Nucleic Acids Research, 2005, Vol. 33, No. 13 ***3985–3993***
doi:10.1093/nar/gki691

Interplay between positive and negative activities that influence the role of Bicoid in transcription

Dechen Fu and Jun Ma*

Division of Developmental Biology, Cincinnati Children's Hospital Research Foundation, Graduate Program in Molecular and Developmental Biology, University of Cincinnati College of Medicine,
3333 Burnet Avenue Cincinnati, OH 45229, USA

Received April 1, 2005; Revised May 20, 2005; Accepted June 16, 2005

ABSTRACT

The *Drosophila* mophogenetic protein Bicoid (Bcd) can activate transcription in a concentration-dependent manner in embryos. It contains a self-inhibitory domain that can interact with the co-repressor Sin3A. In this report, we study a Bcd mutant, Bcd(A57–61), which has a strengthened self-inhibitory function and is unable to activate the *hb-CAT* reporter in *Drosophila* cells, to analyze the role of co-factors in regulating Bcd function. We show that increased concentrations of the co-activator dCBP in cells can switch this protein from its inactive state to an active state on the *hb-CAT* reporter. The C-terminal portion of Bcd(A57–61) is required to mediate such activity-rescuing function of dCBP. Although capable of binding to DNA *in vitro*, Bcd(A57–61) is unable to access the *hb* enhancer element in cells, suggesting that its DNA binding defect is only manifested in a cellular context. Increased concentrations of dCBP restore not only the ability of Bcd(A57–61) to access the *hb* enhancer element in cells but also the occupancy of the general transcription factors TBP and TFIIB at the reporter promoter. These and other results suggest that an activator can undergo switches between its active and inactive states through sensing the opposing actions of positive and negative co-factors.

INTRODUCTION

Regulation of gene transcription plays a critical role in many biological processes that range from cell growth and differentiation to embryonic patterning (1,2). Genes that participate in these biological processes need to be specifically turned on or off by transcription factors at the appropriate time and location. It is becoming increasingly clear that many transcription factors can act as both activators and repressors in a context-dependent manner [reviewed in (3)]. Promoter/enhancer architecture and cellular levels of other proteins have been suggested to play roles in influencing a transcription factor's regulatory functions, but the precise mechanisms in most cases remain largely unclear. For proteins that can work as both activators and repressors, they have three distinct activity states: active, repressive and inactive (neither active nor repressive). In contrast, for proteins that work only as activators, such as the *Drosophila* protein Bicoid (Bcd), they only have two activity states: active and inactive. Analysis of these proteins can thus help us understand the important question of how the simple on–off switches of activator activities are achieved. Bcd is a well-documented protein that undergoes such on–off activity switches in a concentration-dependent manner (see below). The experiments described here suggest another mechanism in which the opposing actions of positive and negative co-factors can facilitate Bcd to switch between its active and inactive states in a manner that is independent of Bcd concentration.

Bcd is a molecular morphogen that plays a critical role in patterning embryonic structures, including the head and thorax (4,5). This 489 amino acids transcription factor contains a homeodomain (residues 92–151) in its N-terminal portion (6). Bcd, which is distributed in the early embryo as an anterior-to-posterior gradient, is responsible for activating specific target genes in a concentration-dependent manner. For example, *orthodenticle* (*otd*), *hunchback* (*hb*) and *knirps* (*kni*) are direct Bcd target genes that are required for patterning the head, thoracic and abdominal structures, respectively (7). These genes are expressed in distinct parts of the embryo by responding to different Bcd concentrations (8–10). Bcd has the ability to bind DNA in a highly cooperative manner (11–14), and it has been suggested that the affinity of Bcd binding sites in an enhancer can determine the concentration of Bcd required

*To whom correspondence should be addressed. Tel: +1 513 636 7977; Fax: +1 513 636 4317; Email: jun.ma@cchmc.org
Present address:
Dechen Fu, Department of Molecular and Cellular Biology, University of California, Berkeley, CA 94720, USA

for activating transcription (9,15,16). Our recent studies suggest that the arrangements of Bcd binding sites in an enhancer can also play a critical role in regulating the activity of Bcd and contributing to its concentration-dependent action (17).

CBP is a co-activator that interacts with many transcription factors and participates in the activation process (18,19). Its histone acetyltransferase (HAT) enzymatic activity is thought to alter chromatin structure by acetylating the histone tails thus increasing the accessibility of DNA for both gene-specific transcription factors and general transcription factors (GTFs). CBP can also play a structural role by bridging between transcription factors and GTFs or by recruiting other HAT activities (18,19). In *Drosophila*, dCBP has been shown to be a co-activator for Ci (20), Mad (21) and Dorsal (22). dCBP also plays a role in facilitating Bcd to activate transcription (23). dCBP and Bcd can interact with each other through distinct domains on different enhancers. In particular, on the *hb* enhancer element the C-terminal portion of Bcd plays an important role in responding to the co-activation function of dCBP, whereas on the *kni* enhancer element, the N-terminal domain plays an important role (23).

In addition to its ability to interact with co-activators, such as dCBP, Bcd can also interact with co-repressors. An analysis of the N-terminal region of Bcd revealed a self-inhibitory domain (residues 52–91) that can dramatically inhibit the ability of Bcd to activate transcription (24). For example, on the *hb-CAT* reporter gene which contains the Bcd-responsive *hb* enhancer element, a Bcd derivative lacking the entire N-terminal domain, Bcd(92–489), exhibits an activity 40 times higher than the full-length protein in *Drosophila* S2 cells. A systematic analysis of the self-inhibitory domain identified a 10 amino acid motif (residues 52–61) that is most critical for the self-inhibitory function. Interestingly, mutations of different residues in this motif can cause drastically opposing effects (25). In particular, the mutant protein Bcd(A52–56), which has residues 52–56 changed to alanines, is 25 times more active than wt Bcd on the *hb-CAT* reporter in S2 cells. In contrast, on the same reporter another mutant, Bcd(A57–61), which has the neighboring five amino acids changed to alanines, is virtually inactive (<2% of wt Bcd activity) at all concentrations. The co-repressor Sin3A has been shown to interact with the evolutionarily conserved N-terminal domain of Bcd, and it is proposed that mutations that alter the 10 amino acid motif can weaken or strengthen this interaction, thus increasing or decreasing, respectively, the activity of Bcd (25). Another component of the Sin3A-HDAC (histone deacetylase) complex, SAP18, has also been shown to interact with Bcd, apparently through multiple Bcd domains [(24,26); see Figure 1A for a schematic diagram of Bcd domains interacting with co-factors].

