A Composite Motif of the *Drosophila* Morphogenetic Protein Bicoid Critical to Transcription Control*

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Bicoid is a molecular morphogen-controlling embryonic patterning in *Drosophila*. It is a homeodomain-containing protein that activates specific target genes during early embryogenesis. Our recent studies have identified a domain of Bcd located outside its homeodomain and referred to as a self-inhibitory domain that can dramatically repress its own ability to activate transcription. Here we present evidence that the self-inhibitory function is evolutionarily conserved. A systematic analysis of this domain reveals a composite 10-amino acid motif with interdigitating residues that regulate Bcd activity in opposite manners. Mutations within the Bcd motif can exert their respective effects when the self-inhibitory domain is grafted to an entirely heterologous activator, but they do not affect DNA binding *in vitro* or subcellular localization of Bcd in cells. We further show that the self-inhibitory domain of Bcd can interact with Sin3A, a component of the histone deacetylase co-repressor complex. Our study suggests that the activity of Bcd is intricately controlled by multiple mechanisms involving the actions of co-repressor proteins.

It is becoming increasingly evident that transcription is a highly coordinated process requiring the actions of both activators and repressors. Meanwhile, the distinction between activators and repressors is less clear-cut than previously thought. For example, a transcription factor can act either as an activator or repressor depending on the enhancer context (1) or availability of specific physiological stimuli (2–5). Enhancer elements also contain recognition sites, sometimes overlapping, for both activators and repressors that work together to control gene transcription (6, 7). In addition, some activators can interact with co-repressor complexes that can repress their own activities (2, 8, 9). Most intriguingly, some repressor proteins can apparently interact with basal transcription factors (10, 11), presumably forming inactive transcription complexes at gene promoters. How the activity of transcription factors is regulated remains a fundamental question in molecular biology because disruption of normal regulatory mechanisms can lead to severe biological consequences. Our recent studies of the *Drosophila* morphogenetic protein Bicoid (Bcd) help illustrate this point.

Bcd is a molecular morphogen required for embryonic patterning along the anterior-posterior axis in *Drosophila* (12, 13). Synthesized from maternally contributed and anteriorly localized *bcd* mRNA, Bcd protein is distributed as an anterior-to-posterior gradient in early embryos, with the highest concentration at the anterior (14, 15). Bcd is a homeodomain-containing transcriptional activator that stimulates the expression of its target genes in a concentration-dependent manner (13). For example, Bcd activates the target gene *hunchback* (*hb*) in the anterior half of the embryo (16, 17), whereas the head-specific gene *orthodenticle* (*otd*) responds to higher concentrations of Bcd with an expression domain restricted to a more anterior portion of the embryo (18). Our recent studies in *Drosophila* tissue culture cells identified a self-inhibitory domain of Bcd that can severely repress its own activity (19). This self-inhibitory domain (residues 52–91) is located in the amino terminus of the protein outside its homeodomain. A deletion derivative lacking this domain is greater than 40 times more active than the wild type protein on a *hb-CAT* reporter in transfected cells. A Bcd mutant (Bcd(A52–56)) bearing alanine substitutions at the critical residues 52–56 causes dominant, gain-of-function defects in embryonic patterning. Embryos from wild type females carrying the *bcd(A52–56)* transgene exhibit patterning defects in both anterior and abdominal segments. In addition, the expression domains of *hb* and *otd* are dramatically expanded toward the posterior (19).

