

# Homeodomain Position 54 Specifies Transcriptional versus Translational Control by Bicoid

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## Summary

Bicoid (BCD), the anterior determinant of *Drosophila*, controls embryonic gene expression by transcriptional activation and translational repression. Both functions require the homeodomain (HD), which recognizes DNA motifs at target gene enhancers and a specific sequence interval in the 3' untranslated region of *caudal* (*cad*) mRNA. Here we show that the BCD HD is a nucleic acid-binding unit. Its helix III contains an arginine-rich motif (ARM), similar to the RNA-binding domain of the HIV-1 protein REV, needed for both RNA and DNA recognition. Replacement of arginine 54, within this motif, alters the RNA but not the DNA binding properties of the HD. Corresponding BCD mutants fail to repress *cad* mRNA translation, whereas the transcriptional target genes are still activated.

## Introduction

In *Drosophila*, the anterior pattern of the embryo is controlled by the graded expression of the homeodomain (HD) protein Bicoid (BCD) (Frohnhöfer and Nüsslein-Volhard, 1986; Driever and Nüsslein-Volhard, 1988a, 1988b). BCD transcriptionally activates zygotic segmentation genes, such as *hunchback* (*hb*), at different threshold concentrations in the anterior region of the embryo (Driever and Nüsslein-Volhard, 1989; Struhl et al., 1989; Burz et al., 1998; Gao and Finkelstein, 1998). In addition, it has recently been shown that BCD acts by repressing the translation of evenly distributed maternal *caudal* (*cad*) mRNA in the embryo (Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996). This second regulatory function of BCD results in a *cad* protein (Caudal) gradient, which forms in the opposite direction to BCD's

(Mlodzik and Gehring, 1987). Mutations of BCD that interfere specifically with transcriptional activation cause a segmentation phenotype, whereas mutations that interfere only with translational control cause a temperature-dependent head involution defect (Niessing et al., 1999). The two regulatory functions of BCD involve the HD, an evolutionarily conserved helix-turn-helix motif composed of three  $\alpha$  helices (Driever and Nüsslein-Volhard, 1989; Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996; Burz et al., 1998; Gao and Finkelstein, 1998).

Despite a high degree of overall conservation, HDs can be subdivided into distinct groups (reviewed by Gehring et al., 1994a). It contains the large paired-like class of HDs (Bopp et al., 1986). A small subgroup is characterized by a lysine residue at position 50 of the HD (Frigerio et al., 1986). This subgroup includes BCD itself (Frigerio et al., 1986; Berleth et al., 1988), Orthodenticle (Finkelstein and Perrimon, 1990), sine oculis (Chayette et al., 1994), and Goosecoid (Goriely et al., 1996; Hahn and Jäckle, 1996) of *Drosophila* as well as their vertebrate homologs (Blumberg et al., 1991; Simeone et al., 1993).

Previous results have shown that the lysine at position 50 of the BCD HD is not only necessary for DNA recognition (Hanes and Brent, 1989; Treisman et al., 1989) but also for the binding to a discrete target sequence in the 3' untranslated region (3'UTR) of *cad* mRNA, termed the BCD-binding region (BBR) (Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996; Chan and Struhl, 1997), providing a link between the DNA and RNA binding properties of BCD. Here we show that the helix III of the BCD HD contains a region similar to the RNA-binding arginine-rich motif (ARM) of the HIV-1 protein REV (Sodroski et al., 1986; Tan et al., 1993; Tan and Frankel, 1995; Battiste et al., 1996). We investigated whether mutations in this motif affect the binding of the BCD HD to its targets and the control of transcription and/or translation by BCD. The results show that the BCD HD is a prototypical nucleic acid-binding unit. It contains a functional arginine-rich motif in the C-terminal portion of helix III, which is characteristic for a class of RNA-binding proteins. Replacement of arginine 54 (R54) within this unit shifts the binding property of the HD to prefer DNA over RNA recognition and abolishes *cad* mRNA translational repression by not affecting transcriptional activation by BCD in vivo. The dual binding property of the HD links two different BCD-dependent processes during early embryogenesis, the establishment of anterior body segments by transcriptional regulation, and proper assembly of head structures by control of *cad* mRNA translation.