In this report, we use Bcd(A57–61), an inactive protein on the *hb-CAT* reporter in S2 cells, as a tool to analyze

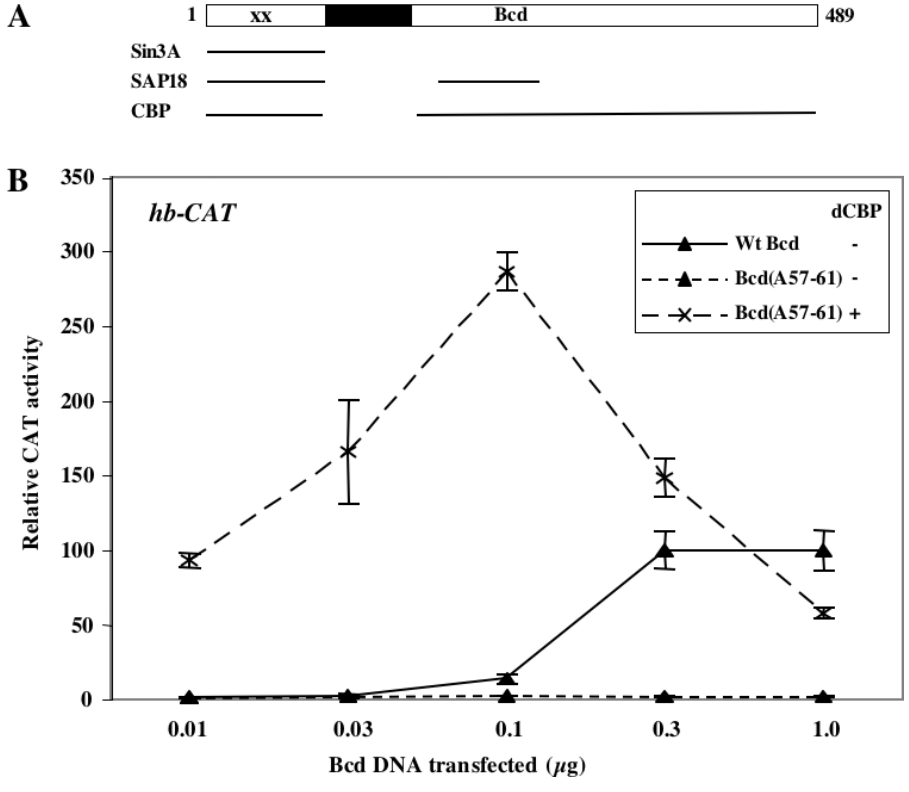

Figure 1. Exogenous dCBP switches the activity states of Bcd(A57–61) in S2 cells. (**A**) Shown is a schematic diagram of Bcd and its interacting domains with co-factors. The homeodomain (residues 92–151) of the 489 amino acid Bcd protein is marked with a black box and the two neighboring mutations discussed in this report, A52–56 and A57–61, are each marked with an 'X'. The interaction information in this diagram is based on (25) for Sin3A, (24,26) for SAP18 and (23) for CBP. The diagram is not drawn to scale. (**B**) Shown are CAT assay results in S2 cells that were transfected with the reporter plasmid *hb-CAT* (1 μg), the indicated amounts of effector plasmids expressing wt Bcd or Bcd(A57–61), with (+) or without (−) another effector plasmid (5 μg) expressing dCBP. Fold activation by wt Bcd (at 1 μg transfected DNA) without exogenous dCBP was set to 100.

the interplay between positive and negative co-factors in regulating Bcd function. This mutant exhibits some special properties. In particular, while it is inactive on the *hb-CAT* reporter gene, it can activate another reporter gene, *kni-CAT* (17). These and other findings suggest that the activity state of this protein is intricately controlled, and we sought to gain a better understanding of this mutant protein by focusing on the roles of, and the interplay between, Bcd interacting co-factors. In this report, we show that increased concentrations of dCBP in S2 cells can switch this protein from an inactive state to an active one on the *hb-CAT* reporter. We further show that the C-terminal domain of Bcd(A57–61) mediates such activity-rescuing function of dCBP. We provide evidence demonstrating that, despite its normal DNA binding ability *in vitro*, Bcd(A57–61) fails to occupy the *hb* enhancer element in cells. High levels of dCBP in S2 cells restore the ability of Bcd(A57–61) to access the *hb* enhancer element and enable this Bcd derivative to recruit the GTFs TBP and TFIIB to the target promoter. We also provide evidence suggesting that dCBP may negatively affect the interaction between Bcd and Sin3A in cells. Together, these results demonstrate that dCBP plays an important role in regulating Bcd function in a dCBP concentration-dependent manner. They suggest that the opposing actions of positive and negative co-factors can facilitate Bcd to switch between its active and inactive states in a manner that is independent on Bcd concentration.

MATERIALS AND METHODS

Plasmid construction

Plasmids expressing Bcd derivatives were generated in two steps as described previously (23). The *bcd* gene was first modified on pFY441, a pGEM3-based plasmid containing wt *bcd* linked to the coding sequence of the hemagglutinin (HA) tag, and then transferred to pFY442, a plasmid expressing HA-tagged wt Bcd from the *Drosophila actin 5C* promoter (24). For Bcd(1–246; A57–61), the pGEM3-based plasmid was pDF333 and the expression plasmid was pFD347. Reporter genes and the effector plasmids pFY443 [Bcd(1–246)] and pFY465 [Bcd(A57–61)] have been described previously (14,24). The expression plasmids of wt and mutant dCBP were kindly provided by Dr S. Smolik.

Transient transfection assays

Drosophila S2 cells were transfected with plasmids by the calcium phosphate co-precipitation method as described by Invitrogen. The total amount of DNA in each transfection was adjusted to 10 μg by salmon sperm DNA. In order to monitor the transfection efficiency, 1 μg control plasmid *pCopia-lacZ* was co-transfected in each experiment, and both CAT assays and western blot analyses were normalized according to the β-galactosidase activity. CAT activity was measured as previously described by using three independently transfected samples for each experiment (14). The protein levels of Bcd derivatives with or without dCBP were detected by western blot using anti-HA antibody (1:500 final dilution, Babco). Double-strand RNA against endogenous dCBP in S2 cells was generated as described previously (23), and the RNAi treatment did not affect the accumulation of Bcd in S2 cells.