In this report we further characterize the self-inhibitory domain of Bcd. We provide evidence that the self-inhibitory function of Bcd is evolutionarily conserved. Our systematic mutagenesis experiments reveal that the most critical region of this domain is localized to a 10-amino acid motif at residues 52–61. Strikingly, mutations at immediately adjacent residues in this motif cause opposite effects on the ability of Bcd to activate a *hb-CAT* reporter gene in *Drosophila* S2 cells. In particular, whereas Bcd(A52–56) has a dramatically increased activity on *hb-CAT*, Bcd(A57–61), which has the immediately adjacent residues 57–61 changed to alanines, was nearly inactive on this same reporter gene. Like the A52–56 mutation, the A57–61 mutation does not affect subcellular localization of Bcd in cells or its DNA binding *in vitro*, and both mutations can confer their respective, opposite effects to an entirely heterologous activator. In addition, Bcd(A57–61) can inhibit efficiently the activity of wild type Bcd in reporter activation assays. A systematic dissection of this 10-amino acid motif suggests that it is composed of interdigitating residues that impact the Bcd function in opposing manners. Our biochemical experiments further show that the paired amphipathic helix (PAH) domains of *Drosophila* Sin3A can interact with the self-inhibitory domain of Bcd. These experiments suggest that the
activity of Bcd is modulated by multiple mechanisms involving the actions of co-repressor proteins such as Sin3A.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—All activator gene constructs for expressing wild-type Bcd or its mutants were based on pAC5.1C (Invitrogen) and were generated in two steps. First, the DNA fragment containing full-length wild-type or mutant bcd genes were cloned as NdeI-XhoI fragments into a modified pGEM3 vector, pFY7002. All the bcd genes have a Xenopus β-globin leader sequence followed by the coding sequence for a single hemagglutinin tag (MYAPYDVPDYA) attached to the 5′ coding; bcd mutations were generated by a PCR-mediated method as described previously (20, 21). Secondly, the DNA fragments including the hemagglutinin-tagged Bcd sequence and the β-globin leader were then taken as HindIII-XhoI fragments and inserted into pFY403, which was derived from pAC5.1C with its KpnI and EcoRV sites converted to HindIII and XhoI sites, respectively (22). The luciferase reporter construct, 5xGa14-TATA-luc, was based on luciferase 3 basic vector (Promega), which contains the E1A TATA box. The CAT reporter construct hb-CAT was described previously (22). Drosophila Sin3A coding fragments were generated by PCR using a CDNA (kindly provided by Drs. Lori Pile and David Wassarman) as a template. They were then cloned into the NdeI-XhoI sites of pFY441 (19), resulting in pAY559 (for PAH1 domain, residues 1–473 of Sin3A), pAY650 (PAH2, residues 419–780), pAY651 (PAH3, residues 910–1318), pAY652 (PAH4, residues 1439–1748), pDF320 (PAH2–3, residues 411–1080), and pDF321 (PAH4, residues 904–1611).

**Transient Transfection and CAT Assays**—Drosophila S2 cells were transfected with plasmids by the calcium phosphate co-precipitation method as described previously (22). For each transfection, a total amount of 10 μg of DNA, including 1 μg of effector plasmid, 1 μg of report plasmid and 1 μg of copia-lacZ transfection efficiency control plasmid was transfected to cells seeded in a 60-mm-diameter tissue culture plate. The luciferase assay was performed according to the protocol provided by Promega, and the CAT activity was measured as described previously (22).

**Cellular Fractionation and Gel Shift Assays**—The cellular fractionation experiments were performed as described previously (19). For gel shifts, in vitro translated proteins and nuclear extracts from transfected Drosophila S2 cells were analyzed for their ability to bind a 26-bp probe containing a consensus A1 site as described previously (19). The in vitro translated proteins were generated by rabbit reticulocyte lysate using Sp6 polymerase (Promega), and the nuclear extracts from S2 cells were generated as described previously (19). The binding reactions were performed in 30 μl of BB Buffer (15 mM HEPES (pH 7.5), 1 mM EDTA, 0.5 mM dithiothreitol, 40 mM KCl) containing 1.0 μg of dI4C on ice for 20 min. See the legends for Figs. 3 and 9 for more details.

