Development 122, 3499-3508 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
DEV5059

Neither the homeodomain nor the activation domain of Bicoid is specifically required for its down-regulation by the Torso receptor tyrosine kinase cascade

Yohanns Bellaïche*,†, Rupa Bandyopadhyay*,¶ Claude Desplan§ and Nathalie Dostatni‡

Howard Hughes Medical Institute, The Rockefeller University, New York NY 10021, USA

*The first two authors have contributed equally to this work
†Present address: HHMI, 200 Longwood Avenue, Boston MA 02115, USA
‡Present address: IBDM, Luminy, Case 907, 13280 Marseille cedex 9, France
§Author for correspondence
¶Present address: SKI, 1275 York Avenue, New York, NY 10021

SUMMARY

Bicoid (Bcd) is a maternal morphogen responsible for patterning the head and thorax of the *Drosophila* embryo. Correct specification of head structure, however, requires the activity of the Torso receptor tyrosine kinase cascade, which also represses expression of Bcd targets at the most anterior tip of the embryo. Here, we investigate the role of both the homeodomain (HD) and the activation domain of Bcd in the anterior repression of its targets. When a Bcd mutant protein whose HD has been replaced by the Gal4 DNA-binding domain is expressed in early embryos, a reporter gene driven by Gal4 DNA-binding sites is first activated in an anterior domain and then repressed from the anterior pole. The down-regulation of Bcd-Gal4 activity requires *torso* function but does not depend on endogenous *bcd* activity, indicating that the Bcd protein alone and none of its targets is required to mediate the effect of *torso*. Functional analysis of a chimeric protein, whose activation domain has been replaced by a generic activation domain, indicates that the activation domain of Bcd is also not specifically required for its down-regulation by Torso. We propose that Torso does not affect the ability of Bcd to bind DNA, but instead directs modification of Bcd or of a potential Bcd co-factor, which renders the Bcd protein unable to activate transcription.

Key words: Bicoid, transcription, RTK signal transduction cascade, *Drosophila*

INTRODUCTION

Determination of the anteroposterior axis of the *Drosophila* embryo requires the activity of the anterior, the posterior and the terminal systems (StJohnston and Nüsslein-Volhard, 1992). The maternal determinants of each system control the patterned expression of a particular set of zygotic genes in the developing embryo (Nüsslein-Volhard et al., 1987). These zygotic genes, in turn, define specific domains along the anteroposterior axis by providing positional information (reviewed in StJohnston and Nüsslein-Volhard, 1992). Formation of the head and the thorax is dependent on the activity of the Bicoid (Bcd) maternal morphogen (Frohnhöfer and Nüsslein-Volhard, 1986). During oogenesis, the maternal *bcd* mRNA is localized at the anterior tip of the egg (Berleth et al., 1988). Upon egg laying, this mRNA is translated, and its protein product, a homeodomain-containing transcription factor, diffuses along the anteroposterior axis of the syncytial blastoderm to form a concentration gradient (Driever and Nüsslein-Volhard, 1988a,b). It has been proposed that *bcd* zygotic target genes respond to different threshold levels of the Bcd protein, depending on the affinity of Bcd-binding sites in their promoters (Driever et al., 1989b; Struhl et al., 1989).

The localized expression of zygotic genes by Bcd in distinct domains is a simple example of how morphogens could control pattern formation at the transcriptional level depending on their concentration and their transcriptional activity. Several observations indicate that other factors are required to allow Bcd to perform its function. First, another morphogen, the product of the maternal *hunchback* (*hb*) gene, acts synergistically with Bcd to activate *bcd* targets (Simpson-Brose et al., 1994). Second, although Bcd levels are highest during the process of cellularization, expression of *bcd* targets, such as *orthodenticle* (*otd*) and *hb*, retracts from the anterior pole (Finkelstein and Perrimon, 1990; Tautz, 1988; Ronchi et al., 1993). The repression of *bcd* targets at the anterior tip of the embryo depends on the activity of the receptor tyrosine kinase Torso (Tor), a member of the terminal system required for the specification of the most anterior and posterior unsegmented regions of the embryo (Klingler et al., 1988). Expression of a Bcd variant immune to the terminal system gives rise to a dominant female sterile phenotype with embryos exhibiting head defects (Ronchi et al., 1993). This strongly suggests that the interaction between the anterior (*bcd*) and the terminal (*Torso*) systems is required for correct head development.

The activity of the terminal system depends on the activa-

tion of the Torso receptor at both poles of the embryo by a ligand locally activated by the polar follicle cells (Sprenger and Nüsslein-Volhard, 1992). This process leads to the sequential activation of the Ras GTPase and the Raf, MEK and MAP protein kinases (Perrimon and Desplan, 1994). This latter kinase is presumed to act on an unknown preexisting transcription factor (product of gene Y) (StJohnston and Nüsslein-Volhard, 1992) leading to the zygotic expression of the terminal gap genes *tailless* (*tll*) and *huckebein* (*hkb*) (Pignoni et al., 1990; Weigel et al., 1990). The maternal terminal genes *Torso*, *D-Raf* and *D-sor* (MEK kinase), but not the zygotic terminal gap genes, *tll* and *hkb*, are required for the anterior repression of *bcd* targets, *otd* and *hb* (Ronchi et al., 1993; N. D. and C.D., unpublished data). Interestingly, down-regulation of Bcd transcriptional activity correlates with hyperphosphorylation of the Bcd protein in response to Torso activation (Ronchi et al., 1993). We have proposed that Torso-dependent phosphorylation of Bcd or a Bcd co-factor is responsible for the down-regulation of Bcd activity at the anterior tip (Ronchi et al., 1993). Alternatively, a transcriptional repressor, activated by the *torso* pathway, or by *bcd*, could directly compete with Bcd for binding and prevent activation of its targets genes. In particular, any homeodomain protein with a lysine at position 50 (K50), such as Orthodenticle (Finkelstein et al., 1990), Sine oculis (Cheyette et al., 1994) or D-Goosecoid (Goriely et al., 1996, Hahn and Jäckle, 1996), could directly compete with Bcd in this way (Hanes and Brent, 1989; Treisman and Desplan, 1989; Treisman et al., 1992). Although *otd* is not required for the *Torso*-dependent down-regulation of Bcd activity (Ronchi et al., 1993), other Bcd target genes encoding homeodomain-containing proteins could still be involved in this process.

In this study, we aimed to distinguish between these hypotheses. We first compared the DNA-binding activities present in nuclear extracts from wild-type embryos versus embryos lacking *torso* activity: no difference was observed in the ability of these two types of extracts to bind to a single Bcd site in a gel shift assay. This suggests that the activity of the *torso* pathway does not affect the ability of Bcd to recognize its target site. It also suggests that it is unlikely that the terminal system induces the binding activity of another protein with the same DNA-binding specificity as Bcd, which could compete with it.

In order to rule out the involvement of a competing repressor, we generated a change-of-specificity mutant of Bcd by replacing its homeodomain with the Gal4 DNA-binding domain (Bcd-Gal4). In the embryo, in response to this chimeric gene, a Gal4 reporter transgene (UAS_{Gal4}-*lacZ*) is first activated in an anterior domain, which later retracts from the anterior at cellularization. The anterior repression of the Gal4 reporter is not observed in embryos lacking *torso* activity and its expression pattern is not affected in embryos lacking endogenous *bcd* activity. We conclude that (i) Bcd activity is down-regulated by *torso*, (ii) no *bcd* target is required to mediate *torso*-dependent down-regulation of Bcd and (iii) the Bcd homeodomain is not required for this effect. We also show that the Bcd activation domain can be replaced by a generic activation domain without affecting the sensitivity of the protein to *torso*. We propose that the mechanism by which the *torso* cascade down-regulates Bcd activity is unlikely to involve Bcd DNA binding and/or competition by another factor for binding to Bcd sites. Instead, it is due to a modification that prevents Bcd from transcriptionally activating its targets. This modification, which does not specifically require the activation domain of Bcd, could be achieved either by direct modification of Bcd or by modification of a Bcd co-factor.

MATERIALS AND METHODS

Nuclear extracts from embryos and transfected Schneider cells

Embryos from wild-type and tor^{PM} homozygous females were collected at 25°C for 0 to 4 hours. Purification of nuclei and nuclear protein extraction were performed as described in Ronchi et al. (1993). The embryos were dechorionated and washed before being homogenized in 300 mM Sucrose, 10 mM Hepes (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM dithiothreitol (DTT), 0.5 mM PMSF, 5 μg/ml leupeptin, 2 μg/ml pepstatin, 15 μg/ml aprotinin, 1% low fat milk. Nuclei were purified by centrifugation on a 1.7 M sucrose cushion in the same buffer. Isolation of nuclei from transfected Schneider cells was performed as described in Müller et al. (1989). Briefly, 48 hours after transfection with 10 μg of producer plasmid (pPAC or pPACBcd), cells were harvested, washed three times with PBS and resuspended in 1 ml of cold lysis buffer (10 mM Hepes, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 5 μg/ml leupeptin, 2 μg/ml pepstatin, 15 μg/ml aprotinin). Deionized Nonidet P-40 was added to the cell suspension at a final concentration of 0.5% and cells were disrupted by vigorous shaking for 30 seconds. Nuclei were recovered by gentle centrifugation (1000 revs/minute) for 5 minutes. Nuclear protein extraction was performed by resuspending the purified nuclei from embryos or transfected cells in NEB (0.42) containing phosphatase inhibitors (20 mM Hepes, 25% glycerol, 0.25 mM EDTA, 0.42 M NaCl, 12,5 mM β-glycerophosphate, 0.05 mM NaF, 0.5 mM DTT, 0.5 mM PMSF, 5 μg/ml leupeptin, 2 μg/ml pepstatin, 15 μg/ml aprotinin). Protein concentration was determined by Bradford assay using BSA as a standard. 15-20 μg of nuclear extract protein were used in each gel-shift reaction.