## Results and Discussion

BCD-dependent activation of transcription and repression of translation involve the HD, which is able to directly interact with DNA of the control region of target genes, such as *hunchback* (*hb*) (Driever et al., 1989), and with the BBR, a short sequence interval within the

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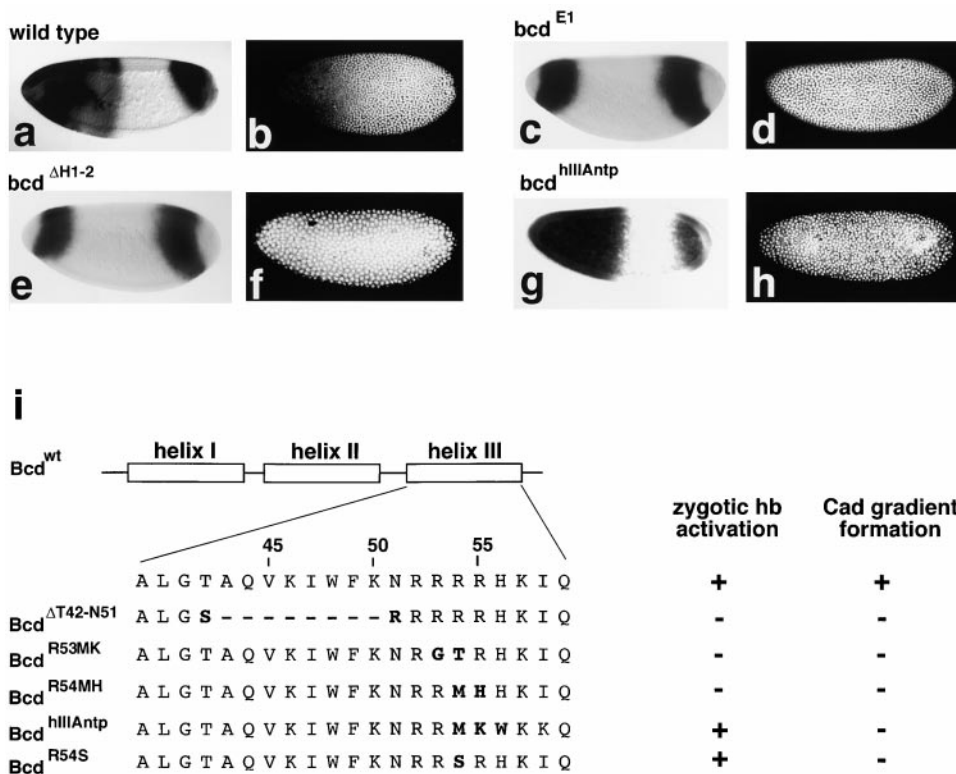


Figure 1. BCD-Dependent Control of Transcriptional Activation and Translational Repression in Response to BCD Mutants in *bcd* Embryos (a–h) Whole-mount preparations of wild-type and mutant embryos showing *hb* expression as monitored by in situ hybridization with an antisense *hb* RNA probe (a, c, e, and g) or CAD by anti-CAD antibody staining (b, d, f, and h). Orientation of embryos is anterior to the left and dorsal up. (a and b) Wild-type embryos showing the *bcd*-dependent anterior expression domain and the *bcd*-independent posterior expression domain of *hb* (a) and the lack of CAD in the anterior region (b). (c and d) Embryos derived from homozygous *bcd<sup>E1</sup>* mutant females (“*bcd*-deficient embryos”). Note that the anterior domain of *hb* expression is absent and replaced by a duplication of the posterior domain (c), and CAD is present in the anterior pole region (d). (e–h) *bcd*-deficient embryos bearing the minigene *bcd<sup>ΔH1-2</sup>* (see text) show no rescued functions (e and f) whereas the minigene-derived BCD<sup>hIII<sup>Antp</sup></sup> expression causes BCD-dependent *hb* expression in the anterior domain (g) but no CAD repression in the anterior pole region (h). (i) Amino acid replacement mutants (left) and their effect on transcription and translational repression (right) after transgene-derived expression in *bcd*-deficient embryos (for examples, see [g] and [h]). For details, see text.

