The Co-activator CREB-binding Protein Participates in Enhancer-dependent Activities of Bicoid*

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Bicoid (Bcd) is a transcriptional activator required for early embryonic patterning in Drosophila. Despite extensive studies, it currently remains unclear how Bcd activates transcription and what proteins participate in its activation process. In this report, we describe experiments to analyze the role of the Drosophila co-activator dCBP in Bcd-mediated activation. In Drosophila S2 cells, the Bcd activity is increased by the co-transfection of plasmids expressing dCBP and reduced by double-stranded RNA-mediated interference against dCBP. We further show that Bcd and dCBP can interact with each other and that Bcd-interacting domains of dCBP can cause dominant negative effects on Bcd activity in S2 cells. Our comparison of two Bcd-responsive enhancers, hunchback (hh) and knirps (kni), reveals a differential role of dCBP in facilitating Bcd activation. A dCBP mutant defective in its histone acetyltransferase activity exhibits a reduced, but not abolished, co-activator function for Bcd. Our chromatin immunoprecipitation experiments show that dCBP can increase not only the occupancy of Bcd itself at the enhancers but also the recruitment of general transcription factors to the promoter. Together, these experiments suggest that dCBP is an enhancer-dependent co-activator of Bcd, facilitating its activation through multiple mechanisms.

Transcription activation is an important and highly coordinated process requiring the actions of both gene-specific activators and co-activators (1–4). Transcriptional activators, upon binding to their target gene enhancers, are thought to achieve their activation function either by directly interacting with general transcription factors (GTFs) or by interacting with co-activators. One of the well-documented co-activators is CREB-binding protein (CBP), which has been shown to interact with many gene-specific activators as well as GTFs (5, 6). One function of CBP that has drawn significant attention in recent years is its ability to acetylate histones through its histone acetyltransferase (HAT) enzymatic activity. It is suggested that activator-recruited CBP can influence local chromatin structure by acetylating histones, thus enhancing promoter accessibility for GTFs. CBP and the related protein p300 can also achieve their co-activator function in a HAT-independent manner (7, 8). It is thought that it can physically bridge between activators and the transcription machinery or act as a scaffold for protein complexes containing other HAT enzymes (6).

The experiments described in this report analyze the role of dCBP in transcriptional activation by the Drosophila morphogenetic protein Bicoid (Bcd). Bcd, a homeodomain protein, is required for instructing embryonic patterning of the anterior structures, including the head and thorax (9, 10). It achieves its morphogenetic activity by activating its direct target genes in early Drosophila embryos (11–14). Despite its critical biological role and extensive previous studies, it remains unclear how Bcd activates transcription and what other factors participate in the activation process (15, 16). Another important issue on Bcd as a molecular morphogen relates to its special ability to activate different target genes in distinctive manners (13, 17–19). Only recent studies have begun to shed light on this important issue, suggesting that enhancer architecture can influence how Bcd binds DNA and activates transcription (20). Enhancer- and promoter-specific actions of transcription factors represent a fundamental issue because many factors including the tumor suppressor protein p53 can often function as both activators and repressors in a context-dependent manner (21, 22). The precise mechanisms governing these switches are not well understood, but, similar to Bcd, enhancer characteristics may play an important role (23–25).

In this report, we describe experiments demonstrating that activation by Bcd in Drosophila S2 cells is dependent on dCBP and that these two proteins physically interact with each other. Activation by Bcd from the enhancer elements of two different target genes of Bcd, hunchback (hh) and knirps (kni), is differentially affected by dCBP, and functional interactions on these enhancers are dependent on distinct Bcd and dCBP domains. A HAT-deficient mutant of dCBP remains its ability, although with a reduced efficiency, to increase the Bcd activity in S2 cells. Our chromatin immunoprecipitation (ChIP) analysis further demonstrates that dCBP can increase the occupancy of Bcd at target enhancers in cells. In addition, dCBP can increase the histone acetylation status and facilitate the recruitment of GTFs to the reporter promoter. These results suggest that dCBP participates in the activation process of Bcd through multiple mechanisms and contributes to enhancer-specific activities of Bcd.

