineated self-inhibitory domain of Bcd, we conducted a GST pull-down analysis. In this assay, bacterially expressed GST-dSAP18, or GST alone, was used to pull down in vitro translated and radioactively labeled Bcd derivatives. Our results (Fig. 8) demonstrate that, as expected, wild-type Bcd can interact with dSAP18 (lanes 1, 2). However, Bcd(A52-56), which has a defective self-inhibitory function, interacted with dSAP18 similarly, suggesting that dSAP18 does not target Bcd through the delineated self-inhibitory domain. Consistent with this suggestion and the findings described in a recent report (Zhu et al., 2001), our experiments further show that dSAP18 can interact with Bcd(92-489), which lacks the entire N-terminal domain (lanes 7, 8). Taken together, these studies suggest that self-inhibitory domain of Bcd delineated in this report represses its own activity by interacting with a novel *Drosophila* factor(s) or complex(es), other than CtBP and dSAP18.

DISCUSSION

The experiments described in this report reveal a self-inhibitory domain of Bcd (residues 52-91) that can dramatically repress its own activity in both *Drosophila* tissue culture cells and embryos. Many transcription factors have been shown to possess domains that have similar self-inhibitory properties. These domains repress their proteins' own activity through a variety of mechanisms. For example, Nkx2-5 and C/EBPβ have been shown to contain self-inhibitory domains that conceal their transcriptional activation functions (Chen and Schwartz, 1995; Durocher et al., 1997; Kowenz-Leutz et al., 1994). Second, both PITX2 and Lab proteins possess inhibitory domains that can affect their DNA-binding function, resulting from proposed intramolecular interactions (Amendt et al., 1999; Chan et al., 1996). Third, an inhibitory motif of the homeodomain protein ESX1 sequesters that protein in the cytoplasmic portion of cells (Yan et al., 2000). Fourth, both the yeast activator GAL4 and the tumor suppressor protein p53 have their activation domains masked by repressor proteins that recognize sequences overlapping their activation domains (Johnston et al., 1987; Ma and Ptashne, 1987a; Oliner et al., 1993; Uesugi and Verdine, 1999). Finally, some activator proteins, such as LIM homeodomain proteins (Bach et al., 1999), adenoviral activator E1A (Schaeper et al., 1995; Sollerbrant et al., 1996) and steroid hormone receptors (Torchia et al., 1998), can recruit co-repressors that in turn actively inhibit transcription.

We currently favor a co-repressor model based on the following results and considerations; in this model, a co-repressor(s) or complex(es) specifically interacts with the self-inhibitory domain of Bcd, thus inhibiting its transcriptional activation function. First, our results argue against a role of the self-inhibitory domain in subcellular localization and DNA binding (Fig. 3). Second, our data show that this domain works as an independent module that does not specifically target any other sequences of Bcd and can repress the activity of heterologous activators (Fig. 4). We note that the magnitude of repression by the self-inhibitory domain in some of our hybrid activators is decreased (Fig. 4), suggesting that Bcd sequences (particularly the neighboring homeodomain), though not necessary, may play a contributory role. Third, our experiments further suggest that the action of the self-inhibitory domain requires a *Drosophila* protein(s) that is missing in yeast cells (Table 2). Our results are consistent with the idea that CtBP and dSAP18 do not directly target Bcd through the self-

inhibitory domain (Figs 7 and 8). These findings suggest the existence of a novel co-repressor(s) or complex(es) that regulates Bcd activity in *Drosophila*. The isolation and characterization of such co-repressor molecule(s) will enhance our future understanding of the molecular mechanisms of transcriptional activation and pattern formation by Bcd during embryonic development.

Our analysis of embryos from *bcd(A52-56)* transgenic females reveals a dominant, gain-of-function effect causing developmental defects in both head and abdominal regions. These phenotypes share resemblance to those caused by a Bcd-VP16 fusion protein which contains the strong activation domain VP16 (Driever et al., 1989). Interestingly, an excessive amount of wild-type Bcd produced from six copies of *bcd* can also cause head and abdominal defects in a fraction of the embryos (Namba et al., 1997). It is relevant to note that a *bcd* cDNA transgene in our P-element vector pCaSpeRBcd*Bgl*II was estimated to produce, on average, approx. half the amount of Bcd protein as an endogenous *bcd* gene (Driever et al., 1990). Compared with wild-type Bcd expressed from six copies of *bcd*, Bcd(A52-56) can cause embryonic defects at a much higher penetrance (100% in line *18A*) and at a much lower concentration (~1/8). We note that two copies of wild-type *bcd* cDNA transgene only caused moderate abdominal defects at a low frequency in most of the lines examined (Table 3). This observation is consistent with the estimate that two copies of our transgene are equivalent to only one copy of genomic *bcd*.