DNA-binding assays

The double-stranded oligonucleotide (5′-CTAGTCTAATCCCA-3′/5′-CTAGTGGGATTAGA-3′ was annealed, labeled with ^{32}P using Klenow enzyme and used as a specific probe in a gel-shift assay. Binding reactions were carried out in a volume of 20 μl in the presence of 10 mM Tris (pH 7.5), 10% glycerol, 0.1 mM EDTA, 0.2 M NaCl, 0.1 % NP40 in the presence of 1 μg of salmon sperm DNA, 1 μg of poly(dI-dC) and 0.5 ng of labeled probe. In competition experiments, 25 ng of unlabelled double-stranded oligonucleotides were added to the incubation. Before use, the double-stranded specific oligonucleotide (5′-CTAGTCTAATCCCA-3′/5′-CTAGTGGGATTAGA-3′) and the double-stranded non-specific oligonucleotide (5′-CTAGTCTAATTGAA-3′/5′-CTAGTTCAATTAGA-3′) were repaired by Klenow. To reduce non-specific binding of nuclear proteins to the probe, preincubations were performed between proteins and DNA competitors (salmon sperm DNA, poly (dI-dC), specific and non-specific double-stranded oligo-competitors) for 10 minutes at room temperature. The specific probe was added for 5 minutes and reactions were then loaded on a low ionic strength 6% acrylamide (29:1) gel containing 0.25× TBE. In experiments with antibodies, 1 μl of a 1/4 dilution of either Bcd antiserum (Driever and Nüsslein-Volhard, 1988b) or preimmune serum was added to the incubation.

Plasmids

To allow swapping of the Bcd homeodomain with the Gal4 DNA-binding domain, *Bcl*I restriction sites have been introduced by site

directed mutagenesis at each side of the homeodomain (position 2301 and 2482) in the Bcd genomic sequence (Berleth et al., 1988). The mutations do not affect protein sequence. To prepare single-stranded DNA for mutagenesis, the Bcd genomic sequence including all the coding sequence from the *Pst*I restriction site (position 1241) to the *Sma*I restriction site (position 4261) has been introduced in the *Xho*I site of pKS using linkers with the following sequences: 5′-TCTAGGGCCGGATCTGCA/5′GATCCGGCCC and 5′-GGGAATTCGGATCCGGCCC/5′-TCGAGGGCCGGATCCGAATTCCC. The sequences of these linkers are such that the Bcd genomic sequence (positions 1241 to 4261) can be easily isolated as a *Bam*HI/*Bam*HI cassette. The sequence of the oligonucleotides used for creating the *Bcl*I restriction sites on each side of the Bcd homeodomain was 5′-CGGGTGCGACGTG**A**TC**AA**CCGCATCACCAGAG-3′ (for the position 2301) and 5′-CCTTGTGCTGATC**A**GATTGGATCTTGTGACG-3′ (for position 2473).

The sequences coding for the Gal4(2-74) and Gal4(2-94) DNA binding domain were amplified by PCR and inserted in the *Bcl*I restriction sites created in the Bcd genomic sequence.

The sequence of the oligonucleotides used for the amplification of these fragments was: 5′-GCCGGTGATCAAGCTACTGTCTTCTATCGAAC-3′ (hybridizing to positions 446 to 466 of the *Gal4* gene corresponding to amino acids 2 to 7), 5′-GGGCCTGATCAATCATGTCAAGGTCTTCTCG-3′ (hybridizing to positions 661 to 680 of the *Gal4* gene corresponding to amino acids 69 to 74) and 5′-TAGCCTGATCATGTTAACAATGCTTTTATATC-3′ (hybridizing with the positions 703 to 724 of the *Gal4* gene corresponding to amino acids 88 to 94). After mutagenesis and swapping, the mutated genomic sequence of Bcd was isolated as a *Bam*HI/*Bam*HI cassette. The sequence was then either introduced into the unique *Bam*HI restriction site of pPAC (Krasnow et al., 1989), the protein producer plasmid used for transfection experiments, or introduced in the unique *Bgl*II site of the pCaSpeRbcdBglII plasmid provided to us by Wolfgang Driever (Driever et al. 1990). The latter plasmid is a pCaSpeR derivative which carries the 2 kb maternal *bcd* promoter fragment from a *Bam*HI site to a *Pst*I site at position 1,244 and the genomic fragment from a *Sma*I site at 4,292 to and *Eco*RI site at 5,900. These fragments span the promoter, 5′ and 3′ non-coding fragments of *bcd.* In pCaSpeRBcdBglII, a unique *Bgl*II site has been generated just 3′ to the transcription start site and can be used to introduce any mutated form of the Bcd coding sequence.

The Bcd responder plasmid (*Bcd3-lacZ*) contains three Bcd DNA-binding sites inserted upstream of the hsp70 promoter driving the CAT gene (Krasnow et al., 1989; Ronchi et al., 1993). The Bcd-Gal4 chimeric protein responder plasmid contains 5 copies of the optimized Gal4 DNA-binding site (Brand and Perrimon, 1993) upstream of the same hsp70 promoter driving the CAT gene.

Tissue culture and transactivation assays

Drosophila Schneider 2 cells were grown in M3 medium supplemented with 12% fetal calf serum. Transfection was done at 50-70% confluence by the calcium phosphate procedure (Wigler et al., 1979) using 10 μg of producer plasmid, 1 μg of reporter plasmid and 1 μg of the hsp82/*lacZ* used as a control for transfection efficiency per 10 cm plate. Cells were harvested 48 hours after the transfection, washed three times with PBS, resuspended in 250 mM Tris (pH 7.5) and lysed by freeze-thawing three times. The supernatant was used in the assay. CAT assays were performed by incubating for 1 hour at 37°C 150 μl of 0.25 M Tris (pH 7.5), 20 μl of extract, 10 μl of 4 mM AcCoA (Boehringer) and 3 μl of [14C] Chloramphenicol (DuPont). The reaction was stopped by extraction with ethyl acetate. The extract was lyophilized and spotted on a TLC plate (Eastman Chromatogram) and run in 19:1 chloroform/methanol. Radiolabeled chloramphenicol and acetylated chloramphenicol were quantified with a PhosphorImager using the integrate volume function. For each transfection, CAT activities were normalized according to β-gal activities measured according to Miller (1972).

Drosophila stocks and transgenic lines

The host strain used for injection was *Df(1)6w⁻y⁻*. The DNA was injected at a concentration of 300 μg/ml in TE, together with 75 μg/ml of the helper plasmid pΔ2-3 (Laski et al., 1986). Mutant alleles used were bcd^{E1} (Frohnhöfer and Nüsslein-Volhard, 1986), tor^{PM} (Klingler et al., 1988) and $Bcd^{E1}tsl^{035}$ (Struhl et al., 1992). The fly lines carrying reporter genes for the Bcd-Gal4 chimeric protein were kindly provided by Andrea Brand and Norbert Perrimon. The Gal4 reporter strain used was UAS*lacZ*-2, which carries the P-element insertion in the 2nd chromosome. The transgene contains the Adh-*lacZ* fusion gene subcloned behind Gal UAS, a fragment containing five optimized Gal4-binding sites and a synthetic TATA box (Brand and Perrimon, 1993).

In situ hybridization

RNA probes for in situ hybridization were prepared as described in (Simpson-Brose et al., 1994). Briefly, the cDNA sequences of *lacZ*, *hb*, *otd* and *tll* have been inserted into the polylinker of pBlueScript KSII (Stratagene). 1 μg of each plasmid was linearized and transcribed with T3 or T7 RNA polymerase to produce antisense RNA in 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 10 mM NaCl, 2 mM Spermidine, 1 mM ATP, GTP, CTP, 0.6 mM UTP, 0.4 mM digoxigenin-UTP (Boehringer Mannheim), 5 mM DTT, 50 U of RNasin for 2 hours at 37°C. The product of the reaction was then hydrolyzed 40 minutes at 65°C in 60 mM Na_2CO_3, 40 mM $NaHCO_3$ (pH 10.2) and precipitated with 0.4 M LiCl and 3 volumes of ethanol. In situ hybridization on whole-mount embryos was performed as originally described by Tautz and Pfeifle (1989). The protocol used was adapted from M. Klinger. Proteinase K treatment was performed for 3 minutes (50 μg/ml). Prehybridization and hybridization were performed at 70°C at pH 5. The anti-digoxigenin antibody (Boehringer) was coupled to alkaline phosphatase. Embryos were mounted in methyl salicilate: Canada Balsam(1:2) and photographed using Nomarski optics.