Gel shift assays

The *hb* enhancer probe for gel shift experiments was released from a plasmid and filled-in with Klenow in the presence of [α-^{32}P]dCTP as described previously (17). Wild-type Bcd and its derivative used in these assays were expressed *in vitro* by using the TnT quick-coupled transcription/translation system (Promega). The experimental procedures and conditions for gel shift assays were described previously (17).

Chromatin-immunoprecipitation (ChIP) assays

ChIP assays were performed according to Fu *et al*. (23). The presence of Bcd, GTFs and acetylated histones at the *hb* enhancer-core promoter region was detected by PCR using primers *hb*-core5 and *hb-CAT*3 as described previously (23).

Co-immunoprecipitation (Co-IP)

Co-IP experiments were performed as described previously (23). Briefly, nuclear extracts prepared from S2 cells were incubated with anti-HA antibody (1:100 final dilution) in IP buffer (20 mM Tris–HCl, pH 8.0, 160 mM MgCl2, 0.1% Nonidet P-40 and 10% glycerol). The precipitated products were resolved by SAS–PAGE gel and detected by western blot using anti-Sin3A antibodies [kindly provided by Drs Lori Pile and David Wassarman (27)]. Quantitation of the co-IP data shown in Figure 5 was conducted as follows. The intensities of input and co-IP Sin3A bands for each sample were measured to obtain an intensity ratio of co-IP product over input. For each experiment, the ratio for Bcd transfection alone (lane 4) was arbitrarily set to 100 to allow comparison of data from independent experiments.

RESULTS

High levels of dCBP switch Bcd(A52–61) to an active state

Our previous transfection experiments in S2 cells have shown that Bcd(A57–61) has a strengthened self-inhibitory function and is nearly completely inactive on the *hb-CAT* reporter gene (17,25). This mutant protein is stably accumulated in cells (25), suggesting that its inability to activate *hb-CAT* reflects a distinct functional state of this protein rather than its defects in protein stability. Unlike wt Bcd, which exhibited a dose-dependent activation function in transfection assays (Figure 1B, solid line), this mutant protein failed to activate *hb-CAT* at all concentrations tested (Figure 1B, dashed line, bottom). To determine whether high concentrations of the co-activator dCBP might counteract the strengthened self-inhibitory function and switch Bcd(A57–61) to an active state, we conducted co-transfection experiments. In these experiments, the ability of Bcd(A57–61) to activate *hb-CAT* was measured in the presence or absence of dCBP exogenously expressed from a transfected plasmid.

Our results showed that exogenous dCBP dramatically rescued the activity of Bcd(A57–61) on *hb-CAT*, increasing its activity by 29 to 110 fold depending on Bcd concentration (Figure 1B, dashed line, top; also see Table 1). In the presence of exogenous dCBP, the activity of Bcd(A57–61) at several concentrations was higher than wt Bcd at its saturating concentrations (without exogenous dCBP). Table 1 lists the effect

of dCBP on wt Bcd and Bcd(A57–61), further indicating that Bcd(A57–61) responds to dCBP much more robustly than wt Bcd does at all concentrations tested. As shown previously, exogenous dCBP has no effect on reporter gene expression in the absence of Bcd and does not alter the amount of Bcd protein in cells (23). Together, these results suggest that dCBP is a limiting co-factor for Bcd(A57–61) and is capable of making this Bcd protein to switch between its inactive and active states on the *hb-CAT* reporter in cells.

Rescue of Bcd(A56–61) activity by dCBP requires the C-terminal domain of Bcd

It has been shown that Bcd and dCBP can physically interact with each other (23). Deletion analysis further suggested that the C-terminal half of Bcd plays an important role in responding to the co-activator function of dCBP on the *hb-CAT* reporter (23). To determine whether this domain is required for mediating the activity-rescuing function of dCBP, we analyzed the effect of exogenous dCBP on a truncated derivative of Bcd, Bcd(1–246). Two versions of Bcd(1–246), with either wt or the A57–61 mutation at its N-terminus, were used in the experiments. As shown previously (23), the truncated derivative Bcd(1–246) responded to dCBP modestly (Figure 2A). However, dCBP failed to rescue the activity of the truncated, mutant protein Bcd(1–246; A57–61) at all concentrations tested (Figure 2A). dCBP did not affect the accumulated levels of the Bcd proteins (Figure 2B). These results suggest that dCBP rescues the activity of Bcd(A57–61) through the C-terminal domain of Bcd.

Table 1. The effect of dCBP on wt Bcd and Bcd(A57–61)

DNA transfected (μg)	Effect of dCBP (fold increase)	
	wt Bcd	Bcd(A57–61)
0.01	17	58
0.03	19	72
0.1	4.8	110
0.3	4.7	61
1.0	7.5	29

Listed is the effect (fold increase) of dCBP on wt Bcd and Bcd(A57–61) in activating the *hb-CAT* reporter gene in S2 cells. The amount of transfected DNA refers to the plasmids expressing the Bcd derivatives. The data for wt Bcd and Bcd(A57–61) are from Fu *et al*. (23) and Figure 1B, respectively.

Defect of Bcd(A57–61) in *hb* enhancer recognition in cells but not *in vitro*

Bcd(A57–61) has a normal ability to bind to a single TAATCC site when analyzed *in vitro* (25). To determine whether this mutant protein might be defective in recognizing natural enhancer elements that contain multiple Bcd binding sites, we conducted gel shift studies using the *hb* enhancer element. As shown previously (17), wt Bcd bound to this enhancer element in a cooperative manner, forming protein–DNA complexes that contained multiple Bcd molecules (Figure 3, lanes 1–4). Our gel shift experiments using Bcd(A57–61) showed that this mutant protein can bind to the *hb* enhancer element in a manner comparable with the wt Bcd protein