**GST Pull-down Assays**—The GST pull-down assays were performed as described previously (19, 23). Briefly, GST-Bcd fusion proteins containing Bcd (1–91) and either wild type or mutants were expressed in Escherichia coli (DH5α) cells and coupled to glutathione-sepharose beads. The amount of all GST-Bcd fusion proteins was adjusted to be 2–3 μg/10 μl bead suspension, as estimated by Coomassie Blue staining. After washing with binding buffer (20 mM Tris (pH 7.5), 30 mM NaCl, 0.012% Nonidet P-40, 10 mM MgCl2, 2 mM dithiothreitol, 10% glycerol, 0.2 mM phenylmethylsulfonyl fluoride, 0.25% milk), 20 μl of GST beads were resuspended in 200 μl of binding buffer and mixed with 6 μl of translation lysate (TNT-couple reticulocyte system; Promega) containing 35S-labeled Sin3A PAH domains. After incubation for 3 h at 4 °C, the beads were washed 3 times with the binding buffer and 1 time with binding buffer without milk. The pull-down products were resolved on a 10% SDS-polyacrylamide gel and analyzed using a Molecular Dynamics PhosphorImager.

**RESULTS**

**The Self-inhibitory Function of Bcd Is Evolutionarily Conserved**—Recent studies have isolated a bcd gene from the primitive cyclorrhaphan fly Megasia abdita (24). Although the carboxyl-terminal portions of the Bcd proteins from Drosophila and M. abdita (Ma-Bcd) show relatively little conservation, their amino-terminal portions share a contiguous stretch of highly conserved sequence (24). This conserved sequence covers the homeodomain and the region immediately amino-terminal to the homeodomain including the self-inhibitory domain (Fig. 1A). To determine whether the self-inhibitory function of Bcd is evolutionarily conserved we compared the abilities of wild type and mutant Ma-Bcd proteins to activate the hb-CAT reporter gene in S2 cells (Fig. 1B). The mutant protein, Ma-Bcd(A43–47), contains alanine substitutions at residues 43–47, corresponding to residues 52–56 of Drosophila Bcd. In our experiments this mutant Ma-Bcd(A43–47) protein exhibited an activity 7.5 times higher than its wild type counterpart (Fig. 1B, lanes 3 and 4), demonstrating that the self-inhibitory function is evolutionarily conserved. Interestingly, compared with Drosophila Bcd, Ma-Bcd proteins are relatively poor activators in Drosophila S2 cells (Fig. 1B; also see Ref. 25) despite their stable accumulation in transfected cells (Fig. 1C).

**Two Critical Regions of the Self-inhibitory Domain of Bcd Are Located Immediately Adjacent to Each Other**—To further define regions within the self-inhibitory domain of Drosophila Bcd important for its action, we generated systematic alanine-scanning mutants of the full-length protein, each bearing five residues converted to alanines (Fig. 2A). In our experiments, 1-μg effecter plasmids were transfected into S2 cells together with the hb-CAT reporter plasmid; because Bcd at this concentration has already reached its maximal activity on this reporter (19), our observed CAT activities are reflective of the activation potentials of the Bcd derivatives. As shown previously (19), a mutation of residues 52–56 increased the activity of Bcd by 25-fold (line 2) on the hb-CAT reporter in S2 cells. Our results shown in Fig. 2A also identify several other regions that when mutated can increase Bcd activity moderately, most notably residues 72–76 (9.7-fold increase, line 6) and residues 67–71 (5.3-fold increase, line 5; also see “Discussion”). Mutations in other regions (except residues 57–61; see below) had relatively little or no effect on Bcd activity in this assay. Fig. 2B shows that all Bcd derivatives were accumulated in S2 cells to comparable levels as determined by a Western blot analysis.

Our alanine-scanning mutation analysis shown in Fig. 2A also reveals another region of Bcd, residues 57–61, that is located immediately adjacent to residues 52–56 but plays an opposite role. Specifically, mutation at residues 57–61 almost completely abolished Bcd activity on the hb-CAT reporter in S2 cells (line 3). The inability of this mutant protein to activate hb-CAT cannot be overcome by expressing it at a higher level in transfected cells (not shown). In addition, Bcd derivatives with critical residues within 57–61 individually mutated also caused a reduction in Bcd activity on hb-CAT (see below). Together, our results identify 2 immediately adjacent regions, residues 52–56 and residues 57–61, that regulate Bcd function in opposing manners.