RESULTS

Bcd DNA-binding activity is not modified by the Torso cascade

To analyze the effect of the Torso cascade on the DNA-binding activity of Bcd, gel-shift assays were performed using nuclear extracts from collections of embryos (0-4 hours) derived from wild-type and tor^{PM} homozygous females. As shown on Fig. 1 (lanes 11 to 20), one major protein-DNA complex (A) was detected in extracts from wild-type embryos and from embryos lacking *torso* activity (lanes 11 and 16) using an 18 bp probe containing a unique Bcd DNA-binding site (Driever and Nüsslein-Volhard, 1989). In both cases, the formation of complex A was reduced by addition of a 50-fold excess of cold oligonucleotide containing the sequence TCTTAATCCC, specific for the binding of a homeodomain with a lysine at position 50 (compare lane 11 to lane 12 and lane 16 to lane 17, Fig. 1). In contrast, the appearance of complex A remained unchanged by addition of a 50-fold excess of a cold oligonucleotide containing the sequence TCTTAATTGA, specific for the binding of a homeodomain with a glutamine at position 50 (compare lane 11 to lane 13 and lane 16 to lane 18, Fig. 1) (Treisman et al., 1989; Hanes and Brent, 1989). Addition of a Bcd polyclonal antiserum (Driever and Nüsslein-Volhard, 1988b) to the incubation almost completely abolished the appearance of complex A (compare lane 11 to lane 14 and lane 16 to lane 19, Fig. 1) whereas addition of a preimmune serum

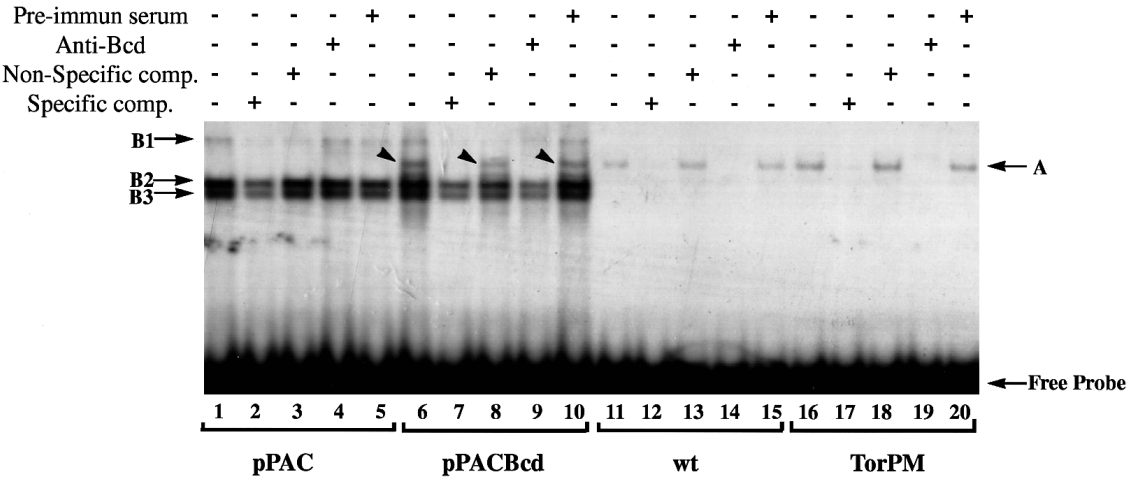

Fig. 1. Bcd DNA-binding activity in nuclear extracts of transfected Schneider cells and embryos. Band shift assays were performed with extracts from wild-type embryos (wt) and extracts from embryos lacking Torso activity (*torso*^{PM}). The DNA probe was the 18-mer double-stranded oligonucleotide CTAGTCTAATCCCACTAG that contained a unique Bcd DNA-binding site (underlined) (Driever et al., 1989b). Each reaction was performed in the presence of 15-20 μg of nuclear proteins and 0.5 ng of the labeled probe. Lanes 2, 7, 12 and 17, 25 ng of the cold specific double-stranded oligonucleotide (5′-CTAGTCTAATCCCA/5′-CTAGTGGGATTAGA) were added to the incubation. Lanes 3, 8, 13 and 18, 25 ng of the cold non-specific double-stranded oligonucleotide (5′-CTAGTCTAATTGAA/5′-CTAGTTCAATTAGA) were added to the incubation. Lanes 4, 9, 14 and 19, 1 μl of Bcd antiserum (Driever and Nüsslein-Volhard, 1988b) (1/4 dilution) was added to the incubation. Lanes 5, 10, 15 and 20, 1 μl of preimmune serum (1/4 dilution) was added to the incubation.

has no effect on the formation of complex A. These experiments strongly suggest that complex A contains the Bcd protein bound to DNA.

To unambiguously determine where the complex of the Bcd protein bound to DNA migrates in gel-shift assays, the binding activity to the Bcd site was detected in nuclear extracts from Schneider cells transfected either by pPAC or the pPACBcd producer (Fig. 1, lane 1 to 10). Three complexes (B1, B2 and B3) were obtained with both extracts (Fig. 1, lanes 1 to 10): since there is no endogenous Bcd protein expressed in Schneider cells (as determined by western blot, data not shown), B1, B2 and B3 likely correspond to the binding of other nuclear proteins to the Bcd site. In contrast, one major band was observed specifically with the nuclear extracts of cells transfected with the Bcd producer (compare lane 1 with lane 6, Fig. 1). This complex migrated at the same position as complex A (arrowheads). As observed with the embryonic nuclear extracts, formation of this complex was inhibited both by addition of an excess of specific DNA competitor (Fig. 1, lane 7) and by the presence of the anti-Bcd antibody (Fig. 1, lane 9). In contrast, its formation was not affected by an excess of non-specific competitor (Fig. 1, lane 8) or in the presence of preimmune serum (Fig. 1, lane 10). Thus, the complex formed by the Bcd protein expressed in transfected Schneider cells migrates at the same position as complex A. This experiment provides additional evidence that complex A is likely to contain the Bcd protein bound to DNA.

Comparison of Bcd DNA-binding activity in wild-type embryos and in embryos lacking *torso* activity showed no detectable differences (compare lane 11 to lane 16, Fig. 1). Furthermore, no additional specific complexes were detected either with extracts from wild-type embryos, or with extracts from embryos lacking *torso* activity. These complexes could have represented DNA-binding activity of other homeoproteins which also contain a lysine at the position 50 of the homeodomain. Such complexes do appear later in development, when genes such as *otd* start to be expressed. This experiment suggests that the *torso*-dependent phosphorylation of Bcd does not affect Bcd ability to bind to DNA, and that activation of the *torso* pathway does not induce the DNA-binding activity of a protein with the same specificity as Bcd that could compete with it.

Swapping the Bcd homeodomain with the Gal4 DNA-binding domain

To test whether Bcd activity is directly regulated by the terminal system, and to exclude the involvement of a competing repressor in the effect of *torso* on Bcd activity, we studied in vivo the transcriptional activity of a variant Bcd protein whose homeodomain has been replaced by the DNA-binding domain of the yeast Gal4 protein. The 60 amino-acid Bcd homeodomain (wt Bcd, Fig. 2A) (Berleth et al., 1988) was replaced with the minimal yeast Gal4 DNA-binding domain (amino-acid 2 to 74) (Bcd-Gal4(2-74), Fig. 2B) (Marmorstein et al., 1992), or with the domain defined as being sufficient for Gal4-specific DNA targeting in vivo (amino-acids 2 to 94) (Bcd-Gal4(2-94), Fig. 2C) (Carey et al., 1989). The transcriptional properties of the resulting chimeric proteins were first tested in a Schneider cell culture assay (Jaynes and O'Farrell, 1988) and compared with the transcriptional activity of the wild-type Bcd protein. The sequences encoding Bcd or the Bcd-Gal4 chimeras (Bcd-Gal4(2-74) and Bcd-Gal4(2-94)) were placed under the control of the Actin5C promoter and co-transfected with their respective CAT reporter plasmids (Krasnow et al., 1989). As shown in Fig. 3, Bcd strongly activated transcription through binding to three strong Bcd sites (lane 2). In contrast, the Bcd-Gal4 chimeras had lost their ability to activate transcription through these sites (lanes 3 and 4, Fig. 3). When tested with a reporter containing five Gal4 responsive elements ($UAS_{Gal4}CAT$), the Bcd-Gal4(2-74) protein produced almost no activation of CAT

reporter (lane 7, Fig. 3). In contrast, the Bcd-Gal4(2-94) protein activated transcription of the UAS_{Gal4}CAT reporter two times better than the wild-type Bcd protein activated transcription of a promoter containing Bcd sites (compare lane 2 to 8, Fig. 3). The reporter containing Gal4 sites was not activated by the wild-type Bcd protein (lane 6, Fig. 3).