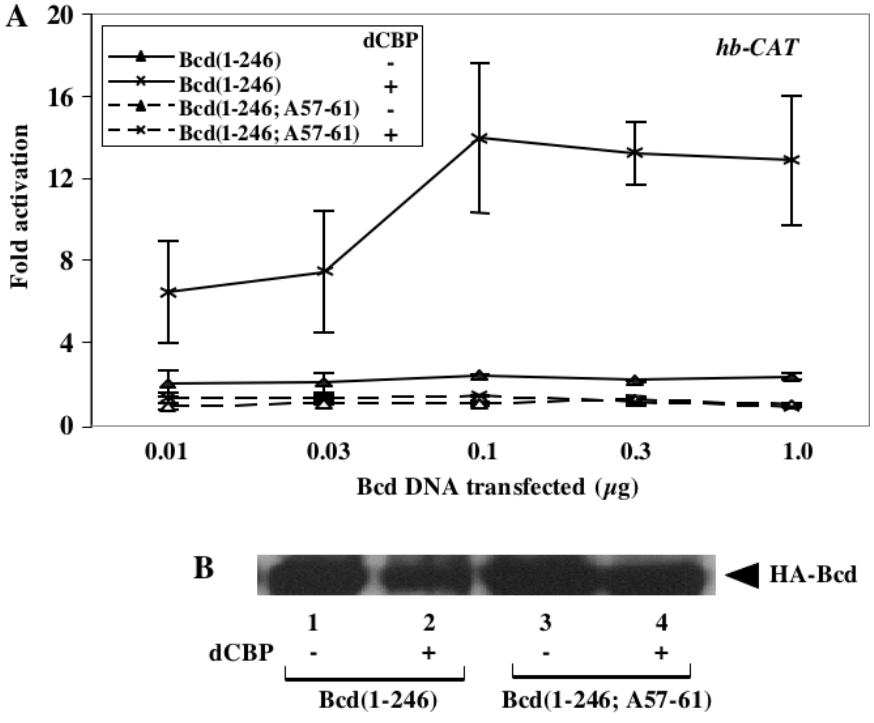

Figure 2. Switch of Bcd(A57–61) activity states by dCBP requires Bcd C-terminal domain. (**A**) Shown are CAT assay results in S2 cells that were transfected with the reporter plasmid *hb-CAT* (1 μg), the indicated amounts of effector plasmids expressing two different Bcd derivatives, with (+) or without (−) the effector plasmid (5 μg) expressing dCBP. The two Bcd derivatives are Bcd(1–246), a truncated Bcd with a wt N-terminus; Bcd(1–246; A57–61), a truncated derivative with the A57–61 mutation in its self-inhibitory domain. Fold activation for each assay, measured by CAT activity, is shown in the figure. (**B**) Western blot data showing the HA-tagged Bcd protein levels (1 μg transfected DNA) in the presence (+) or absence (−) of dCBP (5 μg transfected DNA).

(Figure 3, lanes 5–8). Bcd(A57–61) also bound to another natural enhancer element, *kni*, in a cooperative manner similar to the wt Bcd protein *in vitro* (data not shown). These results further support our conclusion that the A57–61 mutation of Bcd does not abolish the protein's ability to recognize DNA *in vitro* (25).

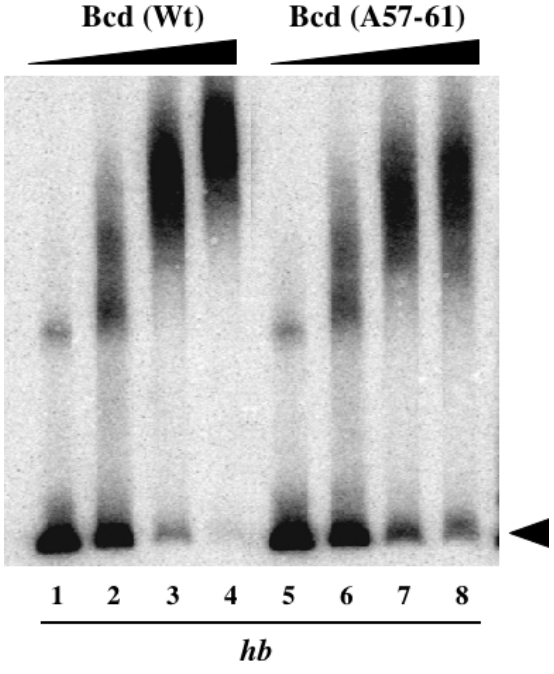

Figure 3. Enhancer element binding by Bcd(A57–61) *in vitro*. Gel shift data showing DNA binding to the *hb* enhancer element by wt Bcd (lanes 1–4) and Bcd(A57–61) (lanes 5–7). Free probe is indicated by an arrowhead (bottom right). In the absence of Bcd, there were no shifted complexes detected (data not shown).

To further dissect the defects of Bcd(A57–61), thus helping understand the mechanisms of the functional rescue by dCBP, we carried out a ChIP analysis in cells. We compared the occupancy of wt Bcd and Bcd(A57–61) at the *hb-CAT* reporter. We specifically chose conditions in which Bcd proteins were expressed at high levels to reveal functional defects of the mutant Bcd that could not be overcome by increased Bcd concentrations (also see Figure 1B for reporter assay data). As shown previously (23), our ChIP experiments detected a significant occupancy of wt Bcd at the *hb* enhancer element of the reporter gene [Figure 4B (a), lane 9]. In contrast, Bcd(A57–61) failed to exhibit an occupancy above background levels at the *hb* enhancer element in the same ChIP assays [Figure 4B (a), lane 11]. Together, these results suggest that Bcd(A57–61), despite its normal ability to bind DNA *in vitro*, has a functional defect in accessing the *hb* enhancer element in cells.

dCBP restores the occupancy of Bcd(A57–61) at *hb* enhancer in cells

To determine whether dCBP can affect the ability of Bcd(A57–61) to access the *hb-CAT* reporter gene in cells, we conducted ChIP experiments in the presence of exogenously expressed dCBP (see Figure 4A for a schematic diagram of the reporter gene). As shown by the ChIP data [Figure 4B (a), lanes 11 and 12], dCBP restored the occupancy of Bcd(A57–61) at the *hb* enhancer element in cells [Figure 4B (a), lane 12]. Under the conditions of high Bcd concentrations, dCBP had little effect on wt Bcd (lanes 9 and 10) as shown previously (23).