The head defects caused by Bcd(A52-56), like those by Bcd-VP16 and excessive amounts of wild-type Bcd (Driever et al., 1989; Namba et al., 1997), are presumably due to a failed or incomplete head involution resulting from the posterior shift of the fate map. It is possible that both Bcd-VP16 and Bcd(A52-56) may have additional molecular consequences associated with their strong activation functions. It remains to be determined whether, for example, Bcd(A52-56) causes the developmental defects, in part, by activating zygotic genes that are normally not targets of Bcd in embryos.

The expression domains of *hb* and *otd* in embryos containing Bcd(A52-56) are expanded dramatically towards the posterior (Fig. 6). Interestingly, we did not observe any obvious increase in the intensity of their expression in these embryos. It is possible that *hb* and *otd* are expressed, in response to the Bcd gradient, at levels that are already maximal in wild-type embryos. According to this idea, the consequence of the stronger activator Bcd(A52-56) is not an elevated level of *hb* and *otd* expression, but rather, a posterior shift of their expression domains. It has been shown that the activating strength of an activator can actually influence its in vivo DNA-binding ability (Tanaka, 1996). In particular, activators with stronger activation domains can bind DNA at lower concentrations in vivo, presumably because a stronger interaction with the basal transcription machinery can facilitate their DNA binding function at low concentrations. Although our experiments demonstrate that both wild type Bcd and Bcd(A52-56) have a similar affinity to a single Bcd binding site in vitro (Fig. 3), a dramatic posterior shift of the *hb* and *otd* expression domains in embryos containing Bcd(A52-56) suggests that Bcd(A52-56) may have a significantly higher in vivo affinity to both enhancers. Furthermore, as Bcd(A52-56) is a much stronger activator, it is possible that, as proposed previously (Arnosti et al., 1996; Lehman et al., 1998; Ma et al., 1999), *hb* and *otd* can be activated by fewer Bcd(A52-56) molecules (than wild-type molecules) in the more posterior part of the embryo.

Another domain of Bcd (residues 300-340, alanine-rich) was shown recently to also exhibit an inhibitory function (Janody et al., 2001). Besides their different physical locations and amino acid compositions, there are several other important differences between the self-inhibitory domain delineated in this report and that newly described domain. First, the self-inhibitory domain described here represses transcription over 20-fold in deletion assays (Fig. 1), whereas a single alanine-rich domain only represses transcription threefold (its effect is significantly enhanced when multimerized). We note that a Bcd derivative lacking the alanine-rich region also causes a posterior shift of the *hb* expression domain in embryo (Schaeffer et al., 1999), though less dramatically than Bcd(A52-56). Second, the self-inhibitory domain can work on heterologous activation domains (Fig. 4B), in addition to those from Bcd, suggesting an active repression mechanism. Third, this domain has been systematically dissected by deletion and point mutations (Figs 1 and 2; C. Z. and J. M., unpublished). Finally and most importantly, while point mutations in the self-inhibitory domain cause severe developmental defects (Fig. 5), the entire C-terminal half of Bcd, including the alanine-rich domain, can be deleted (Schaeffer et al., 1999).

Although our transgenic studies demonstrate that the self-inhibitory function of Bcd is important for proper embryonic pattern formation in *Drosophila*, it is not completely clear how this function is regulated by other developmental cues. Our results show that the action of the self-inhibitory domain is not responsible for Tor-dependent repression upon cellularization (Fig. 6), although we cannot rule out the possibility that the self-inhibitory domain may play a contributory role in this process. In addition, it has been shown that self-inhibitory domains of other proteins are involved in synergistic activation with co-factors (Amendt et al., 1999; Durocher et al., 1997). The self-inhibitory domain of Bcd may similarly participate in synergistically activating transcription with other *Drosophila* factors, such as Hb (Simpson-Brose et al., 1994). Furthermore, as the N-terminal region of Bcd is also engaged in self-association and cooperative DNA binding (Yuan et al., 1996; Zhao et al., 2000), enhancer architecture [i.e. the arrangement of DNA sites for Bcd (Yuan et al., 1999) and other factors] may influence how Bcd molecules are positioned on different enhancers and, thus the availability of the self-inhibitory domain for interacting with the proposed co-repressor(s). Given its intricate morphogenetic role in instructing embryonic patterning, an intriguing possibility exits that Bcd itself may function as an active repressor in a context-dependent manner during embryonic development.

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