Bicoid directly controls anterior activation and later retraction in the embryo

Transgenic lines expressing the Bcd-Gal4(2-74) or Bcd-Gal4(2-94) chimeric proteins in the embryo were obtained using P-element transformation. The genomic sequences encoding these chimeras were placed under the control of the *bcd* regulatory sequences (Berleth et al., 1988; Struhl et al., 1989), including the 3′ region necessary for mRNA localization (Macdonald and Struhl, 1988). The ability of these maternal transgenes to activate a UAS_{Gal4}-*lacZ* reporter gene was tested in embryos. The reporter transgene contained the same $(UAS_{Gal4})_5$ target sequence used in the cell culture assay, inserted upstream of the heat-shock (hsp70) TATA box and promoter sequences (Ronchi et al., 1993). Females from several independent transgenic lines carrying the Bcd-Gal4(2-94) chimeric gene were crossed with transgenic males carrying the UAS_{Gal4}-*lacZ* reporter gene. *LacZ* mRNA expression was detected by in situ hybridization on embryos derived from these females. In these embryos, *lacZ* was first expressed as an anterior cap, from 75 to 100% egg length (EL) (Fig. 4C), which then retracted to give rise to a stripe of expression from 90 to 75% EL at the end of cellularization (Fig. 4D). This pattern of expression was qualitatively similar to the pattern of expression of a Bcd reporter gene (Bcd3-*lacZ*) in response to the wild-type Bcd protein (compare Fig. 4A,B to Fig. 4C,D; Ronchi et al., 1993). The wild-type chimeric Bcd proteins have very different DNA-binding specificities, suggesting that both the activation of the Bcd3-*lacZ* and UAS_{Gal4}-*lacZ* reporter genes and the later anterior repression of the *bcd* target genes are directly mediated by the Bcd proteins and do not involve binding of other factors to the Bcd-binding sites. No expression of *lacZ* was induced by the Bcd-Gal4(2-74) protein in the same assay, indicating that the minimal Gal4 DNA-binding domain is not sufficient to mediate Gal4 DNA targeting in vivo in the embryo or in cell culture (Carey et al., 1989).

Torso activity but not Bcd activity is required for the down regulation of the Bcd-Gal4 chimera

Because of reports of a weak anterior background expression from the pCaSpeR vectors containing *lacZ* (Driever et al., 1989b), and also to rule out any interaction between the endogenous Bcd protein and the Bcd-Gal4(2-94) chimera, we analyzed the activity of the chimeric Bcd-Gal4(2-94) protein in a bcd^- background. Embryos lacking *bcd* activity did not express any *lacZ* mRNA from an unrelated CaSpeR vector containing *lacZ*, or from the Bcd3-*lacZ* construct (Ronchi et al., 1993). However, in the presence of the Bcd-Gal4(2-94) chimeric protein, the expression of the UAS_{Gal4}-*lacZ* reporter was not affected by the removal of the *bcd* endogenous activity: it was expressed at syncytial blastoderm as an anterior cap (Fig. 4E) and retracted from the anterior pole during cellularization (Fig. 4F). In embryos lacking both *bcd* and *torso* activities (from bcd^- tsl^- females), Bcd-Gal4(2-94) directed expression of the UAS_{Gal4}-*lacZ* reporter as an anterior cap (Fig. 4G) but it was not repressed at the anterior pole during cellularization (Fig. 4H). The same observation was made in embryos lacking *torso* activity only (data not shown). Thus, when expressed at the anterior pole of the embryo, the Bcd-Gal4(2-94) protein is sufficient to mediate initial activation and later repression of a reporter gene. This regulation also occurs in the absence of endogenous *bcd* activity, showing that no *bcd* target gene is required for the *torso*-mediated effect on the activity of the Bcd-

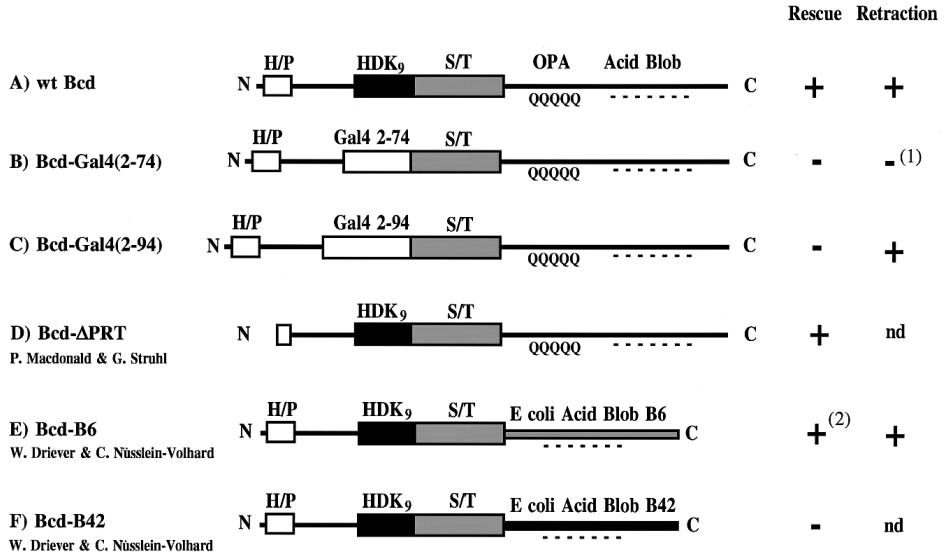

Fig. 2. Structure of wt Bcd protein and the different chimeric Bcd proteins expressed in the embryo. (A) The wild-type Bcd protein is composed of a PRD repeat (H/P), a 60 amino-acid homeodomain with a lysine at position 50 (HDK50), a serine/threonine rich domain (S/T) and a long activation domain composed of a glutamine-rich domain (OPA) and an acid blob. (B) The Bcd-Gal4(2-74) chimeric protein is identical to Bcd except that the 60 amino-acid homeodomain has been swapped with the DNA-binding domain of Gal4 (amino-acids 2 to 74). (C) The Bcd-Gal4(2-94) chimeric protein is identical to Bcd except that the 60 amino-acids homeodomain has been swapped with the DNA-binding domain of Gal4 (amino-acids 2 to 94). (D) In Bcd-ΔPRT, a region encompassing the PRD repeat rich in histidine and proline (position 1 to 29) has been deleted. This construct was generated by P. Macdonald and G. Struhl. (E) In Bcd-B6, the activation domain of Bcd has been replaced by the random acidic sequence from E. coli (B6) (Ma and Ptashne, 1987). This construct, as well as Bcd-B42, has been generated and generously provided by W. Driever and C. Nüsslein-Volhard (Driever et al., 1989a). (F) In Bcd-B42, the activation domain of Bcd has been replaced by the random acidic sequence from *E. coli* (B42) (Ma and Ptashne, 1987). Proteins were expressed in the embryo under the control of the *bcd* regulatory sequences. Their ability to rescue the lack of Bcd activity (rescue) and to be down-regulated by Torso (retraction) is indicated. (1)Retraction could not be determined because the Bcd-Gal4(2-74) protein did not induce expression of the UAS_{Gal4}-*lacZ* transgene. (2)Four copies of the transgene are required for rescue. nd is not determined.