Our ChIP experiments also revealed a restored occupancy of GTFs at the *hb-CAT* reporter caused by high levels of dCBP. In the absence of exogenous dCBP, Bcd(A57–61) failed to enhance the occupancy of either TBP or TFIIB at the promoter region [Figure 4B (b and c), compare lanes 7, 9 and 11]. In the presence of exogenous dCBP, Bcd(A57–61) increased the

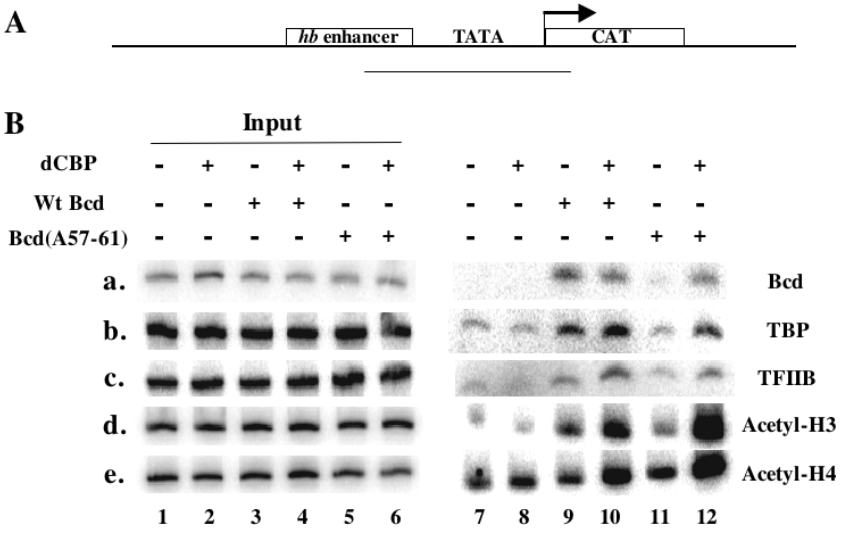

Figure 4. Restored occupancy of Bcd(A57–61) and GTFs by exogenous dCBP. (**A**) Shown is a schematic diagram of the reporter gene, marking the promoter region (thin line) used for detection by PCR in ChIP assays. The diagram is not drawn to scale. (**B**) Shown are ChIP data from S2 cells that were transfected with plasmids expressing the indicated effectors [dCBP proteins, 5 μg; Bcd(A57–61), 1 μg] and the *hb-CAT* reporter plasmid (1 μg). Antibodies used for ChIP assays were: HA to detect HA-tagged Bcd (panel a), TBP (panel b), TFIIB (panel c), acetyl-H3 (panel d) and H4 (panel e). Lanes 1–6 show input controls, which represent the PCR product of 1% of the total isolated DNA used in the ChIP assays.

occupancy of both TFIIB and TBP (lane 12). Finally, our ChIP experiments showed that dCBP increased the acetyl-H3 and H4 levels at the reporter in the presence of either wt Bcd or Bcd(A57–61) [Figure 4B (d and e), lanes 9–12]. In all the cases, the effects of dCBP required the presence of Bcd or its derivative (compare lanes 7 and 8), indicating that these observed effects represent Bcd-dependent functions of dCBP. Together, these results reveal not only a restored occupancy, caused by increased dCBP levels in cells, of Bcd(A57–61) at the *hb-CAT* reporter but also an elevated recruitment of GTFs and an increased histone acetylation level at the reporter.

dCBP may negatively affect Bcd–Sin3A interaction in cells

As further detailed in Discussion (below), several models are consistent with our finding that high levels of dCBP can restore activity to Bcd(A57–61). For example, it is possible that dCBP and Sin3A may compete for Bcd interaction, thus representing antagonistic forces to influence Bcd function. To determine whether the interaction between Bcd and Sin3A might be affected by dCBP, we conducted co-IP experiments in cells with altered dCBP levels. To reduce cellular levels of dCBP, we used an RNAi approach, which has been shown to specifically affect Bcd activity without altering the amount of Bcd in cells (23). We used exogenously expressed dCBP to increase its cellular levels. As shown in our co-IP experiments (Figure 5A), the amount of Sin3A precipitated by Bcd was increased by dCBP RNAi treatment (lane 6) and marginally affected by dCBP overexpression (lane 5). Figure 5B shows the quantitation of the data from three independent experiments (relative amounts of co-IP Sin3A for lanes 4, 5 and 6 are 100, 87 ± 16 and 276 ± 88, respectively). These results suggest that dCBP may negatively affect the interaction between Bcd and Sin3A. The modest effect of dCBP on Sin3A–Bcd interaction suggests that such an antagonistic effect may represent only one of the several individually weak mechanisms by which dCBP rescues the activity of Bcd(A57–61) (see Discussion for further details).

The HAT-deficient mutant dCBP can partially rescue Bcd(A57–61) activity

Our previous experiments have shown that dCBP can increase the activity of wt Bcd through both HAT-dependent and -independent mechanisms (23). A HAT-independent action of dCBP suggests a structural role of this protein in regulating Bcd activity, a suggestion consistent with the observed negative effect of dCBP on Bcd-Sin3A interaction (Figure 5). To specifically determine whether the HAT activity of dCBP is required for its ability to restore function to Bcd(A57–61) on the *hb-CAT* reporter, we used a HAT-deficient mutant of dCBP (28); both wt dCBP and this mutant protein are accumulated to similar levels when expressed in S2 cells (23). As shown in Figure 6, this mutant dCBP increased partially the activity of wt Bcd on the *hb-CAT* reporter [lanes 4–6; also see (23)]. It also rescued, though with a reduced efficiency, the activity of Bcd(A57–61) on the *hb-CAT* reporter (lanes 7–9). Together, these results suggest that dCBP can play an enzyme activity-independent role in rescuing the activity of Bcd(A57–61) on the *hb-CAT* reporter in cells.

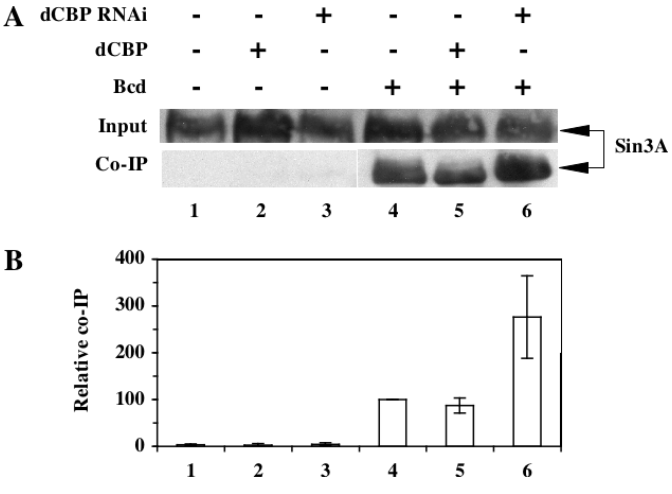

Figure 5. Interaction between Bcd and Sin3A may be affected by dCBP. (**A**) The interaction between HA-tagged Bcd and the endogenous Sin3A in S2 cells was detected by a co-IP analysis (see Materials and Methods for details). Sin3A co-precipitated by anti-HA antibodies was detected by western blot using anti-Sin3A antibodies. S2 cells were either transfected (+) or not (−) with the indicated plasmids expressing HA-Bcd (1 μg) and dCBP (5 μg), and had either been subject (+) or not (−) to dCBP RNAi treatment (25 μg dsRNA). In this figure, lanes 1–3 are controls showing that no Sin3A was precipitated in the absence of HA-Bcd. Input represents one-tenth of total nuclear extract used in the co-IP assay as described previously (23). (**B**) The relative amounts of the co-IP Sin3A product in three independent experiments were quantified (see Materials and Methods) and the results are shown (mean ± SD); all lanes in this graph correspond to those in (A).