* This study was supported by grants from the American Heart Association Grant 0255347N and National Science Foundation Grant 0323957 (to J. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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* The abbreviations used are: GTF, general transcription factor; CBP, CREB-binding protein; CREB, cAMP-response element-binding protein; HAT, histone acetyltransferase; GAT, chloramphenicol acetyltransferase; IP, immunoprecipitation; ChIP, chromatin immunoprecipitation; HA, hemagglutinin; RNAi, RNA interference; dsRNA, double-stranded RNA.
**Bcd Interacts with dCBP**

### EXPERIMENTAL PROCEDURES

**Plasmid Construction**—Plasmids used in this study and their sources are listed in Table I. Effector plasmids for Bcd and dCBP derivatives generated in this study are based on the pAc5.1/V-5-His vector (Invitrogen) and encode an N-terminal hemagglutinin (HA) tag for each protein. To construct pFY408 and pFY409, bcd coding sequences, generated as PCR products, were first cloned into the NdeI-XbaI sites of the pGEM3-based vector pFY441 (26). The HindIII-XbaI fragments were then isolated and cloned into the expression vector pFY442 (26). The GST-Bcd fusion plasmid pDF364 was generated by cloning appropriate PCR products into the EcoRI-XbaI sites of the pGEX-KG-based vector pFY441 (26). The total amount of DNA in each transfection was 10 μg as adjusted by salmon sperm DNA. Both CAT assays and quantification of PCR products used to detect the presence of the precipitated DNA sequences were as follows: hh-E5 (5′-GTCGGAC-TCTTCTACGCGACAGACGCTT-3′) and hh-E3 (5′-GGGATATATCAACGGTGGGC-3′) for the hh enhancer; hh-Core5 (5′-GTCACCTCTGCCCATCTA-3′) and hh-CAT3 (5′-CATTGCCGATATCACCACGGTGACAGACGCT-3′) for the E1b core promoter of the hh-Bcd reporter gene; and kni-E5 (5′-CTAG-GATAACATGCAGCTTGGACAT-3′) and kni-E3 (5′-TGGTCTTTATCGATGCGTTGACAT-3′) for the kni enhancer/promoter region.

### RESULTS

**Bed Activity is Regulated by dCBP in S2 Cells**—dCBP, encoded by *neji* (nej), is a co-activator for several activators including Dorsal (Dl) (34), Cubitus interruptus (Ci) (28), Mad (nedj) (32), and Deformed (Dfd) (36). Our analysis of early *even-skipped* (eve) expression revealed that a fraction of the embryos (30%) from *neji* (+) females had weaker expression for several stripes, including stripe 2 (data not shown). Since eve stripe 2 is activated by both Bed and Hb (37), it is possible that dCBP may play a role in activation by either Bed or Hb or both.

To directly analyze the role of dCBP in Bed-mediated activation, we conducted a co-transfection analysis in *Drosophila* S2
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To determine the role of endogenous dCBP on Bcd function in S2 cells, we used the double-stranded RNAi technique to inhibit endogenous dCBP expression. In this analysis, dCBP dsRNA was transfected into S2 cells prior to the transfection of the hb-CAT reporter and Bcd effector plasmids. Our experiments showed that dCBP RNAi treatment reduced the CAT reporter activity at all Bcd concentrations tested (Fig. 1C). The observed effect of dCBP RNAi treatment on reporter activity is direct and Bcd-dependent: the RNAi treatment did not alter reporter activity in the absence of Bcd (Fig. 1C, lanes 1 and 2) and had no adverse effect on the accumulated cellular levels of Bcd (Fig. 1D). In addition, dsRNA sequences that were unrelated to dCBP did not affect Bcd-induced reporter activity (Fig. 1C, inset). Together, these experiments suggest that dCBP plays a co-activator role for Bcd, directly increasing its ability to activate transcription from the hb-CAT reporter gene in S2 cells.