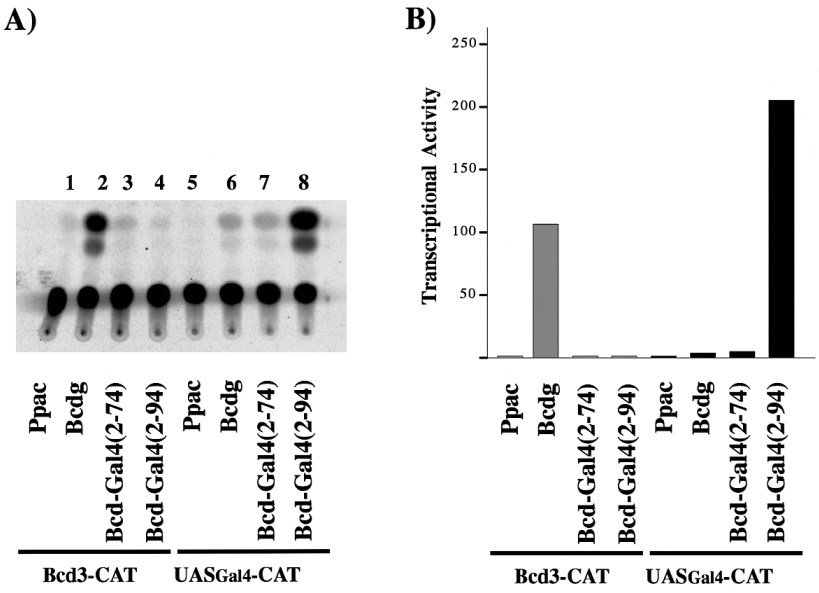

Fig. 3. Transcriptional activity of wt Bcd and Bcd-Gal4 proteins in tissue-culture. (A) CAT activity in Schneider cells transiently transfected with reporter plasmids (1 μg) in which CAT was driven by the hsp 70 minimal promoter containing either three Bcd DNA-binding sites (Bcd3-CAT, lanes 1 to 4) (Ronchi et al., 1993) or five copies of the Gal4-UAS sequences (UASGal4-CAT, lanes 5 to 8) (Brand and Perrimon, 1993). Wild-type Bcd or Bcd-Gal4 proteins were supplied by co-transfecting 10 μg of the pPAC producer plasmids containing the respective coding sequences driven by the Actin 5C promoter (Krasnow et al., 1989). Cells were co-transfected with pPAC alone, lanes 1 and 5; with a pPAC construct expressing the wild-type Bcd protein (Bcdg), lanes 2 and 6; with a pPAC construct expressing the Bcd-Gal4(2-74) chimera, lanes 3 and 7; with a pPAC construct expressing the Bcd-Gal4(2-94) chimera, lanes 4 and 8. Efficiency of transfection was controlled by co-transfecting 1 μg the hsp82/*lacZ* construct. (B) transcriptional activity of the wild-type Bcd and the Bcd-Gal4 chimeras measured as the ratio of the percent of 14C Chloramphenicol converted to the monoacetylated form, to the β-galactosidase activity obtained from the same extract.

Gal4(2-94) protein. The experiments described in Fig. 4 also show that the posterior border of expression of the UAS$_{Gal4}$-*lacZ* reporter is shifted anteriorly in the absence of torso (compare the position of the posterior border of the transgene in Fig. 4F and H). This effect can also be seen in the context of the wild-type Bcd protein (data not shown). Interestingly, a similar observation has recently been described for the regulation of *otd* expression (Gao et al., 1996). It is likely that the terminal system has a positive effect on Bcd transcriptional activity which has been missed so far because of its weak effect (the position of the posterior border of expression of the Bcd target genes is only shifted from 2 to 5%). The molecular mechanism leading to this effect is unknown, however our swapping experiment with the Bcd-Gal4(2-94) shows that the Bcd homeodomain is not required for this effect.

The Bcd acidic activation domain is not a specific target of the Torso cascade

The swapping experiment described above demonstrates that the Bcd homeodomain is not essential for the *torso*-mediated down-regulation of Bcd activity. The Bcd protein, however, contains several other recognizable domains which could be the target of the *torso* pathway (Fig. 2). At the N terminus, a His-Pro repeat (called the PRD repeat) (Frigerio et al., 1986) precedes the 60 amino acid long homeodomain. The Bcd homeodomain is followed by a serine/threonine-rich region, which by itself has only a weak transactivation ability (Driever et al., 1989a). A C-terminal domain, which contains a poly-glutamine repeat and an acidic domain, behaves as a strong

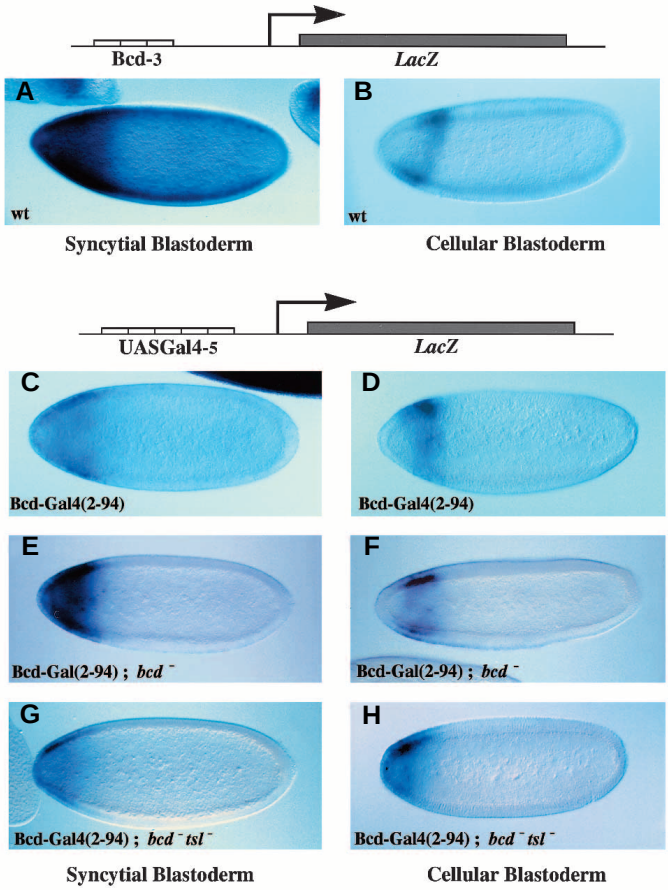

Fig. 4. Transcriptional activity of wt Bcd and Bcd-Gal4 proteins in the embryo. In situ hybridizations of whole-mount embryos stained using *lacZ* antisense RNA probe. In all figures showing embryos, anterior is to the left. (A,B) Expression of *lacZ* in wild-type embryos carrying the Bcd3-*lacZ* transgene. The transgene is expressed in an anterior cap at syncytial bastoderm (A) and it is repressed form the anterior pole at cellular blastoderm (B). (C-H) Expression of *lacZ* in embryos carrying the UASGal4-*lacZ* transgene and expressing the Bcd-Gal4(2-94) protein anteriorly. In wild-type embryos (Bcd-Gal4), *lacZ* is expressed in an anterior cap at syncytial blastoderm (C) and is repressed from the anterior pole at cellular blastoderm (D); in embryos lacking Bcd activity (Bcd-Gal4; *bcd*$^{-}$), *lacZ* is expressed in an anterior cap at syncytial blastoderm (E) and it is still repressed from the anterior pole at cellular blastoderm (F); in embryos lacking Bcd and Torso activity (Bcd-Gal4; *bcd*$^{-}$ *tsl*$^{-}$), *lacZ* is expressed in an anterior cap at syncytial blastoderm (G) but it is not repressed from the anterior pole at cellular blastoderm (H).

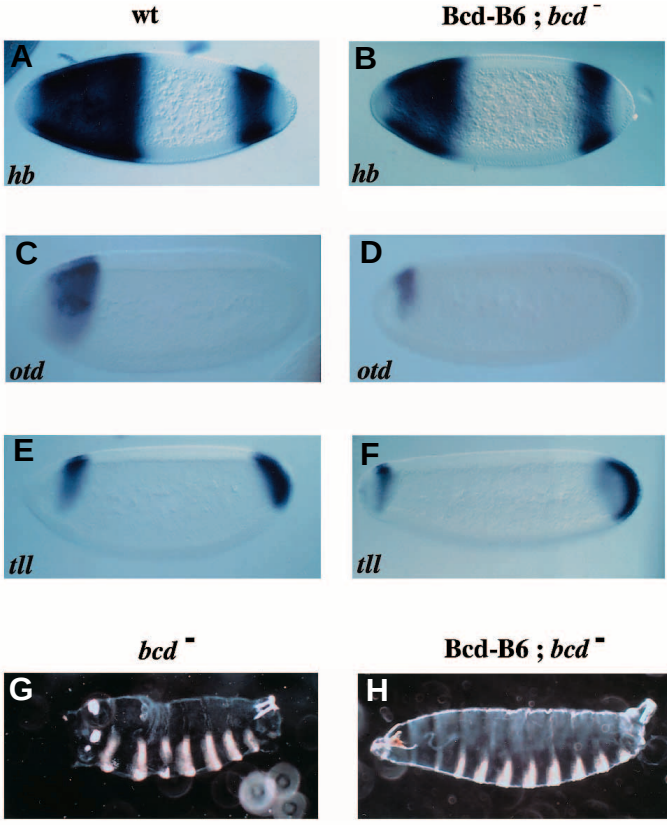

Fig. 5. Activity of the Bcd-B6 chimera in the embryo. (A-F) *In situ* hybridizations of whole-mount embryos stained with antisense RNA probes for the Bcd target genes *hb* (A,B), *otd* (C,D) and *tll* (E,F). Wild-type embryos (A,C,E) and embryos lacking Bcd activity and expressing two copies of the Bcd-B6 transgene under the control of the *bcd* regulatory sequences (B,D,F). Bcd-B6 is able to activate the *bcd* target genes in an anterior domain and its activity is repressed at the anterior pole. (G) Cuticle preparation of embryos lacking Bcd activity: additional spiracles (posterior structures) develop at the anterior. (H) Cuticle preparation of embryo lacking Bcd activity and partially rescued by the presence of two copies of the Bcd-B6 transgene under the control of the *bcd* regulatory sequences. Development of the thorax and part of the head is rescued. The two white dots at the anterior of the embryo in G are duplication of the posterior spiracles.

activation domain (Driever et al., 1989a). To test whether specific sequences within this activation domain were required for the down-regulation of Bcd activity, we took advantage of transgenic lines containing *bicoid* chimeric genes in which the Bcd C-terminal activation domain (Poly-Q and acidic domain) has been swapped with artificial activation domains from random E. coli sequences (B6 or B42; Ma and Ptashne, 1987). In a yeast assay, the activation potential of the Bcd-B6 chimera is slightly weaker than that of Bcd, while that of Bcd-B42 chimera is stronger (Driever et al., 1989a). However, injection of mRNA coding for the Bcd-B6 chimeric protein is able to rescue the lack of anterior structures in embryos derived from *bcd* mutant mothers, while Bcd-B42 mRNA cannot do so (Driever et al., 1989a).