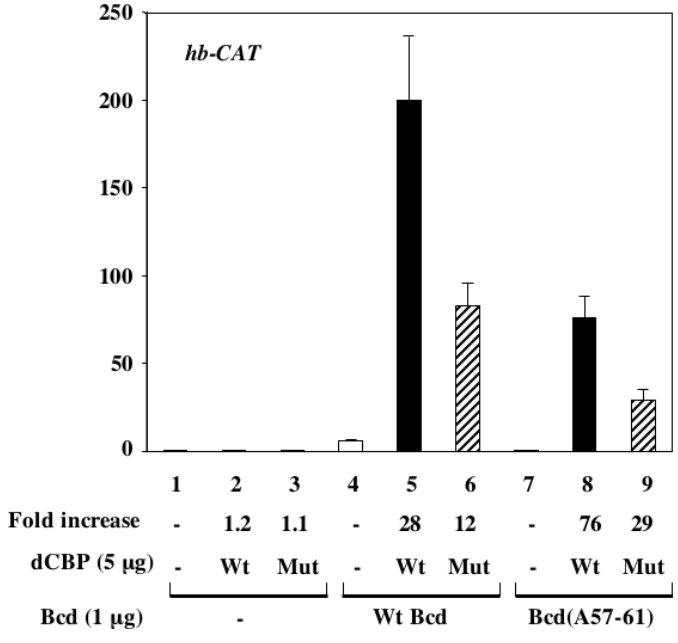

Figure 6. HAT-deficient dCBP can partially rescue the activity of Bcd(A57–61). Shown are CAT assay results in S2 cells that were transfected with the reporter plasmid *hb-CAT* (1 μg), the effector plasmids (1 μg) expressing the indicated Bcd proteins, with (+) or without (−) another effector plasmid (5 μg) expressing dCBP. The increase of Bcd activity by wt and mutant dCBP proteins is also indicated in the figure as fold increase. The exogenously expressed wt and mutant dCBP proteins are accumulated at similar levels in S2 cells as described previously (23).

DISCUSSION

As a molecular morphogen, Bcd can undergo switches, in a concentration-dependent manner, between its active and inactive states in activating transcription of its target genes. The experiments described in this report suggest another mechanism that can facilitate on–off switches of Bcd activity in a Bcd concentration-independent manner. In particular, the mutant Bcd(A57–61) is incapable of activating the *hb-CAT* reporter gene in S2 cells at all concentrations tested (Figure 1B). The inability of this mutant Bcd to activate the *hb-CAT* reporter reflects a distinct functional state of this protein rather than its defects in protein stability. In fact, this same mutant protein is only modestly weaker than the wt protein on another reporter gene, *kni-CAT*, which contains the Bcd-responsive *kni* enhancer element (17). These and other results suggested that the A57–61 mutation may cause its functionally inactive state on *hb-CAT* by more efficiently interacting with a co-repressor protein(s), such as Sin3A and its associated complex(es) (24,25). The experiments described in this report show that increased concentrations of dCBP can restore activity to Bcd(A57–61) on the *hb-CAT* reporter in cells. These results suggest that the opposing actions of positive and negative co-factors can facilitate Bcd to switch between its active and inactive states in a manner that is Bcd concentration-independent.

Although Bcd(A57–61) can bind to both a single site and natural enhancer elements *in vitro*, it is unable to access the *hb* enhancer element in cells (Figures 3 and 4). These results suggest that the DNA binding defect of this mutant protein is only manifested in a cellular context. This notion is consistent with our finding that the PAH domains of Sin3A do not exhibit any increased ability to reduce DNA binding by Bcd(A57–61) *in vitro* when compared with wt Bcd (data not shown) (25). We propose that other co-repressors or those that are associated with Sin3A, such as the HDACs, can reduce the ability of Bcd to access a natural enhancer in cells. It is possible that the enzymatic HDAC activity that is more stably associated with Bcd(A57–61) makes it unable to negotiate with histones for accessing DNA. It is also possible that a more stable Bcd-co-repressor complex may sterically hinder the interaction between Bcd(A57–61) molecules and prevent cooperative binding to the enhancer element in cells.

The most striking finding of this report is that high levels of dCBP can switch Bcd(A57–61) from its inactive state to an active one on the *hb-CAT* reporter in cells. Our ChIP data further show that dCBP increases both the ability of Bcd(A57–61) to access the *hb* enhancer element in cells and the occupancy of GTFs at the reporter promoter (Figure 4B). How does dCBP switch the activity states of Bcd(A57–61) on *hb-CAT* in cells? Since Bcd and dCBP can physically interact with each other through multiple domains (23) (Figure 1A), it is possible that dCBP may increase the DNA binding ability of Bcd in cells by stabilizing the interaction between Bcd molecules and thus enhancing its cooperativity. It is also possible that dCBP may physically compete with co-repressor complexes in interacting with Bcd. Our co-IP results suggest that dCBP may negatively affect the interaction between Bcd and Sin3A in cells (Figure 5). dCBP could also play a role in facilitating the interaction between Bcd and the transcription machinery. For all these actions, dCBP may play a structural (rather than enzymatic) role (Figure 6). Finally, the fact that the HAT-defective mutant of dCBP does have a reduced ability to restore activity to Bcd(A57–61) (Figure 6) indicates that its enzymatic activity has a positive role, possibly through modifications of histones. It is likely that dCBP can affect the Bcd(A57–61) activity through multiple mechanisms that may be weak individually (Figures 5 and 6) but, when combined, can lead to a dramatic switch from its inactive state to an active one on the *hb-CAT* reporter in cells.