3'UTR of *cad* mRNA (Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996; Chan and Struhl, 1997). We investigated whether mutations in the HD affect the binding of the BCD HD to its targets and the control of transcription and/or translation by BCD.

### The BCD HD Contains an ARM Necessary for Translational Repression

In order to characterize portions and individual amino acid residues of the BCD HD that are specifically required for one or both BCD regulatory functions, we placed transgenes expressing wild-type or mutant *bcd* cDNAs into the genome of homozygous *bcd* mutant females and assayed their ability to rescue wild-type zygotic *hb* activation and *cad* mRNA translation in their embryos (Figures 1a and 1b). Such embryos, referred to as “*bcd* embryos,” fail to exert BCD-dependent transcriptional activation of the zygotic target gene *hb* (Driever and Nüsslein-Volhard, 1989; Burz et al., 1998; Gao and Finkelstein, 1998) in their anterior half. Instead, the embryos show a duplication of the posterior BCD-independent stripe of *hb* expression in the anterior region (Figures 1a and 1c). They also fail to form the CAD

gradient, indicating that the translation of *cad* mRNA is not repressed (Figures 1b and 1d).

Transgene-dependently expressed BCD mutants that lack the helices I and II of the HD (BCD<sup>ΔH1-2</sup>) or the amino acid interval between positions 42 and 51 in helix III (BCD<sup>T42-N51</sup>) failed to restore BCD-dependent *hb* transcriptional activation and translational repression of *cad* mRNA in the anterior region of *bcd* embryos (Figures 1e and 1f; summarized in Figure 1i). This indicates that the integrity of the BCD HD is necessary for the control of transcription and translation. Transgene-dependent expression of BCD<sup>hIII<sup>Antp</sup></sup>, in which the C-terminal half of the BCD HD was exchanged for the corresponding sequence of the *Antennapedia* (*Antp*) HD (Figure 1i), rescued BCD-dependent *hb* expression in the anterior region of *bcd* embryos (Figure 1g), but no CAD gradient was formed (Figure 1h). BCD mutations in which two adjacent arginines at positions 53–54 and 54–55 of the HD, respectively, were replaced failed to control BCD-dependent transcription and translation (Figure 1i). Thus, helix III of the BCD HD is necessary for both transcriptional activation and translational repression, and amino acids within helix III are essential for specifying not only DNA binding but also RNA recognition by the