Bed and dCBP Interact with Each Other—To determine whether Bcd and dCBP can interact with each other, we carried out a co-IP analysis. Nuclear extracts were prepared from S2 cells that had been transfected with plasmids expressing both HA-tagged full-length Bcd and V5-tagged full-length dCBP. The extracts were subjected to co-IP using an antibody against the V5 tag, and the precipitated products were analyzed in a Western blot using an anti-HA antibody. Our results showed that Bed was co-precipitated efficiently by dCBP (Fig. 2A, lane 7). Under the same conditions, Bed was also co-precipitated efficiently by a full-length dCBP mutant (Fig. 2A, lane 8; also see below), which is defective in its HAT enzymatic activity (29).

To further analyze the interaction between Bed and dCBP, we carried out a GST pull-down analysis. Full-length Bed was expressed as a GST fusion protein in bacteria and used to pull down in vitro synthesized and radioactive labeled fragments of
dCBP. Our results showed that Bcd could pull down efficiently all three dCBP fragments tested (Fig. 2B, lane 3): 383–1090, 1677–2608, and 2040–3190 (see Fig. 2C for diagrams of dCBP derivatives). GST alone failed to pull down efficiently any of these dCBP fragments (Fig. 2B, lane 2), indicating that the detected interaction is Bcd-specific. Our deletion analysis of Bcd further revealed that the homeodomain alone was insufficient to interact with these dCBP fragments (data not shown), suggesting that Bcd protein sequences outside its homeodomain are required for efficient interaction with dCBP in vitro.

**Differential Effect of dCBP on the hh and kni Enhancer Elements**—Our recent experiments have shown that Bcd can exhibit distinctive behaviors on different enhancer elements (20). To determine whether dCBP may participate in enhancer-specific activities of Bcd, we took advantage of another reporter gene, kni-CAT, which contains the 64-bp Bed-responsive kni enhancer element (17). Our co-transfection experiments showed that, whereas dCBP increased the activity of Bcd on hh-CAT reporter by 19 fold (Fig. 3A, lanes 1 and 2), the co-activation role of dCBP on the kni-CAT reporter was only 3-fold (lanes 3 and 4). Our experiments with dCBP RNAi further confirmed that the Bcd activity on the hh-CAT reporter was more dependent on endogenous dCBP than on the kni-CAT reporter (Fig. 3B). Neither dCBP transfection nor RNAi treatment altered the reporter activities in the absence of Bcd (Fig. 1 and data not shown). Together, these results suggest that Bcd can differentially respond to the co-activator function of dCBP on different enhancers.

**Distinct Bcd and dCBP Domains Are Engaged in Functional Interactions on hh and kni Enhancer Elements**—To define Bcd domains mediating the dCBP co-activation function, we analyzed different Bcd deletion derivatives in co-activation assays (see Fig. 4C for schematic diagrams of the derivatives). We also compared the behaviors of these derivatives on hh-CAT and kni-CAT reporter genes. Whereas the entire C-terminal portion of Bcd (residues 152–489) was important in responding to dCBP on both hh-CAT and kni-CAT reporters (Fig. 4, A and B, lanes 5–8), subdomains within this portion appeared to play different roles on these two reporters. For example, Bcd-(1–246) responded to dCBP as efficiently as WT Bcd did on kni-CAT, but such response was reduced on hh-CAT (Fig. 4, A and B, compare lanes 9 and 10 with lanes 1 and 2). The N terminus of Bcd (residues 1–91) also exhibited different roles on these two reporters. Whereas the removal of this domain had little effect on the hh-CAT reporter (Fig. 4A, lanes 3 and 4), it significantly reduced both the activity of Bcd and its responsiveness to dCBP on the kni-CAT reporter (Fig. 3B, lanes 3 and 4). Further analysis suggested that residues 42–91 of Bcd were important for mediating the dCBP co-activation function on the kni-CAT reporter (Fig. 4B, compare lanes 13 and 14 with lanes 3 and 4). Together, these results suggest that different Bcd domains are differentially utilized to respond to dCBP on hh-CAT and kni-CAT reporters.