Currently, it is poorly understood how precisely Bcd activates transcription. Previous studies suggest that much of its activation function is conferred by the C-terminal portion of Bcd (16,29). This portion of the protein contains several domains, including the acidic, glutamine-rich and alanine-rich domains, that are characteristic of activation domains capable of interacting with components of the transcription machinery (16,29–31). Interestingly, the alanine-rich domain previously thought to play an activation role was shown recently to exhibit an inhibitory function instead (32). The C-terminal domain of Bcd can also interact with dCBP (23), and our results show that this domain is responsible for mediating the activity-switching function of dCBP (Figure 2). Although much of the activation function of Bcd is provided by its C-terminal domain, the N-terminal portion of the protein also contains some activation function. Studies have shown that Bcd(1–246), a derivative lacking the entire C-terminal portion of Bcd, can rescue the bcd^{-} phenotype when expressed at high levels (33). These results suggest that Bcd can achieve its activation function through multiple domains presumably by interacting with different proteins, including co-activators and components of the transcription machinery. The results described in this report further support the importance of dCBP in facilitating activation by Bcd.

Bcd is a morphogenetic protein whose behavior can be regulated not only by its own concentration but also by the enhancer architecture (17). Our recent experiments show that, on the *kni* and *hb* enhancer elements, the N-terminal domain of Bcd is preferentially used for either cooperative DNA binding or self-inhibition, respectively (17). We propose that the interaction between Bcd molecules bound to the *kni* enhancer element, through its N-terminal domain, can interfere with its interaction with co-repressors, such as Sin3A. As described in this report, co-activators such as dCBP and co-repressors such as Sin3A can also functionally antagonize each other, possibly by competing for Bcd interaction as part of the mechanisms (Figure 5). Bcd is more sensitive to the self-inhibitory function on the *hb* enhancer element than on the *kni* enhancer element (17); consistent with dCBP's antagonistic role, dCBP increases the activity of Bcd more robustly on the *hb* enhancer element than on the *kni* enhancer element (23). However, the interplay between positive and negative activities that regulate Bcd functions is probably far more complex than the simple physical competition: as already discussed above, dCBP can affect Bcd activity through multiple mechanisms in both HAT-dependent and independent manners (Figure 6) (23). Moreover, in the presence of exogenous dCBP, high levels of Bcd(A57–61) cause a reduction in its activity on the *hb-CAT* reporter in cells (Figure 1B), a reduction that is not observed with wt Bcd (23), suggesting that the optimal concentration ratio between Bcd and dCBP may vary depending on the strengths of the self-inhibitory function and

interaction with co-repressors. In addition, high concentrations of dCBP can rescue the inactive derivative Bcd(A57–61), but not another inactive derivative lacking the C-terminal portion, Bcd(1–246; A57–61), suggesting that the Bcd–dCBP interaction strength can also influence the balance between positive and negative activities that regulate Bcd function.

The experiments described in this report suggest that an activator's function is subject to intricate controls by both positive and negative activities in cells. A fine balance between these activities is critical for normal cellular and developmental processes. Our transgenic experiments show that both Bcd(A57–61), which has a strengthened self-inhibitory function, and Bcd(A52–56), which has a weakened self-inhibitory function, cause embryonic defects [(24) and unpublished data]. In addition, embryos with reduced dCBP activity exhibit defects in early expression patterns of a Bcd target gene, even-skipped [(23) and Y. Wen, A. York and J. Ma, unpublished data]. Finally, a recent study reveals that mutations affecting SAP18, a component of the Sin3A-HDAC complex, can alter Bcd function and anterior patterning in embryos (34). In addition to the co-factors discussed here (Sin3A, dCBP and SAP18), Bcd likely has the ability to interact with many other proteins, including not only regulatory proteins but also components of the transcription machinery (30,31). Precisely how all these different proteins harmoniously regulate and facilitate the execution of Bcd functions during development remains to be determined. Recent studies have shown that the Bcd gradient in embryos possesses a strikingly sophisticated ability to activate its target genes in a precise manner (35–37). These findings further underscore the need of intricate control mechanisms that facilitate Bcd to switch between its active and inactive states in target gene activation. Our studies suggest that on–off switches of Bcd activity can be achieved not only in a Bcd concentration-dependent manner but also in a Bcd concentration-independent manner. It remains to be investigated whether and how Bcd interacting proteins, including those yet to be identified, participate in the precision control of target gene activation during development.

ACKNOWLEDGEMENTS

The authors thank Dr S. Smolik for providing the dCBP clones used in this study, Drs Lori Pile, David Wassarman and James Kadonaga for providing necessary antibodies, and Dr D. Wiginton, members of this laboratory, and anonymous reviewers for comments on the manuscript. This work was supported in part by grants from NSF, NIH, AHA and DOD. Funding to pay the Open Access publication charges for this article was provided by NSF.

Conflict of interest statement. None declared.