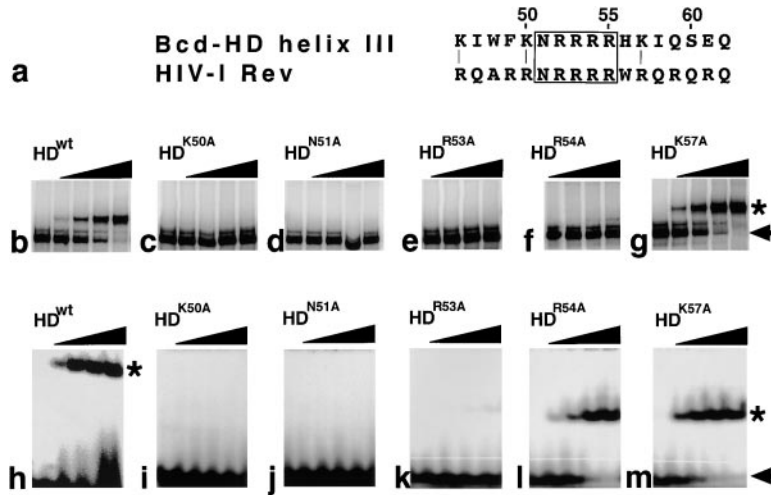


Figure 2. Binding Properties of BCD HD Helix III Mutants In Vitro

(a) Sequence comparison of BCD HD helix III and the arginine-rich motif of the RNA-binding domain of HIV-1 REV (taken from Sodroski et al. [1986] and Tan et al. [1993]). Numbers refer to positions of amino acid residues in the BCD HD. (b-m) RNA binding to the BBR (b-g) and DNA binding to the DNA target sequence ATCTAATCCC (h-m) of the wild-type BCD HD (HD<sup>wt</sup>) and single amino acid replacement mutants. Arrow, position of free nucleic acid targets; asterisk, nucleic acid/protein complexes. Closed triangles, increase (5-fold steps) of protein concentrations. Denomination of the HD mutants refers to amino acid residue, its position in the HD, and replacement by alanine. For details, see text.

HD. This proposal is consistent with the observation that part of the helix III of the BCD HD has characteristics of an arginine-rich motif (ARM; see Figure 2a), an  $\alpha$ -helical RNA-binding motif common to a subclass of RNA-binding proteins such as HIV-1 proteins REV or TAT (Sodroski et al., 1986; Mattaj, 1993; Tan et al., 1993; Burd and Dreyfuss, 1994; Tan and Frankel, 1995; Battiste et al., 1996).

**Arginine at Position 54 Specifies DNA versus RNA Recognition**

To test whether the conserved amino acids of BCD's ARM are indeed required for RNA target recognition and

whether single amino acid replacements may allow the DNA and RNA binding properties to separate, we generated alanine replacement mutants of the BCD HD and assayed their in vitro binding properties. Results summarized in Figure 2 indicate that the BCD HD (HD<sup>wt</sup>) binds both DNA and RNA (Figures 2b and 2h), whereas HD<sup>K50A</sup> (Figures 2c and 2i), HD<sup>N51A</sup> (Figures 2d and 2j), HD<sup>R53A</sup> (Figures 2e and 2k), and HD<sup>R55A</sup> (not shown) failed to bind to both targets. BCD HD<sup>R54A</sup>, which contains alanine in place of arginine in position 54 of the HD, bound DNA properly, but its RNA binding was reduced by more than one order of magnitude (Figures 2f and 2l). The binding properties of HD<sup>K57A</sup> (Figures 2g and 2m)

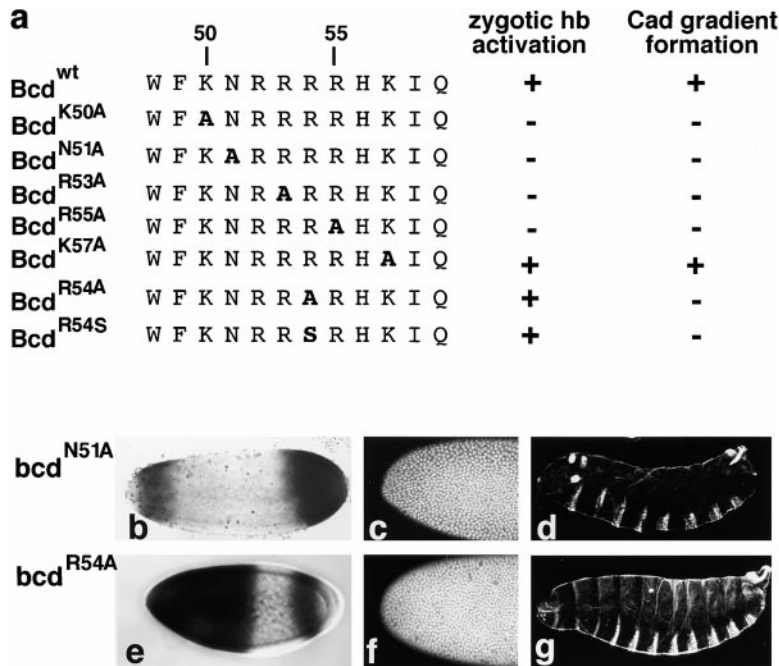


Figure 3. Transcriptional Activation, Translational Repression, and Larval Cuticle Phenotype in Response to Transgene-Expressed Helix III Mutations with Known In Vitro DNA and RNA Binding Properties