**The A57–61 Mutation Does Not Affect Subcellular Distribution or DNA Binding**—Our previous studies show that the A52–56 mutation does not affect the subcellular distribution or DNA binding properties of Bcd in vitro (19). To determine whether the two adjacent regions located at residues 57–61 and 52–56 exert their regulatory activities on Bcd function through a common mechanism(s) we analyzed the effect of the A57–61 mutation on subcellular distribution and DNA binding properties of Bcd. Our results (Fig. 3A) show that, similar to both wild type Bcd (lanes 1 and 2) and Bcd(A52–56) (19), Bcd(A57–61) is predominantly localized to the nucleus (lanes 3 and 4). In addition, our gel shift analysis using both in vitro translated proteins (Fig. 3B) and extracts made from transfected S2 cells (Fig. 3C) revealed a normal DNA binding by Bcd(A57–61) in vitro. Dissociation constant measurements using extracts generated from transfected S2 cells (Fig. 3D) fur-

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1 The abbreviations used are: CAT, chloramphenicol acetyltransferase; GST, glutathione S-transferase; SID, Sin3A interaction domain; HDAC, histone deacetylase; PAH, paired amphipathic helix; TSA, trichostatin A.
A.

![Sequence alignment of Bcd proteins from different species. Only the sequences highly conserved between the Bcd proteins from Drosophila melanogaster (Dm-Bcd) and M. abdita (Ma-Bcd) are shown. A proline-histidine PRD repeat (not shown) is located amino-terminal to the exhibited sequences for each protein. Bcd sequences from two species that are much closer to D. melanogaster than M. abdita is, Drosophila pseudoobscura (Dp-Bcd (44)) and Musca domestica (Md-Bcd (45)), are also included in the alignment. Amino acid residues that are identical between any two proteins are shown in black. Included in the alignment.](image)

**Fig. 1.** The self-inhibitory function of Bcd is evolutionarily conserved. A, sequence alignment of Bcd proteins from different species. Only the sequences highly conserved between the Bcd proteins from Drosophila melanogaster (Dm-Bcd) and M. abdita (Ma-Bcd) are shown. A proline-histidine PRD repeat (not shown) is located amino-terminal to the exhibited sequences for each protein. Bcd sequences from two species that are much closer to D. melanogaster than M. abdita is, Drosophila pseudoobscura (Dp-Bcd (44)) and Musca domestica (Md-Bcd (45)), are also included in the alignment. Amino acid residues that are identical between any two proteins are shown in black. B, three helices of the homeodomain and the self-inhibitory domain are marked at the top. B, shown are CAT assay results from S2 cells that were transfected with the \(h_b\)-CAT reporter and various effector plasmids. *Lanes 1–4* represent the results from cells containing no activator, Bcd, Ma-Bcd, and Ma-Bcd(A43–47), respectively. To detect the weak activity of Ma-Bcd and its mutant, the CAT assay results shown here were obtained with a longer reaction time (60 min). The standard error for Ma-Bcd and Ma-Bcd(A43–47) was 5.2 and 6.3%, respectively. C, Western blot analysis showing the total amount of proteins in transfected cells.

ther revealed a comparable DNA binding affinity for wild type Bcd and Bcd(A57–61). Together these experiments demonstrate that the A57–61 mutation does not disrupt Bcd activity by altering its in vitro DNA binding or subcellular distribution properties.