Our GST pull-down experiments described above showed that multiple dCBP domains can interact with Bcd in vitro (Fig. 2B). To determine whether individual Bed-interacting domains of dCBP may cause dominant negative effects in S2 cells, we conducted co-transfection experiments using dCBP fragments (Fig. 5). As shown in Fig. 5A, the dCBP-(1677–2608) fragment reduced the Bcd activity on the hh-CAT reporter gene in a concentration-dependent manner, up to nearly 6-fold (lane 5). In the absence of Bcd, dCBP-(1677–2608) had no effect on reporter activity (data not shown). A comparison of such dominant negative effect of three dCBP fragments revealed signif-

**Fig. 2. Bed and dCBP interact with each other.** A, shown are co-IP assay results from S2 cells transfected with plasmids expressing HA-tagged WT Bcd (1 μg) and V5-tagged dCBP (5 μg). Full-length dCBP was either WT or an HAT-deficient mutant (see below for further details). Nuclear extracts generated from transfected S2 cells were precipitated with anti-V5 antibody, and the precipitated products were subjected to a Western blotting analysis using the anti-HA antibody to detect HA-Bcd (arrowhead). Input represents one-tenth of the amount of proteins used in co-IP. B, GST pull-down assay results. The GST-Bcd fusion protein expressed in E. coli was used to pull down the dCBP fragments that had been in vitro translated and radioactively labeled. Input (lane 1) represents one-twentieth of the dCBP fragments used in the pull-down experiments. C, schematic diagrams of dCBP derivatives used in the GST pull-down experiments shown in B. The zinc domains (Zn), CREB binding domain, bromodomain (Br), and the glutamine-rich (Q-rich) domain of dCBP are also marked.
significant differences on *hb-CAT* and *kni-CAT* reporters (Fig. 5, B and C). Together, these experiments suggest that Bcd and dCBP may use different interaction modes on the *hb* and *kni* enhancer elements, relying on distinct Bcd and dCBP domains.

**Enhancer Characteristics Influence the Role of dCBP on Bcd Activity—** Both *hb* and *kni* enhancer elements contain six Bcd binding sites, but these sites are arranged differently (Fig. 6A). Whereas all of the Bcd binding sites in the *kni* enhancer element are arranged in symmetric pairs separated by short spacing (17, 38, 39), most of the Bcd binding sites in the *hb* enhancer element are arranged in a tandem fashion and are separated by long spacing (13, 38, 40). In addition, whereas all six Bcd binding sites in the *kni* enhancer element deviate from the TAATCC consensus, three of the Bcd binding sites in the *hb* enhancer element are of the consensus type. Our previous experiments have shown that increasing the spacing between the symmetric pairs of Bcd binding sites can alter the Bcd protein domains required for cooperative DNA binding and the sensitivity to a self-inhibitory function of the protein (20). These experiments suggested that enhancer architecture can influence how Bcd utilizes its domains for different functions (20, 41).

To determine whether the differential responsiveness of Bcd to dCBP on *hb* and *kni* enhancer elements is caused by enhancer characteristics, we took advantage of three modified enhancer elements (Fig. 6B). One of these elements, *kni*(6A), maintains the precise arrangement of all Bcd binding sites in the *kni* enhancer element but has all of the nonconsensus sites converted to the consensus TAATCC sites (26). The other two modified enhancer elements, *3HH* and *3TT*, contain three tightly spaced symmetric pairs of consensus Bcd binding sites; these pairs (38), either head-to-head (HH) or tail-to-tail (TT), are separated by long spacing (20). Bcd exhibited an increased sensitivity to dCBP on all three modified enhancer elements when compared with the *kni* enhancer element (Fig. 6A, lanes 5–10), with the most dramatic effect on *3TT-CAT*. In the absence of Bcd, dCBP did not increase the reporter activities (data not shown). These results suggest that the characteristics of enhancers, including both the types of the Bcd binding sites and their arrangements, contribute to enhancer-dependent responses of Bcd to dCBP.