Flies carrying P-element constructs expressing the Bcd-B6 and Bcd-B42 chimeric proteins under the control of *bcd* regulatory sequences were generously provided to us by W. Driever. In agreement with previous results (W. Driever and C. Nüsslein-Volhard, personal communication), two copies of the Bcd-B42 transgene are not sufficient to rescue the cuticle of embryos from *bcd* mutant mothers. In these embryos, the expression of three *bcd* target genes, *otd*, *tll* and *hb*, was very weak and the posterior border of their expression domain was shifted to the most anterior region of the embryo (data not shown). In contrast, four copies of the Bcd-B6 transgene are able to rescue the lack of Bcd activity to viability (W. Driever, personal communication). Two copies of the Bcd-B6 transgene were able to rescue the cuticular phenotype of embryos from bcd^{-} mutant mothers, though these embryos were not viable (compare Fig. 5G and H). Significant rescue of the expression pattern of *bcd* target genes was observed: in the absence of endogenous Bcd activity and in the presence of two copies of the Bcd-B6 transgene, *hb* (Fig. 5B), *otd* (Fig. 5D) and *tll* (Fig. 5F) were first expressed as an anterior cap with a posterior border more anterior than in wild-type embryos, indicating a reduced Bcd activity. The expression then retracted from the anterior pole. Thus, despite its weaker activation potential, the Bcd-B6 chimeric protein is down-regulated in a *torso*-dependent manner. The C-terminal activation domain of Bcd can thus be replaced by a generic acidic E. coli domain (Bcd-B6, Fig. 2E) without affecting the sensitivity of the protein to the Torso system. This suggests that there are no specific sequences within the strong Bcd activation domain that mediate the *Torso* effect, or that the target domain of the *Torso* pathway is redundant within the Bcd protein.

DISCUSSION

Down-regulation of Bcd does not involve a competing DNA-binding repressor

Several *Drosophila* homeoproteins that share with Bcd the same critical lysine at position 50 of their homeodomain (e.g. orthodenticle (Finkelstein et al., 1990), sine oculis (Cheyette et al., 1994) and Goosecoid (Blumberg et al., 1991; Goriely et al., 1996; Hahn and Jäckle, 1996), may be able to recognize Bcd-binding sites. These genes are all expressed in the anterior region of the developing embryo in response to *bcd*, and could in principle be involved in the down-regulation of the *bcd* targets by the terminal system. However, their late expression patterns as well as genetic experiments show that neither *otd* nor *sine oculis* play a role in this regulation. Swapping the DNA-binding domain of Bcd and targeting the protein to different binding sites allowed us to rule out the possibility that another repressor protein recognizing the Bcd-binding sites or sequences overlapping the Bcd-binding sites is induced by the terminal system (Fig. 6A). Finally, the endogenous Bcd activity is dispensable for the down-regulation of the Bcd-Gal4 chimera by Torso, indicating that no *bcd* target gene is involved in this process. Instead, Bcd activity is a direct target of the Torso RTK system.

The DNA-binding activity of Bcd is not regulated by Torso

Transcription factors are usually composed of separate DNA-binding and transcriptional activation domains (Ptashne, 1988). Down-regulation of their transcriptional activity could

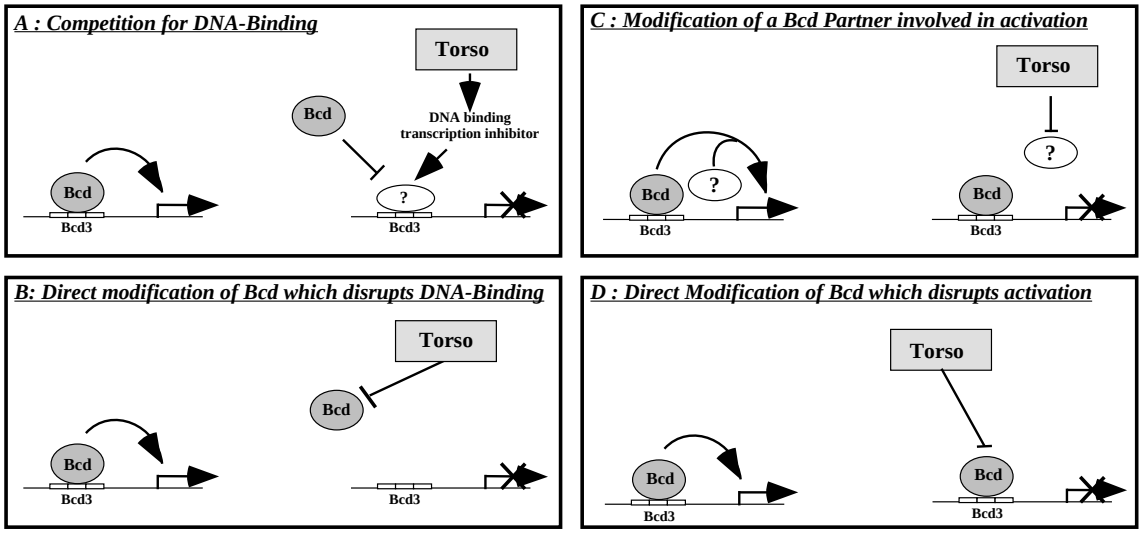

Fig. 6. Possible mechanisms for down-regulation of Bcd activity by the terminal system. (A) Activation of the Torso RTK cascade induces the expression of a factor competing with Bcd for DNA binding. The swapping of the homeodomain with the Gal4 DNA-binding domain excludes this possibility. (B) Activation of the Torso RTK cascade directly modifies Bcd and prevents it from binding to DNA. Gel-shift experiments and the non-requirement of the homeodomain for the down-regulation of Bcd activity strongly argue against this possibility. (C,D) The down-regulation of Bcd by the Torso RTK cascade involves the activation process, either by modification of a Bcd co-factor (C) or by direct modification of the Bcd protein (D).

be achieved by disrupting the function of one of these domains. If the Bcd down-regulation by Torso involved Bcd DNA binding, this could be achieved through direct modification of the Bcd protein (Fig. 6B), most likely through phosphorylation. In many cases where phosphorylation modifies DNA binding of transcription factors, the sites of modification are located either nearby or within the DNA-binding domain: in vitro phosphorylation of Oct1 by PKA in the N-terminal tail of the homeodomain leads to a loss of DNA binding (Segil et al., 1991) and phosphorylation of a region N-terminal to the DNA-binding domain of c-*Jun* and c-*Myb* negatively affects binding to DNA (for review, see Hunter and Karin, 1992; Karin and Hunter, 1995). In the case of the Bcd homeoprotein, swapping of the homeodomain with the Gal4 DNA-binding domain showed that the homeodomain is not required for Bcd down-regulation by the terminal system. Bcd DNA binding is thus probably not altered by the terminal system. It is still possible that modification of a domain of Bcd distinct from the homeodomain affects the structure of the protein and prevents DNA binding, even when the homeodomain has been replaced by the Gal4 DNA-binding domain. Such a mechanism has been described for the negative action of the steroid binding domain of the glucocorticoid receptor, which can affect an unrelated DNA-binding domain placed in *cis*, likely through an interaction with a chaperone protein (Picard et al., 1988). We do not favor this possibility for two reasons. First, our gel-shift analysis suggests that the Bcd DNA-binding activity is not affected by the Torso pathway. Second, the Gal4 DNA-binding domain (amino-acids 2 to 94) is totally unrelated to the Bcd homeodomain : it is composed of zinc clusters and binds efficiently to DNA as a dimer whereas the Bcd homeodomain binds to DNA as a monomer. It is thus unlikely that modifications outside of the homeodomain would alter the Gal4 DNA-binding activity.