Embryos derived from transgenic *bcd*<sup>fl</sup> females ("bcd-deficient embryos"; see Figures 1c and 1d) were analyzed as described in Figure 1; orientation of embryos and larvae is anterior to the left and dorsal up. (a) Summary of BCD replacement mutants (left) and their effect on zygotic *hb* transcriptional activation and *cad* mRNA translational repression (right) as illustrated in (b)-(g). (b-d) *bcd*-deficient embryos expressing BCD<sup>N51A</sup> lack the anterior *hb* expression domain and show instead a duplication of the posterior, BCD-independent *hb* expression domain (b). CAD is present and remains in the anterior pole region even during the syncytial blastoderm stage (c), indicating that translational repression of *cad* mRNA does not occur. Such embryos show a *bcd* larval cuticle phenotype, indicating the lack of rescue in response to BCD<sup>N51A</sup> expression (d). (e-g) *bcd*-deficient embryos expressing the BCD<sup>R54A</sup> mutant show a BCD-dependent anterior *hb* expression domain (e), but translation of *cad* mRNA is not repressed anteriorly (f). Larval segmentation appears to be normal, but the head pattern elements are not properly assembled (g); for details of the head phenotype, see Figure 4).

were indistinguishable from HD<sup>wt</sup>. In summary, arginine at position 54 of the HD is critical for specifying RNA versus DNA binding, and its replacement shifts the binding property of the HD to prefer DNA over RNA recognition.

#### R54 Mutations Prevent BCD-Dependent Translational Repression of *cad* mRNA

In order to test the *in vivo* relevance of our binding studies, we examined the corresponding BCD HD mutants by transgene-dependent expression in *bcd* embryos. The BCD mutants were generated in the context of an 8.7 kb genomic DNA fragment spanning the entire *bcd* locus, which fully rescues *bcd* embryos after P element-mediated transformation (Figure 3a) (Berleth et al., 1988). The transgene-expressed BCD<sup>K57A</sup> protein, which contains an HD with normal DNA and RNA binding properties (see Figures 2g and 2m), caused BCD-dependent *hb* expression and CAD gradient formation (Figure 3a), and the embryos developed into normal-looking larvae and fertile adults (not shown). BCD<sup>N51A</sup>, BCD<sup>R53A</sup>, and BCD<sup>R55A</sup>, which contain HD mutations that caused the loss of DNA and RNA binding properties *in vitro* (see Figure 2), failed to activate BCD-dependent *hb* transcription (Figures 3a and 3b) and to repress translation of *cad* mRNA (Figures 3a and 3c); such embryos developed a *bcd* mutant phenotype (Figure 3d). The BCD<sup>R54A</sup> mutant, which contains an HD with DNA, but no RNA, binding properties, was able to activate the transcription of *hb* (Figure 3e), but not to repress the translation of *cad* mRNA (Figure 3f). This observation is consistent with the result obtained using the transgene bearing the BCD<sup>R54S</sup> mutation, which contains a serine residue in place of arginine at position 54 (Figure 1i). Thus, both BCD mutants that contain a replacement of arginine at position 54 of the HD fail to control *cad* mRNA translation but activate transcription of *hb*.

#### R54 Mutation Causes a Head Mutant Phenotype

Mutations of *bcd* that interfere with the control of *cad* mRNA translation but not with the activation of transcription cause temperature-dependent head involution defects (Niessing et al., 1999). The corresponding larvae develop the normal number and identity of head segments, which, however, fail to be properly assembled (Niessing et al., 1999). The same phenotype would be expected for the BCD<sup>R54A</sup> mutant embryos, ensuring that the replacement affects only *cad* mRNA translational control. *bcd* embryos expressing the BCD<sup>R54A</sup> mutant developed a normal segment pattern at 18°C and gave rise to normal-looking and fertile adults. At 29°C, however, the majority of the embryos (more than 90%) died as unhatched larvae, and all of them expressed a strong head defect (Figure 3g). The embryos showed a normal expression pattern of the segment polarity gene *engrailed* (*en*) (DiNardo et al., 1985) at stages 9–11 (stages according to Campos-Ortega and Hartenstein [1997]), indicating that segments were generated normally (Figures 4a and 4c). Furthermore, all discernible head markers (Campos-Ortega and Hartenstein, 1997) could be observed in larval cuticle preparations, but, as observed with mutations affecting the translational repressor region of BCD (Niessing et al., 1999), the assembly of the