REFERENCES

1. Levine,M. and Tjian,R. (2003) Transcription regulation and animal diversity. *Nature*, **424**, 147–151.
2. Kadonaga,J.T. (2004) Regulation of RNA polymerase II transcription by sequence-specific DNA binding factors. *Cell*, **116**, 247–257.
3. Ma,J. (2005) Crossing the line between activation and repression. *Trends Genet*., **21**, 54–59.
4. Driever,W. (1992) The Bicoid morphogen: concentration dependent transcriptional activation of zygotic target genes during early *Drosophila* development. In McKnight,S.L. and Yamamoto,K. (eds), *Transcriptional Regulation*. Cold Spring Harbor Laboratory Press, NY, pp. 1221–1250.
5. Ephrussi,A. and Johnston,D.S. (2004) Seeing is believing: the bicoid morphogen gradient matures. *Cell*, **116**, 143–152.
6. Berleth,T., Burri,M., Thoma,G., Bopp,D., Richstein,S., Frigerio,G., Noll,M. and Nüsslein-Volhard,C. (1988) The role of localization of bicoid RNA in organizing the anterior pattern of the *Drosophila* embryo. *EMBO J*., **7**, 1749–1756.
7. Rivera-Pomar,R. and Jackle,H. (1996) From gradients to stripes in *Drosophila* embryogenesis: filling in the gaps. *Trends Genet*., **12**, 478–483.
8. Driever,W. and Nüsslein-Volhard,C. (1988) The bicoid protein determines position in the Drosophila embryo in a concentration-dependent manner. *Cell*, **54**, 95–104.
9. Gao,Q. and Finkelstein,R. (1998) Targeting gene expression to the head: the Drosophila orthodenticle gene is a direct target of the Bicoid morphogen. *Development*, **125**, 4185–4193.
10. Rivera-Pomar,R., Lu,X., Perrimon,N., Taubert,H. and Jackle,H. (1995) Activation of posterior gap gene expression in the *Drosophila* blastoderm. *Nature*, **376**, 253–256.
11. Ma,X., Yuan,D., Diepold,K., Scarborough,T. and Ma,J. (1996) The *Drosophila* morphogenetic protein Bicoid binds DNA cooperatively. *Development*, **122**, 1195–1206.
12. Yuan,D., Ma,X. and Ma,J. (1996) Sequences outside the homeodomain of bicoid are required for protein–protein interaction. *J. Biol. Chem*., **271**, 21660–21665.
13. Burz,D.S., Pivera-Pomar,R., Jackle,H. and Hanes,S.D. (1998) Cooperative DNA-binding by Bicoid provides a mechanism for threshold-dependent gene activation in the *Drosophila* embryo. *EMBO J*., **17**, 5998–6009.
14. Zhao,C., Dave,V., Yang,F., Scarborough,T. and Ma,J. (2000) Target selectivity of bicoid is dependent on non-consensus site recognition and protein–protein interaction. *Mol. Cell. Biol*., **20**, 8112–8123.
15. Driever,W., Thoma,G. and Nüsslein-Volhard,C. (1989) Determination of spatial domains of zygotic gene expression in the *Drosophila* embryo by the affinity of binding site for the bicoid morphogen. *Nature*, **340**, 363–367.
16. Struhl,G., Struhl,K. and Macdonald,P.M. (1989) The gradient morphogen bicoid is a concentration-dependent transcriptional activator. *Cell*, **57**, 1259–1273.
17. Fu,D., Zhao,C. and Ma,J. (2003) Enhancer sequences influence the role of the amino-terminal domain of Bicoid in transcription. *Mol. Cell. Biol*., **23**, 4439–4448.
18. Goodman,R.H. and Smolik,S. (2000) CBP/p300 in cell growth, transformation, and development. *Genes Dev*., **14**, 1553–1577.
19. Chan,H.M. and La Thangue,N.B. (2001) p300/CBP proteins: HATs for transcriptional bridges and scaffolds. *J. Cell Sci*., **114**, 2363–2373.
20. Akimaru,H., Chen,Y., Dai,P., Hou,D.X., Nonaka,M., Smolik,S.M., Armstrong,S., Goodman,R.H. and Ishii,S. (1997) *Drosophila* CBP is a co-activator of cubitus interruptus in hedgehog signalling. *Nature*, **386**, 735–738.
21. Waltzer,L. and Bienz,M. (1999) A function of CBP as a transcriptional co-activator during Dpp signalling. *EMBO J*., **18**, 1630–1641.
22. Akimaru,H., Hou,D.X. and Ishii,S. (1997) *Drosophila* CBP is required for dorsal-dependent twist gene expression. *Nature Genet*., **17**, 211–214.
23. Fu,D., Wen,Y. and Ma,J. (2004) The co-activator CREB-binding protein participates in enhancer-dependent activities of Bicoid. *J. Biol. Chem*., **279**, 48725–48733.
24. Zhao,C., York,A., Yang,F., Forsthoefel,D.J., Dave,V., Fu,D., Zhang,D., Corado,M.S., Small,S., Seeger,M.A. and Ma,J. (2002) The activity of the *Drosophila* morphogenetic protein Bicoid is inhibited by a domain located outside its homeodomain. *Development*, **129**, 1669–1680.
25. Zhao,C., Fu,D., Dave,V. and Ma,J. (2003) A composite motif of the *Drosophila* morphogenetic protein bicoid critical to transcription control. *J. Biol. Chem*., **278**, 43901–43909.
26. Zhu,W., Foehr,M., Jaynes,J.B. and Hanes,S.D. (2001) *Drosophila* SAP18, a member of the Sin3/Rpd3 histone deacetylase complex, interacts with Bicoid and inhibits its activity. *Dev. Genes Evol*., **211**, 109–117.

27. Pile,L.A. and Wassarman,D.A. (2000) Chromosomal localization links the SIN3-RPD3 complex to the regulation of chromatin condensation, histone acetylation and gene expression. *EMBO J.*, **19**, 6131–6140.
28. Ludlam,W.H., Taylor,M.H., Tanner,K.G., Denu,J.M., Goodman,R.H. and Smolik,S.M. (2002) The acetyltransferase activity of CBP is required for wingless activation and H4 acetylation in *Drosophila melanogaster*. *Mol. Cell Biol.*, **22**, 3832–3841.
29. Driever,W., Ma,J., Nusslein-Volhard,C. and Ptashne,M. (1989) Rescue of bicoid mutant *Drosophila* embryos by bicoid fusion proteins containing heterologous activating sequences. *Nature*, **342**, 149–154.
30. Sauer,F., Hansen,S.K. and Tjian,R. (1995) DNA template and activator-coactivator requirements for transcriptional synergism by *Drosophila* bicoid. *Science*, **270**, 1825–1828.
31. Sauer,F., Hansen,S.K. and Tjian,R. (1995) Multiple TAFIIs directing synergistic activation of transcription. *Science*, **270**, 1783–1788.
32. Janody,F., Sturny,R., Schaeffer,V., Azou,Y. and Dostatni,N. (2001) Two distinct domains of Bicoid mediate its transcriptional downregulation by the Torso pathway. *Development*, **128**, 2281–2290.
33. Schaeffer,V., Janody,F., Loss,C., Desplan,C. and Wimmer,E.A. (1999) Bicoid functions without its TATA-binding protein-associated factor interaction domains. *Proc. Natl Acad. Sci. USA*, **96**, 4461–4466.
34. Singh,N., Zhu,W. and Hanes,S.D. (2005) Sap18 is required for the maternal gene bicoid to direct anterior patterning in *Drosophila melanogaster*. *Dev. Biol.*, **278**, 242–254.
35. Houchmandzadeh,B., Wieschaus,E. and Leibler,S. (2002) Establishment of developmental precision and proportions in the early *Drosophila* embryo. *Nature*, **415**, 798–802.
36. Spirov,A.V. and Holloway,D.M. (2002) Making the body plan: precision in the genetic hierarchy of Drosophila embryo segmentation. *In Silico. Biol.*, **3**, 89–100.
37. Lucchetta,E.M., Lee,J.H., Fu,L.A., Patel,N.H. and Ismagilov,R.F. (2005) Dynamics of *Drosophila* embryonic patterning network perturbed in space and time using microfluidics. *Nature*, **434**, 1134–1138.