**dCBP Increases Bcd Binding to Enhancer Elements—** An important feature of CBP is that it has an HAT activity (42), which has been suggested to play a critical role in co-activation (5, 6). To determine whether the HAT activity of dCBP is required for its co-activator role for Bcd, we took advantage of a mutant form of dCBP that is deficient in its HAT enzymatic activity (29). This mutant has a Phe-to-Ala alteration at residue 2161 (P2161A), abolishing its acetyl CoA binding site (29). As shown in Fig. 2A (lane 8), the dCBP(F2161A) mutant protein had a reduced, but not abolished, co-activator function (lane 3). Neither WT nor mutant dCBP altered reporter activities in the absence of Bcd (data not shown).

The finding that the HAT-deficient mutant of dCBP retains, at least partially, its co-activator function suggests that dCBP can increase Bcd activity through an HAT-independent mechanism(s). It is possible that dCBP may act as a co-factor of Bcd to facilitate its recognition of enhancer sequences in cells. To test this idea, we conducted a ChIP analysis to directly measure the occupancy of Bcd on the *hb* and *kni* enhancer elements in S2 cells. We varied the dCBP levels either by overexpressing dCBP or by dCBP RNAi treatment. Our experiments showed that, at low Bcd concentrations, ChIP-detected Bcd occupancy at the *hb* enhancer element was increased by dCBP overexpression but reduced by dCBP RNAi treatment (Fig. 8A, a, lanes 8...
At high Bcd concentrations, the effect of dCBP on Bcd binding to the \( \text{hb} \) enhancer element became less significant (Fig. 8A, b). The effect of dCBP on enhancer occupancy of Bcd was also reduced on the \( \text{kni} \) enhancer element regardless of the Bcd concentration (lanes 8–10, c and d).

**CBP is a co-activator interacting with many different gene-specific activators as well as GTFs (5, 6, 43).** The experiments described in this report suggest that the activity of Bcd is regulated by dCBP. Our results reveal that the co-activator role of dCBP is influenced by enhancer characteristics, suggesting that dCBP can contribute to enhancer-specific functions of Bcd. Similar to several other examples (5, 6, 43), multiple domains of Bcd and dCBP can engage in interactions (Figs. 2, 4, and 5). Interestingly, different Bcd and dCBP domains can be differ-
entially utilized on the \textit{hb} and \textit{kni} enhancer elements (Figs. 4 and 5). One of the Bcd domains, namely its N-terminal domain (residues 1–91), plays a particularly important role in contributing to enhancer-specific properties of the protein. As we have shown previously, this domain is strictly required for cooperative binding to the \textit{kni} enhancer element, but it is largely dispensable for cooperative binding to the \textit{hb} enhancer element (20). This domain also provides a self-inhibitory function that is much more robust on the \textit{hb} enhancer element than on the \textit{kni} enhancer element (20). Our results described in this report suggest that this domain may also play a more important role in responding to dCBP on the \textit{kni-CAT} reporter than on the \textit{hb-CAT} reporter. Since DNA binding is a prerequisite to activation by Bcd, we suggest that the manner in which Bcd molecules interact with each to achieve cooperative binding to a given enhancer can affect how Bcd responds to co-factors such as Sin3A and dCBP.