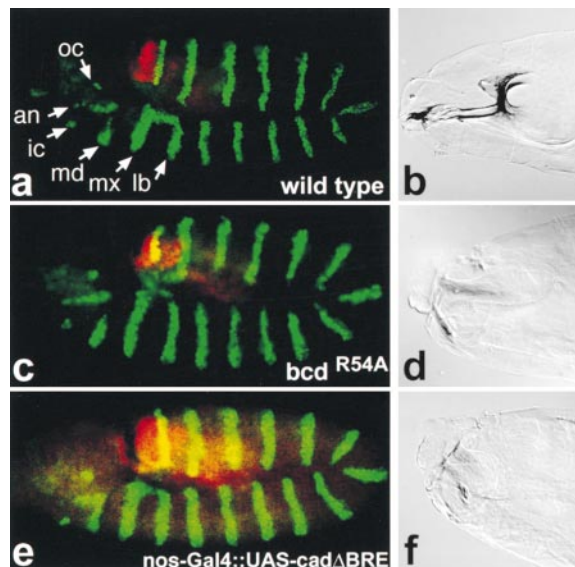


Figure 4. CAD Activity in the Anterior Pole Region of the Embryo Affects Head Morphogenesis

(a and b) Engrailed expression (anti-Engrailed antibody staining; green) in germband extended (stage 11) wild-type embryos (a) and enlarged larval head region (b). The head segments are indicated with arrows. oc, ocular; an, antennal; ic, intercalar; md, mandibular; mx, maxillar; lb, labial. (c and d) Corresponding Engrailed expression pattern in BCD<sup>R54A</sup>-expressing *bcd*-deficient embryos (see also Figures 3e–3g) (c) and enlarged larval head region (d). Note that the head pattern elements are present, but head morphogenesis is defective, as described in the text. (e and f) A normal Engrailed expression pattern was observed in all embryos that had received anterior CAD activity by the UAS/GAL4 system (see Experimental Procedures). Such embryos develop similar head defects (f) as BCD<sup>R54A</sup> mutant embryos (d). CAD expression in early embryos (not shown) and its absence from later embryos (a, c, and e) were examined by immunofluorescence using anti-CAD antibodies (red staining).

head elements was strongly perturbed (Figures 4b and 4d). The same temperature-dependent phenotype was observed when *cad* cDNA lacking the BCD-responsive BBR in the 3'UTR was expressed in the preblastoderm embryo using the GAL4/UAS system (Brand and Perrimon, 1993) (Figures 4e and 4f). Taken together, the *in vivo* transgene studies and the *in vitro* binding results establish that a single amino acid replacement in the ARM of the BCD HD specifically interferes with BCD-dependent RNA binding and translational repression of *cad* mRNA, without affecting DNA binding and transcriptional activation. The finding is consistent with the observation that an arginine residue at this position is conserved in ARMs (Mattaj, 1993; Burd and Dreyfuss, 1994; Tan and Frankel, 1995) but rare in HDs (Gehring et al., 1994a).

#### Binding Features of the BCD HD

The results provide strong evidence that the BCD HD functions as a nucleic acid-binding unit that enables BCD to function in transcriptional and translational control. In addition, the findings establish that the direct interaction of BCD with the BBR of *cad* mRNA shown *in vitro* is necessary to prevent CAD activity from interfering with head morphogenesis. We identified helix