The activation domain of Bcd is not specifically affected by Torso

Since the DNA-binding activity of Bcd is not affected by Torso, the ability of the Bcd activation domain to activate transcription might have been the target of the terminal system. The Bcd region responsible for most of the transactivation (a Q-rich region and an acidic region, both located at the C terminus) can be replaced by an unrelated *E. coli* acidic activation domain and still support *bcd* function, including its ability to be down-regulated by the terminal system. It is formally possible that the transcriptional activity of both the Bcd activation domain and the B6 activation domain can be down-regulated by the terminal system. This could be due to fortuitous similar phosphorylation sites in Bcd and B6 activation domains. Alternatively, the mechanism of inhibition of transcription may involve a general control of the transcriptional activity of certain types of activation domains by RTK cascades, through for instance a specific TAF (TATA-binding protein associated factor). Direct interaction between Bcd and particular TAFs has recently been reported (Sauer et al., 1996). We do not favor this hypothesis because it does not seem that there is a general decrease in the transcriptional activity at the pole of the embryo. In particular, expression of *hkb* is strongly induced during the cellularization process. Finally, another domain of Bcd might be the target of the terminal system and affect the activation domain. A similar observation has been made in the case of the ADR1 yeast transcription factor: repression of ADR1 activity by glucose is correlated with direct phosphorylation of ADR1 by PKA (Cherry et al., 1989). This phosphorylation occurs in a small domain located outside the DNA-binding and activation domains and does not affect DNA binding (Taylor and Young, 1990). Several models have been proposed to explain the catabolic repression of ADR1 : upon phosphorylation, the PKA target domain can directly contact another domain of ADR1 and block its activation function (Denis et al., 1992); alternatively, the

phosphorylated domain may interact with an extragenic repressor protein that reduces the function of the ADR1 protein (Cook et al., 1994). Both mechanisms are also possible in the case of Bcd (Fig. 6C,D).

Direct modification of Bcd by the terminal system?

Clustering of Bcd-binding sites has been shown to be required for activation of transcription by Bcd monomers (Driever and Nüsslein-Volhard, 1989; Struhl et al., 1989) and the spacing between the sites has been reported to be critical (Hanes et al., 1994; Rivera-Pomar et al., 1995). It is possible that synergy between the activation domains of several Bcd proteins is required for Bcd function. Direct modification of Bcd by the Torso system may prevent this intermolecular interaction or any other function required for Bcd transcriptional activity (Fig. 6D). However, neither the homeodomain nor the C-terminal activation domain of Bcd is specifically involved. The PRD repeat, another conserved region in Bcd is also unlikely to be required: a truncated *bcd* gene lacking a large portion of the PRD repeat (Bcd-ΔPRT, Fig. 2D) is able to rescue the phenotype of *bcd* mutant flies (Dubnau and Struhl, 1996)

Activation of the terminal system induces phosphorylation of the Bcd protein (Ronchi et al., 1993). The only recognizable region that has not been deleted or replaced in the experiments described in this study is a S/T-rich region located C-terminal to the homeodomain. This domain appears to contain all but one of the putative MAP-kinase sites identified by searching the primary sequence of Bcd. The *rolled* gene, encoding the *Drosophila* MAP-kinase, is likely to be an effector of the Torso RTK pathway (Brunner et al., 1994). It is thus possible that activation of the Torso RTK leads to activation of MAP-kinase, which may then phosphorylate the S/T-rich region of Bcd and modify its transcriptional activity (N. D. and C. D., unpublished data).

Modification of a Bcd partner involved in activation?

Alternatively, an essential Bcd co-factor may be modified and inactivated by the Torso pathway (Fig. 6C). A similar phenomenon has been described in the case of Elk-1, which acts in conjunction with the SRF transcription factor (Marais et al., 1993). The EGF/PDGF signal transduction cascade modifies Elk-1, which is a transcription factor constitutively bound to DNA in association with SRF and becomes active upon phosphorylation (Hill et al., 1993; Marais et al., 1993). The DNA-binding specificity of Elk-1 (an ETS family member) is minimal and it acts only as a co-factor of SRF (Hill et al., 1993). Bcd may have a function similar to SRF, and an unknown co-factor may play the role of Elk-1. In this case, modification of this co-factor by the RTK cascade should lead to its inactivation. In the *Drosophila* eye, two genes encoding proteins from the ETS family, *pointed* and *Yan*, have been shown to act downstream of the Sevenless pathway and to be modified by the RTK/Ras signal transduction cascade in the R7 cell (O'Neill et al., 1994). These genes have early phenotypes that have not yet been characterized in detail. Another possible candidate for a Bcd co-factor could be the product of the *hb* gene. The Hb protein is a transcription factor with a zinc-finger DNA-binding domain. A strong synergistic effect has been observed between Hb and Bcd activities in vivo for the regulation of most of the Bcd target genes (Simpson-Brose et al., 1994). However, the *torso*-mediated anterior repression of the *bcd* target genes is still observed in embryos lacking maternal as well as zygotic components of *hb* (Simpson-Brose et al., 1994), indicating that *hb* is not required for the down-regulation of Bcd activity by the terminal system.

Conclusion

The graded distribution of the Bcd protein along the anteroposterior axis of the embryo is essential for setting up the anterior pattern of the larva. Interactions between the terminal and anterior systems modify the shape of the gradient of Bcd activity at the most anterior tip of the embryo. Our in vivo dissection of the Bcd protein demonstrates that the transcriptional activity of the Bcd protein is down-regulated by the RTK terminal system. This effect is unlikely to be mediated by a change in DNA binding but rather may involve the activation process. Direct phosphorylation of Bcd, possibly by an activated MAP-kinase, may render the protein unable to activate transcription without affecting DNA binding (Fig. 6D). Alternatively, phosphorylation may inactivate a co-factor necessary for Bcd activity which itself is not dependent on *bcd* (Fig. 6C).

We are indebted to Paul Macdonald, Wolfgang Driever and Gary Struhl for fly stocks, unpublished results and helpful comments about this project. Andrea Brand and Norbert Perrimon provided us with the UAS_{Gal4}-*lacZ* transgenic lines. Cathy Snook-Branda and Rachel Sturny were extremely helpful with technical assistance during injections and fly stock maintenance or gel-shift assays, respectively. We are very grateful to the members of the Desplan, DiNardo and Gaul labs for their constant support during this work. We especially appreciate the help from Anne Goriely, Susie Jun, Carolina Mailhos, Guojun Sheng, Marcia Simpson, David Wilson and Ernst Wimmer. Y.B. would like to address special thanks to Scott Dougan and Anne Morineau for their help and support during the project. Y. B. was a DEA student from the 'Ecole Normale Supérieure de Lyon' (France). N. D. was on leave from INSERM and was supported by a long-term fellowship from the Human Frontier Science Program Organization.

REFERENCES

Berleth, T., Burri, M., Thoma, G., Bopp, D., Richstein, S., Frigerio, G., Noll, M. and Nusslein-Volhard, C. (1988). The role of localization of bicoid RNA in organizing the anterior pattern of the *Drosophila* embryo. *EMBO J.* **7,** 1749-1756.

Blumberg, B., Wright, C. V. E., DeRobertis, E. M. and Cho, K. W. Y. (1991). Organizer-specific homeobox genes in *Xenopus laevis* embryos. *Science* **253,** 194-196.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118,** 401-15.

Brunner, D., Oellers, N., Szabad, J., Biggs, W. H., Zipursky, S. L. and Hafen, E. (1994). A gain-of-function mutation in Drosophila MAP kinase activates multiple Receptor Tyrosine Kinase signaling pathways. *Cell* **76,** 875-888.

Carey, M., Kakidani, H., Leatherwood, J., Mostashari, F. and Ptashne, M. (1989). An amino-terminal fragment of GAL4 binds DNA as a dimer. *J. Mol. Biol.* **209,** 423-432.

Cherry, J., Johnson, T., Dollard, C., Shuster, J. and Denis, C. (1989). Cyclic AMP-dependent protein kinase phosphorylates and inactivates the yeast transcriptional activator ADR1. *Cell* **56,** 409-419.

Cheyette, B., Green, P., Martin, K., Garren, H., Hartenstein, V. and Zipursky, L. (1994). The *Drosophila sine oculis* locus encodes a homeodomain-containing protein required for the development of the entire visual system. *Neuron* **12,** 977-996.

Cook, W. J., Chase, D., Audino, D. C. and Denis, C. L. (1994). Dissection of the ADR1 protein reveals multiple, functionally redundant activation domains interspersed with inhibitory regions: evidence for a repressor binding to the ADR1c region. *Molec. Cell. Biol.* **14,** 629-40.

Denis, C. L., Fontaine, S. C., Chase, D., Kemp, B. E. and Bemis, L. T. (1992). ADR1c mutations enhance the ability of ADR1 to activate transcription by a

mechanism that is independent of effects on cyclic AMP-dependent protein kinase phosphorylation of Ser-230. *Molec. Cell. Biol.* **12,** 1507-14.

Driever, W., Ma, J., Nüsslein-Volhard, C. and Ptashne, M. (1989a). Rescue of bicoid mutant drosophila embryos by bicoid fusion proteins containing heterologous activating sequences. *Nature* **342,** 149-154.

Driever, W. and Nüsslein-Volhard, C. (1988a). The Bicoid protein determines position in the Drosophila embryo in a concentration-dependent manner. *Cell* **54,** 95-104.

Driever, W. and Nüsslein-Volhard, C. (1988b). A gradient of bicoid protein in Drosophila embryos. *Cell* **54,** 83-93.

Driever, W. and Nüsslein-Volhard, C. (1989). The bicoid protein is a positive regulator of hunchback transcription in the early Drosophila embryo. *Nature* **337,** 138-143.