**Bcd(A57–61) Can Efficiently Inhibit the Activity of Wild Type Bcd**—Because Bcd(A57–61) was virtually inactive on the \(h_b\)-CAT reporter in S2 cells (Fig. 2A, line 3), we wanted to further rule out the possibility that it is a completely defective protein due to, e.g., inadequate protein folding or subcellular localization. When co-transfected into S2 cells, Bcd(A57–61) inhibited in a dose-dependent manner the ability of wild type Bcd to activate the \(h_b\)-CAT reporter (Fig. 4). Such an inhibition was significant (5-fold) even when Bcd(A57–61) and the wild type protein were expressed from the same amount of transfected effector DNA (lane 3). These results demonstrate a dominant negative (and potentially an active repression) role of Bcd(A57–61), suggesting that this mutant protein retains its ability to access the \(h_b\)-CAT reporter in cells but is specifically defective in activating transcription.

**Mutations in Adjacent Regions of Bcd Can Exert Their Respective Effects on a Heterologous Activator**—It has been shown previously that the self-inhibitory domain of Bcd works as a functional module that does not target any specific Bcd domains and can repress the activity of heterologous activators (19). To determine whether the observed “inactivating” effect of the A67–61 mutation is a transferable property of the self-inhibitory domain of Bcd, we analyzed the activity of a hybrid activator protein, Bcd-GAL4-B6, in S2 cells. This fusion protein contains the self-inhibitory domain of Bcd (residues 1–91) linked to the heterologous activator GAL4-B6 that has the DNA binding domain of GAL4 (residues 1–147) fused to the bacterially derived activation domain B6 (26). In addition to the wild type self-inhibitory domain of Bcd, we also assayed the activity of fusion derivatives bearing the A52–56 or A57–61 mutations. These proteins were assayed for their abilities to activate a 5xGAL4-Luc reporter gene containing five GAL4 binding sites upstream of the luciferase reporter (Fig. 5A). Our results shown in Fig. 5B demonstrate that GAL4-B6 can respond to both A52–56 and A57–61 mutations. Specifically, the A52–56 mutation increased the activity of Bcd-GAL4-B6 by more than 13-fold (line 2; also see Ref. 19), whereas the A57–61 mutation reduced the activity by more than 30-fold (line 3). These results demonstrate that the two immediately adjacent regions of Bcd can exert their respective, opposite effects on an entirely heterologous activator.

**Systematic Mutation Analysis Defines Critical Residues Controlling Bcd Activity**—To further dissect individual residues that are responsible for controlling the activity of Bcd, we conducted a systematic mutagenesis analysis of the 10-amino acid motif (residues 52–61). It should be noted that this entire 10-amino acid region has an overall inhibitory function on the \(h_b\)-CAT reporter; either deletion of this region or mutation of all 10 amino acids to alanines in full-length Bcd dramatically increased its activity (Ref. 19 and data not shown). In our mutation analysis, full-length Bcd derivatives bearing single or multiple amino acid mutations were assayed for their abilities to activate the \(h_b\)-CAT reporter in S2 cells. These Bcd derivatives were accumulated in transfected cells to comparable levels as determined by a Western blot analysis (not shown).
Our mutational analysis shown in Fig. 6 reveals the following results. First, three residues of Bcd (Phe-53, Leu-56, and Phe-57) play a most critical role in repressing its activity. Mutations at these residues either individually (Mut 7, 8, 16) or in combination with other residues (Mut 1, 2, 4, and 13), significantly increased Bcd activity in S2 cells. Second, residues Asp-54, Asp-58, and Glu-59 appear to contribute positively to Bcd activity, and mutating these residues further reduced Bcd activity (Mut 9, 14, and 17–20). These results show that the 10-amino acid motif of Bcd is composed of interdigitating residues that have opposite effects on Bcd function (for a summary see Fig. 7A). Mutations in other residues only had relatively minor effects.

Our mutagenesis analysis shown in Fig. 6 also suggests that the inhibitory function conferred by the 10-amino acid motif may require the formation of an α-helix; mutation of Leu-55 to proline (L55P) weakened the inhibitory function, resulting in a Bcd derivative that is almost 6 times more active than the wild type protein (Mut 11). In addition, mutation of Asp-54 to proline (D54P; Mut 10) resulted in higher Bcd activity than mutation of this residue to alanine (D54A; Mut 9), although D54P was not more active than wild type Bcd. Finally, the mutagenesis analysis shows that a consensus C-terminal-binding protein (CtBP)-interacting motif (PLDLS) led to an increase rather than decrease in Bcd activity (Mut 12), further confirming our previous suggestion that the co-repressor CtBP is not responsible for mediating self-inhibition of Bcd (19).