III of the BCD HD as a region in which a single amino acid replacement shifts the *in vitro* binding property of the HD to prefer DNA over RNA recognition and abolishes *cad* mRNA translational repression not affecting transcriptional activation by BCD *in vivo*. The  $\alpha$ -helical structure and sequence comparison between HIV-1 REV and the third helix of the BCD HD indicate that it formally fits as a member of the ARM family of RNA-binding proteins that show a low degree of amino acid sequence identity (Tan and Frankel, 1995, 1998). The sequence similarity between the ARMs of HIV-1 REV and the BCD HD is therefore remarkable (Figure 2a). However, there is no corresponding sequence similarity observed between the RNA target sequences to which they bind. Furthermore, REV fails to bind the BBR, and BCD-HD does not recognize the REV response element (R. R.-P and D. N.; unpublished results). Thus, the high degree of amino acid identity and conservation of the critical arginine residue in the ARMs of the BCD HD and HIV-1 REV is not correlated with similarity at the level of the targets.

Asparagine is absolutely conserved at position 51 of HDs (Gehring et al., 1994a, 1994b; Billeter, 1996) and is also found in the corresponding position in ARM family members (Tan et al., 1993; Tan and Frankel, 1998). It has been shown to provide base contacts in DNA/HD complexes (Gehring et al., 1994b; Billeter, 1996) and RNA target recognition by ARM proteins (Tan et al., 1993; Battiste et al., 1996; Tan and Frankel, 1998), respectively. Consistently, mutation of arginine in position 51 of the BCD HD abolished DNA binding as well as RNA binding. In contrast, the 52–57 region of HDs interacts with DNA electrostatically, whereas some of the corresponding REV arginine residues are hydrogen bonded to bases (Battiste et al., 1996). Mutating arginine at position 54, which is rare in other HDs (Gehring et al., 1994a; Billeter, 1996), affects RNA binding without altering the DNA binding. In summary, these and earlier findings with respect to the DNA binding properties of HDs (Gehring et al., 1994a, 1994b; Billeter, 1996) support the proposal that the ARM within the helix III of the BCD HD is necessary for both RNA and DNA target recognition, and that individual amino acids within this portion of the HD specify RNA versus DNA binding.

Although the BCD HD is by now the only known HD with RNA binding properties, it has been noted that the ARM-containing RNA-binding domain of EIAV-TAT and the ribosomal protein L11 can fold into HD-like structures with the RNA-binding domain exposed as an helix III equivalent (Rösch and Willbold, 1996; Markus et al., 1997; Xing et al., 1997). The recently solved crystal structure of this protein bound to a ribosomal RNA fragment shows binding to the minor groove of RNA that is similar in width to a DNA major groove. The results also indicate that L11 uses the same surface as the HD does in binding DNA (Conn et al., 1999; Wimberly et al., 1999). The structural similarities and the fact that helix III regions of HDs are generally rich in basic amino acids (Billeter et al., 1993; Gehring et al., 1994a, 1994b) suggest that HDs hold a high potential to either exert or to adopt RNA binding properties during evolution. The possibility that other HDs also bind RNAs and thereby provide HD proteins with dual regulatory functions is a challenging proposal.

## Experimental Procedures

### Mutagenesis, Expression, and Purification of Proteins

pRSN-HisbcdHD (spanning amino acids 89–154; positions according to Berleth et al. [1988]) was generated by PCR. It creates an NdeI site at the methionine in position 89 and a stop codon at position 155, followed by a BamHI site. DNA fragments were cloned into a blunted NheI site of pRSETa (Invitrogen), generating an in-frame amino tag of six N-terminal histidines. Mutated HDs were generated by PCR amplification; proteins were produced in *E. coli* BL21 (DE3) and purified as described (Rivera-Pomar et al., 1995). Purity (>90%) was determined on silver-stained SDS-PAGE gels. A frequent 24 kDa contaminant protein was removed by chromatofocusing using Mono P columns (Pharmacia, Uppsala, Sweden) and 25 mM triethanolamine-HCl (pH 7.0).