Driever, W., Thoma, G. and Nüsslein-Volhard, C. (1989b). Determination of spatial domains of zygotic gene expression in the Drosophila embryo by the affinity of binding sites for the bicoid morphogen. *Nature* **340,** 363-367.

Driever, W., Siegel, V. and Nüsslein-Volhard, C. (1990). Autonomous determination of anterior structures in the early *Drosophila* embryo by the Bicoid morphogen. *Development* **109**, 811-820.

Dubnau J. and Struhl G. (1996). RNA recognition and translational repression by a homeodomain protein. *Nature* **379**, 694-699.

Finkelstein, R. and Perrimon, N. (1990). The orthodenticle gene is regulated by bicoid and torso and specifies *Drosophila* head development. *Nature* **346,** 485-488.

Finkelstein, R., Smouse, D., Capaci, T. M., Spradling, A. C. and Perrimon, N. (1990). The orthodenticle gene encodes a novel homeo domain protein involved in the development of the Drosophila nervous system and ocellar visual structures. *Genes Dev.* **4,** 1516-1527.

Frigerio, G., Burri, M., Bopp, D., Baumgartner, S. and Noll, M. (1986). Structure of the segmentation of gene paired and the Drosophila PRD gene set as part of a gene network. *Cell* **47,** 735-746.

Frohnhöfer, H. G. and Nüsslein-Volhard, C. (1986). Organization of anterior pattern in the Drosophila embryo by the maternal gene bicoid. *Nature* **324,** 120-125.

Gao Q., Wang Y. and R. Finkelstein (1996). *orthodenticle* regulation during embryonic head development in Drosophila. *Mech. Dev.* **56**, 3-15.

Goriely, A., Stella, M., Coffinier, C., Kessler, D., Mailhos, M., Dessain S. and Desplan C. (1996). A functional homologue of *goosecoid* in *Drosophila*. *Development* **122**, 1641-1650.

Hahn, M. and Jäckle, H. (1996). *Drosophila goosecoid* participates in neural development but not in body axis formation. *EMBO J.* **15**, 3077-3084.

Hanes, S. D. and Brent, R. (1989). DNA specificity of the bicoid activator protein is determined by homeodomain recognition helix residue 9. *Cell* **57,** 1275-1283.

Hanes, S. D., Riddihough, G., Ish-Horowicz, D. and Brent, R. (1994). Specific DNA recognition and intersite spacing are critical for action of the Bicoid morphogen. *Mol. Cell. Biol.* **14,** 3364-3375.

Hill, C. S., Marais, R., John, S., Wynne, J., Dalton, S. and Treisman, R. (1993). Functional analysis of a growth factor-responsive transcription factor complex. *Cell* **73**, 395-406.

Hunter, T. and Karin, M. (1992). The regulation of transcription by phosphorylation. *Cell* **70,** 375-387.

Jaynes, J. B. and O'Farrell, A. P. H. (1988). Activation and repression of transcription by homeodomain-containing proteins that bind a common site. *Nature* **336,** 744-749.

Klingler, M., Erdelyi, M., Szabad, J. and Nüsslein-Volhard, A. C. (1988). Function of Torso in determining the terminal anlagen of the Drosophila embryo. *Nature* **335,** 275-277.

Karin, M. and Hunter, T. (1995). Transcriptional control by phosphorylation: signal transmission from the cell surface to the nucleus. *Current Biology* **5**, 747-757.

Krasnow, M. A., Saffman, E. E., Kornfeld, K. and Hogness, A. D. S. (1989). Transcriptional activation and repression by Ultrabithorax proteins in cultured Drosophila cells. *Cell* **57,** 1031-1043.

Laski, F. A., Rio, D. C. and Rubin, G. M. (1986). Tissue specificity of Drosophila P element transposition is regulated at the level of mRNA splicing. *Cell* **44,** 7-19.

Ma, J. and Ptashne, M. (1987). Deletion analysis of GAL4 defines two transcriptional activating segments. *Cell* **48,** 847-853.

Macdonald, P. M. and Struhl, G. (1988). Cis-acting sequences responsible for anterior localization of bicoid mRNA in Drosophila embryos. *Nature* **336,** 595-598.

Marais, R., Wynne, J. and Treisman, R. (1993). The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. *Cell* **73,** 381-393.

Marmorstein, R., Carey, M., Ptashne, M. and Harrison, S. C. (1992). DNA recognition by GAL4: structure of a protein-DNA complex. *Nature* **356,** 408-414.

Miller J. H. ed. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, New-York: Cold Spring Harbor Laboratory.

Müller M. M., Schreiber E, Schaffner W and Matthias P. (1989). Rapid test for in vivo stability and DNA binding of mutated octamer binding proteins with 'mini-extracts' prepared from transfected cells. *Nucleic Acids Res* **17**, 6420.

Nüsslein-Volhard, C., Frohnhofer, H. G. and Lehmann, R. (1987). Determination of anteroposterior polarity in Drosophila. *Science* **238,** 1675-1681.

O'Neill, E. M., Rebay, I., Tjian, R. and Rubin, G. M. (1994). The activities of two Ets-related transcription factors required for Drosophila eye development are modulated by the Ras/MAPK pathway. *Cell* **78,** 137-147.

Perrimon, N. and Desplan, C. (1994). Signal transduction in the early *Drosophila* embryo: When genetics meets biochemistry. *Trends Biochem. Sci.* **19,** 509-513.

Picard, D., Salser, S. J. and Yamamoto, K. R. (1988). A movable and regulable inactivation function within the steroid binding domain of the glucocorticoid receptor. *Cell* **54,** 1073-1080.

Pignoni, F., Baldarelli, R., Steingrimsson, E., Diaz, R., Patapoutian, A., Merriam, J. and Lengyel, J. (1990). The Drosophila gene tailless is expressed at the embryonic termini and is a member of the steroid receptor superfamily. *Cell* **62,** 151-163.

Ptashne, M. (1988). How eukaryotic transcriptional activators work. *Nature* **335,** 683-689.

Rivera-Pomar, R., Lu, X., Perrimon, N., Taubert, H. and Jäckle, H. (1995). Activation of posterior gap gene expression in the Drosophila blastoderm. *Nature* **376,** 253-6.

Ronchi, E., Treisman, J., Dostatni, N., Struhl, G. and Desplan, C. (1993). Down-regulation of the *Drosophila* morphogen bicoid by the Torso receptor-mediated signal transduction cascade. *Cell* **74,** 347-355.

Sauer F, Hansen S and Tjian R. (1996). Multiple TAFIIs directing synergistic activation of transcription. *Science* **270**, 1783-1788.

Segil, N., Roberts, S. B. and Heintz, N. (1991). Mitotic phosphorylation of the Oct-1 homeodomain and regulation of Oct-1 DNA binding activity. *Science* **254,** 1814-1816.

Simpson-Brose, M., Treisman, J. and Desplan, C. (1994). Synergy between the Hunchback and Bicoid morphogens is required for anterior patterning in Drosophila. *Cell* **78,** 855-865.

Sprenger, F. and Nüsslein-Volhard, C. (1992). Torso receptor activity is regulated by a diffusible ligand produced at the extracellular terminal regions of the Drosophila egg. *Cell* **71,** 987-1001.

StJohnston, D. and Nüsslein-Volhard, C. (1992). The origin of pattern and polarity in the Drosophila embryo. *Cell* **68,** 201-219.

Struhl, G., Johnston, P. and Lawrence, P. A. (1992). Control of Drosophila body pattern by the hunchback morphogen gradient. *Cell* **69,** 237-249.

Struhl, G., Struhl, K. and Macdonald, P. M. (1989). The gradient morphogen bicoid is a concentration-dependent transcriptional activator. *Cell* **57,** 1259-1273.

Tautz, D. (1988). Regulation of the Drosophila segmentation gene Hunchback by two maternal morphogenetic centres. *Nature* **332,** 281-284.

Tautz, D. and Pfeifle, C. (1989). A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. *Chromosoma* **98,** 81-85.

Taylor, W. E. and Young, E. T. (1990). cAMP-dependent phosphorylation and inactivation of yeast transcription factor ADR1 does not affect DNA binding. *Proc. Natl. Acad. Sci. USA* **87,** 4098-4102.

Treisman, J. and Desplan, C. (1989). The products of the Drosophila gap genes hunchback and Kruppel bind to the hunchback promoters. *Nature* **341,** 335-7.

Treisman, J., Harris, E., Wilson, D. and Desplan, C. (1992). The homeodomain: A new face for the helix-turn-helix? *BioEssays* **14,** 145-150.

Weigel, D., Jürgens, G., Klingler, M. and Jäckle, H. (1990). Two gap genes mediate maternal terminal pattern information in Drosophila. *Science* **248,** 495-498.

Wigler, M., Sweet, R., Sim, G. K., Wold, B., Pellicer, A., Lacy, E., Maniatis, T., Silverstein, S. and Axel, R. (1979). Transformation of mammalian cells with genes from procaryotes and eucaryotes. *Cell* **16,** 777-85.

(Accepted 31 July 1996)