The Self-inhibitory Domain of Bcd Interacts with PAH Domains of Sin3A—A careful examination of the self-inhibitory motif of Bcd indicates that it shares similarity to motifs previously shown to interact with Sin3A (27), most notably the amino-terminal half of the Sin3A interaction domain (SID) of the transcription factor P1 (Fig. 7B; also see below). Sin3A is a component of the histone deacetylase (HDAC) complex that is recruited to promoters by many transcription repressors and co-repressors including Mad1, N-CorR, SMRT, p53, MeCp2, and Ikaros (for reviews, see Refs. 27–29). Sin3A contains four paired amphipathic helix (PAH) domains that are involved in specific protein-protein interactions. Structural studies of PAH2 of mammalian Sin3A and SID of Mad1 showed that both components undergo a conformational transition upon complex formation (27). In the complex, the amphipathic helix formed from the Mad1 SID rests in a deep hydrophobic pocket of Sin3A PAH2 (27). The structural data also suggested an important role of two alanine residues on the carboxyl-terminal side of the Mad1 SID, which due to their short side chains allow close packing against the narrow pocket of PAH2. Although the self-inhibitory motif of Bcd lacks such alanine residues on its carboxyl-terminal side, mutations that make the Bcd motif more similar to the known SIDs exhibited further enhanced self-inhibitory function. For example, Mut 19 (D58A/E59A), which makes the Bcd motif more similar to the Pf1 SID, reduced Bcd activity by 15-fold (Fig. 6). In addition, Mut 14 (D58E/E59A/R60A), which has a motif more similar to the Mad1 SID, was 30 times less active than the wild type protein (Fig. 6). Furthermore, Mut 20 (E59A/R60A), which has the two important alanine residues, decreased the activity of Bcd by more than 5-fold (Fig. 6).

To determine whether the self-inhibitory domain of Bcd can interact with the PAH domains of Sin3A, we carried out a GST pull-down analysis. The self-inhibitory domain of Bcd (residues 1–91) was expressed in bacterial cells as a GST fusion protein and used to pull down in vitro translated and radioactively labeled individual PAH domains of Drosophila Sin3A. Our
results show that the self-inhibitory domain of Bcd was able to interact with PAH3 and PAH4 of Sin3A (Fig. 8, C–D, lanes 3) but not with PAH1 and PAH2 under the same conditions (Fig. 8, A–B, lanes 3). Our GST pull-down experiments using Bcd mutants further revealed a correlation between the self-inhibitory strength and its ability to interact with the PAH domains of Sin3A. First, the self-inhibitory domain of Bcd(A52–56), which has a reduced self-inhibitory function (Fig. 2), exhibited a reduced ability to interact with PAH4 (Fig. 8D, lane 4). This mutant also had a modestly reduced ability to interact with PAH3 (Fig. 8C, lane 4). Second, Mut 14, which has an enhanced self-inhibitory function (Fig. 6), exhibited an increased ability to interact with both PAH3 and PAH4 of Sin3A (Fig. 8, C–D, lanes 5). These results suggest that the self-inhibitory activity of Bcd may require the interaction between the Bcd motif and Sin3A.