### In Vitro Binding Assays

DNA-labeling reactions were performed by using [ $\gamma$ - $^{32}$ P]ATP and polynucleotide kinase (Burz et al., 1998). Electrophoretic mobility shift assays were performed using a double-stranded oligonucleotide (15-mer) that carries a BCD consensus binding site (5'-TCTTAATCCC-3') (Driever and Nüsslein-Volhard, 1989). In brief, 1–10 pmol of protein was incubated with 10–100 fmol of labeled DNA in 20  $\mu$ l for 15 min on ice in reaction buffer (25 mM HEPES K<sup>+</sup> [pH 7.5]; containing 100 mM KCl, 12.5 mM MgCl<sub>2</sub>, 0.1% NP-40, and 20% glycerol). Reactions were loaded onto 15% polyacrylamide gels and electrophoresed for 3 hr at 15 V/cm. Labeled RNA was produced by *in vitro* transcription using [ $\alpha$ - $^{32}$ P]ATP and T7 RNA polymerase. The plasmid used for generating the transcript (pBS-BBR2) bears a 110-nucleotide *cad* 3' UTR cDNA containing the BBR (Rivera-Pomar et al., 1996). The transcript was ethanol precipitated and dissolved in water; purity was determined by denaturing polyacrylamide gel electrophoresis. Prior binding reaction the BBR was heated (70°C; 5 min) in binding buffer (10 mM HEPES K<sup>+</sup> [pH 7.6], 100 mM KCl, 1 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 1 mM DTT, 10% glycerol, 2.5  $\mu$ g/ $\mu$ l heparin, and 5  $\mu$ g/ $\mu$ l *E. coli* tRNA). HD-RNA interactions were monitored by EMSA using 1–10 pmol of protein and 2000 cpm of *in vitro* transcribed BBR (0.1 ng). Binding reactions were done in a 20  $\mu$ l volume (room temperature; 5 min). Free DNA or RNA targets and protein/nucleic acid complexes were separated by PAGE (7.5%, acrylamide:bisacrylamide: 29:1, 0.5 $\times$  TBE; 6 hr at 10V/cm; 4°C) and analyzed by a phosphorimager (Molecular Dynamics).

### Transgenic Flies and Embryo Analysis

Mutant *bcd* minigenes (Driever et al., 1990) were generated by PCR. External primers included the PstI and Sall restriction sites present at helix II of the homeodomain and the amino acid residue 246. Mutant primers (5' to 3') generated restriction sites at the site of mutation that allowed subsequent cloning. PstI-Sall fragments containing the mutation were inserted into PstI-Sall sites of *bcd* cDNA to substitute the wild-type sequence. The mutant cDNAs contained the 5'UTR of the *Xenopus*  $\beta$ -globin leader. Mutant cDNAs were inserted into the *bcd* minigene P transformation vector pCaSpeRbcdBgIII (Driever et al., 1990). Cloning and DNA analysis were done according to standard protocols. Mutagenesis of the entire *bcd* gene was carried out in a pBluescript-based plasmid bearing the genomic fragment spanning 8.7 kbp of the *bcd* gene (Berleth et al., 1988) using the Quik Change kit (Stratagene). Mutations were confirmed by sequencing. Mutant genes were subsequently transferred to the P transformation vector pCaSPeR4 (Thummel and Pirrotta, 1992) to generate transgenic lines by P element-mediated germline transformation (Rivera-Pomar et al., 1995). Transformants were crossed to *bcd*<sup>E1</sup> mutants. The GAL4/UAS system (Brand and Perrimon, 1993) involved the UAS-*cad* $\Delta$ BBR (Moreno and Morata, 1999) and a maternal-expressing Gal4 line (gift from P. Gergen). Transgene-dependent zygotic *hb* expression, CAD gradient formation, and rescue of the *bcd*<sup>E1</sup> mutant phenotype were scored as described in the text. Fixation and antibody staining of embryos were done as described (Mlodzik et al., 1990) using fluorescent secondary antibodies labeled with the dyes Cy3 (Jackson Laboratories), Alexa488, and Alexa546 (Molecular Probes). Embryos were embedded in Mowiol (Hoechst); fluorescent images were taken by

a Zeiss Laser-Scanning Microscope. Whole-mount in situ hybridization of staged embryos was performed as described (Klingler and Gergen, 1993).

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