The experiments described in this report suggest that transcriptional activation by Bcd may be achieved through multiple mechanisms. First, several Bcd derivatives can activate (although poorly) transcription from the \textit{kni-CAT} reporter but are unable to respond to dCBP (Fig. 4B, \textit{lanes} 5–8), suggesting that Bcd may achieve its activation function through both dCBP-dependent and -independent mechanisms. In addition, dCBP itself may function as a co-activator for Bcd through multiple mechanisms. Our ChIP results show that dCBP can increase the occupancy of Bcd at the \textit{hb} enhancer element, particularly at low Bcd concentrations (Fig. 8). It can also increase the histone acetylation status and the recruitment of GTFs to the promoter. Some of these functions of dCBP (e.g. histone acetylation) reflect the direct presence of the HAT enzymatic activity of dCBP (Fig. 8B, c and d, \textit{lanes} 12 for the HAT-deficient dCBP). But other functions may not require this enzymatic activity; in particular, the occupancy of Bcd at the \textit{hb} enhancer element at low concentrations was similarly increased by the HAT-deficient mutant dCBP (data not shown). Our finding that a HAT-deficient dCBP mutant has a reduced, but not completely abolished, co-activator function supports the notion that dCBP can achieve its co-activator function through both HAT-dependent and -independent mechanisms. In our ChIP experiments, Bcd can increase the promoter occupancy of both TFIIB and TBP (Fig. 8B, a and b, \textit{lanes} 7 and 10); however, dCBP overexpression increased promoter occupancy of TFIIB more appreciably than of TBP (\textit{lanes} 11 and 12). Previous studies have shown that, whereas TBP recruitment is an important mechanism in activation in many cases examined (44–46), TBP binding to the core promoter does not always correlate with a gene’s transcription status (47–50). Since CBP and TFIIB can interact with each other (51), it is possible that dCBP can also participate in the direct recruitment of GTFs such as TFIIB.

\begin{figure}
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\includegraphics[width=\textwidth]{figure5.png}
\caption{Different dominant negative profiles of dCBP fragments on \textit{hb-CAT} and \textit{kni-CAT} reporters. A, CAT assay results from S2 cells transfected with 1 \(\mu\)g of \textit{hb-CAT} reporter plasmid, 0.1 \(\mu\)g of Bcd effector plasmid, and the indicated amounts of an effector plasmid expressing the dCBP fragment (residues 1677–2608). The dominant negative effect of this dCBP fragment is indicated as -fold change. B and C, CAT assay results from S2 cells transfected with 1 \(\mu\)g of \textit{hb-CAT} (B) or \textit{kni-CAT} (C) reporter plasmid, 0.1 \(\mu\)g of Bcd effector plasmid, and effector plasmids expressing the indicated dCBP fragments (5 \(\mu\)g). See Fig. 2D for diagrams of the dCBP fragments.}
\end{figure}
One of our major motivations for performing this current study was our earlier finding that the activity of Bcd is inhibited by co-repressor complexes such as Sin3A-HDAC (27, 52). We reasoned that the inhibitory function of the HDAC complexes most likely would counteract the positive role of a HAT co-activator activity (or activities). Our current results support this suggestion and help identify dCBP as an important co-activator for Bcd. Our experiments show that overexpression of dCBP can partially restore the activity of Bcd(A52–56) on the hb-CAT reporter in S2 cells.2 This derivative is normally inactive on this reporter due to a strengthened self-inhibitory func-

2 D. Fu and J. Ma, unpublished data.
performed in S2 cells to detect the occupancy of Bcd on the regulating transcription.

Without (c) or 1.0 μg (panels b and d) Bcd effector plasmid, with (+) or without (−) the dCBP effector plasmid (5 μg). Lane 10 represents results from cells treated with dCBP dsRNA. Anti-HA antibody was used to precipitate the cross-linked Bcd-DNA complexes, and the precipitated enhancer DNA was detected by PCR. Input represents the RNAi protocol; and Chun Han for the pBSK-Dfz2 control vector.

ACKNOWLEDGMENTS—We thank Dr. Sarah Smolik for nep1 flies and dCBP clones; Dr. Jim Kadonaga for antibodies against GTFs; Drs. Sarah Smolik, Hailan Zhang, Steve Small, Dan Wiginton, and Tso-Pang Yao for discussions; Dr. Sean Li for the ChiP protocol; Dr. Lori Pile for the RNAi protocol; and Chun Han for the pBSK-Dfz2 control vector.

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J. Biol. Chem. 2004, 279:48725-48733.
doi: 10.1074/jbc.M407066200 originally published online September 8, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M407066200

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