The Activity of Bcd Is Increased by the HDAC Inhibitor Trichostatin A (TSA)—Because Sin3A is a component of the HDAC complex (28, 29), one possible mechanism of the self-inhibitory function of Bcd is the recruitment of HDAC activity. Previous studies show that the expression of transiently transfected reporters is sensitive to histone acetylation status (30–33). To determine whether HDAC activity is involved in inhibiting Bcd function, we performed activation assays in S2 cells in the presence or absence of the HDAC inhibitor TSA. Our results show that, consistent with a recent report (34), Bcd activity was increased by more than 6-fold in the presence of TSA (Table I). More importantly, our experiments further showed that the effect of TSA correlated with the self-inhibitory function of Bcd. In particular, TSA only modestly increased (less than 3-fold) the activity of Bcd(A52–56), which has an attenuated self-inhibitory function, whereas the activity of Bcd(A57–61), which has a strengthened self-inhibitory function, was increased by 15-fold in the presence of TSA. Together, these results suggest that HDAC activity plays an important role in repressing Bcd function.
The effect of TSA treatment was relatively modest (6-fold increase for wild type Bcd) compared with that caused by the removal or mutations of the self-inhibitory domain (up to 40-fold increase; Figs. 2 and 6, also see Ref. 19). These results suggest that Bcd activity may be repressed by both HDAC (TSA-sensitive)-dependent and -independent mechanisms. It is possible that other co-repressors in addition to Sin3A can interact with the self-inhibitory domain of Bcd (see “Discussion”). Furthermore, Sin3A or other co-repressor proteins may be able to exert their effects in both HDAC-dependent and -independent manners. The experiments shown in Fig. 3, B–D, have already demonstrated that mutations affecting the self-inhibitory function are transferable.
tory function of Bcd did not affect its DNA binding ability in the absence of co-factors, but these experiments did not address whether Bcd-interacting co-repressors such as Sin3A could affect its DNA binding. The results of a gel shift analysis shown in Fig. 9 reveal that DNA binding of Bcd was reduced by either PAH3 or PAH4 of Sin3A (lanes 1–6) but not by PAH1 or PAH2 (not shown). The repressive effect was most significant when a Sin3A fragment containing both PAH3 and PAH4 was used in the analysis (lanes 11–13). Together these results suggest that Sin3A and possibly additional factors can inhibit Bcd activity through both HDAC-dependent and -independent mechanisms.

DISCUSSION

The experiments described in this report demonstrate that Bcd activity is intricately controlled. In particular, the amino terminus of Bcd contains a self-inhibitory domain that can dramatically reduce its ability to activate the hb-CAT reporter in S2 cells. The most critical region of the self-inhibitory domain is a 10-amino acid motif composed of interdigitating residues that control Bcd activity in opposing manners (Fig. 7A). The experiments described here suggest a model in which the self-inhibitory domain of Bcd inhibits its own ability to activate transcription by recruiting a co-repressor or complex. First, mutations within this domain do not affect subcellular localization of Bcd in cells (Fig. 3); they also fail to exert intrinsic effects without co-factors on the DNA binding activity of Bcd in vitro (Fig. 3). In addition, these mutations can exert their respective effects on an entirely heterologous activator (Fig. 5). Moreover, Bcd(A67–61) can cause a dominant negative effect, inhibiting the ability of wild type Bcd to activate the hb-CAT reporter (Fig. 4). Finally, PAH domains (3 and 4) of the Drosophila Sin3A co-repressor can interact with the self-inhibitory domain of Bcd in vitro (Fig. 8, C and D).

Sin3A is a component of the HDAC co-repressor complex recruited by transcription factors to deacetylate histones at target gene promoters (27–29). Our experiments show that TSA treatment can increase Bcd activity, suggesting that the Sin3A-HDAC co-repressor complex may play a role in repressing Bcd activity when it is recruited by the self-inhibitory domain. Interestingly, another component of the Sin3A-HDAC co-repressor complex, SAP18, has been isolated as a Bcd-interacting factor (34, 35). Although the self-inhibitory domain of Bcd is not required to interact with SAP18 (19), it is possible that efficient recruitment of the Sin3A-HDAC co-repressor complex by Bcd is facilitated by multiple contacts between Bcd and the components of the complex; even for Sin3A itself, two separate domains, PAH3 and PAH4, can interact with the self-inhibitory domain of Bcd. It is also possible that a coordi-
nation of the interactions between Bcd and multiple components of the Sin3A-HDAC co-repressor complex may permit a more intricate control of Bcd activity.

Our study suggests that the activity of Bcd may be regulated by multiple mechanisms. In particular, the effect of TSA treatment is relatively modest compared with the effect of mutations in the self-inhibitory domain of Bcd. Our DNA binding experiments show that the PAH domains of Sin3A can also inhibit the DNA binding function of Bcd in a gel shift assay (Fig. 9), suggesting that Sin3A can affect Bcd activity through both HDAC-dependent and -independent mechanisms. Interestingly, the amino-terminal domain of Bcd is not essential for responding to the effect of Sin3A PAH domains in DNA binding (not shown), suggesting that multiple regions of Bcd can interact with Sin3A. Our finding is similar to a previous study demonstrating that the mammalian transcription factor Pf1 contains two separate domains interacting with PAH domains of Sin3A (36). In this context we would like to emphasize that, although our experiments suggest a role of Sin3A in regulating Bcd activity, Sin3A is probably not the only protein recognizing the self-inhibitory domain of Bcd. Recent studies reveal that a growing number of transcription factors use hydrophobic surfaces in protein interactions, relying on a common motif of 4XXΦΦ, where Φ represents hydrophobic residues (27, 37–39). The identification of three similarly situated hydrophobic residues critical to self-inhibition (FDLLE, Fig. 7A) suggests that this motif may be targeted by multiple proteins. A short motif of p53, FSDLW, has been shown to be recognized by both MDM2 and TAFII31 (38). Our finding that interdigitating residues within the Bcd motif contribute to its activity in opposing manners can also be explained by a model in which two counteracting co-factors, a co-repressor such as Sin3A and a positive co-factor, compete with each other by recognizing overlapping determinants.

In addition to self-inhibition, the amino terminus of Bcd also plays an important role in self-association and cooperative DNA binding (20, 22, 40). A recent comparison of two natural Bcd-responsive enhancers, hb and knirps (kni), demonstrates that although this domain is required for cooperative binding to the kni enhancer, it is dispensable for cooperative binding to the hb enhancer (41). Interestingly, unlike on the hb enhancer, the self-inhibitory effect is muted on the kni enhancer, suggesting that the amino-terminal domain of Bcd is differentially utilized for distinct functions on these enhancers (in this context it is worth noting that Bcd(A57–61), although virtually inactive on the hb-CAT reporter (Fig. 2), is nearly fully active on the kni-CAT reporter in S2 cells (41), further demonstrating that it is not just a “dead” protein.) We propose that the cooperative DNA binding and self-inhibitory functions conferred by the amino-terminal domain of Bcd are executed in a coordinated manner (41). On the kni enhancer, self-association between the Bcd molecules through the amino-terminal domain “masks” the determinants for the self-inhibitory function. In contrast, on the hb enhancer, this domain is exposed to interact with co-repressors such as Sin3A, leading to a reduction in the ability of Bcd to activate transcription.

A recent study suggests that the amino terminus of Bcd is also involved in inhibiting translation of the caudal (cad) mRNA in embryos (42). It is striking that residues 68–73 that are suggested to interact with the initiation factor eIF4E are very close to, but distinct from, our 10-amino acid self-inhibitory motif (residues 52–61). Interestingly, our transfection experiments reveal that mutations affecting residues 68–73 can also increase Bcd activity in S2 cells (Fig. 2, lanes 5 and 6). It remains to be determined whether these residues and our delineated 10-amino acid motif regulate Bcd transcription activity through similar or distinct mechanisms. Regardless, the finding that the amino terminus of Bcd provides multiple regulatory functions both in transcription and translation further underscores the importance of this domain in development (43). As shown by our experiments (Fig. 1) at least one such regulatory function, namely, self-inhibition, is evolutionarily conserved. Precisely how a short domain of Bcd accommodates and likely coordinates the actions of different proteins remains to be understood.

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