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Sequence interval within the PEST motif of Bicoid is important for translational repression of *caudal* mRNA in the anterior region of the *Drosophila* embryo

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The *Drosophila* body organizer Bicoid (Bcd) is a maternal homeodomain protein. It forms a concentration gradient along the longitudinal axis of the preblastoderm embryo and activates early zygotic segmentation genes in a threshold-dependent fashion. In addition, Bcd acts as a translational repressor of maternal *caudal* (*cad*) mRNA in the anterior region of the embryo. This process involves a distinct Bcd-binding region (BBR) in the 3′ untranslated region (UTR) of *cad* mRNA. Using cotransfection assays, we found that Bcd represses translation in a cap-dependent manner. Bcd-dependent translational repression involves a portion of the PEST motif of Bcd, a conserved protein motif best known for its function in protein degradation. Rescue experiments with Bcd-deficient embryos expressing transgene-derived Bcd mutants indicate that amino acid replacements within the C-terminal portion of the PEST motif prevent translational repression of *cad* mRNA but allow for Bcd-dependent transcriptional activation. Thus, Bcd contains separable protein domains for transcriptional and translational regulation of target genes. Maternally-derived *cad* protein in the anterior region of embryos interferes with head morphogenesis, showing that *cad* mRNA suppression by Bcd is an important control event during early *Drosophila* embryogenesis.

Keywords: Bicoid/cap-dependence/head morphogenesis/PEST/translational control

Introduction

The *Drosophila* homeodomain protein Bicoid (Bcd) acts as a maternal anterior determinant during embryogenesis (Frohnhofer and Nusslein-Volhard, 1986; Berleth *et al*., 1988). *bicoid* (*bcd*) mRNA is expressed during oogenesis and becomes localized at the anterior pole region of the egg. Following egg deposition, *bcd* mRNA is translated and the protein forms an anterior-to-posterior concentration gradient in the developing embryo (reviewed in Driever, 1993; St Johnston, 1995). Bcd mediates the control of gene expression in the anterior region of the embryo. It activates transcription of zygotic segmentation genes, such as *hunchback* (*hb*) (Tautz *et al*., 1987; Tautz, 1988; Driever and Nusslein-Volhard, 1989; Struhl *et al*., 1989) and suppresses the translation of ubiquitous maternal *caudal* (*cad*) mRNA (Dubnau and Struhl, 1996; Rivera-Pomar *et al*., 1996). Bcd-dependent translational repression of *cad* mRNA results in a concentration gradient of the protein product (Mlodzik *et al*., 1985; Macdonald and Struhl, 1986; Mlodzik and Gehring, 1987) which is complementary to that of Bcd. The protein Caudal (Cad) is a homeodomain (HD) transcription factor (Mlodzik *et al*., 1985) necessary for proper zygotic segmentation gene activation in the posterior region of the embryo (reviewed in Rivera-Pomar and Jackle, 1996).

Most of our present knowledge concerning the regulated translation of mRNAs in the *Drosophila* embryo is derived from studies with the maternal polarity determinants encoded by *nanos* (*nos*) and *oskar* (*osk*) (reviewed in St Johnston, 1995). The mRNAs of these genes are expressed in nurse cells and then transferred to, and localized within, the adjacent oocyte (reviewed in St Johnston, 1995; Rongo and Lehmann, 1996). Translation of these mRNAs is silenced during their transport until the protein is required in the embryo (reviewed in Curtis *et al*., 1995; Hake and Richter, 1997). The role of Bcd in translational repression of *cad* mRNA, the regions of Bcd required to mediate this process and the biological significance of *cad* mRNA repression are still unknown.

Previous results have shown that Cad is evenly distributed throughout embryos lacking functional *bcd* activity (Mlodzik and Gehring, 1987). Failure of gradient formation is caused by impaired translational repression of *cad* mRNA in the anterior region of the embryo. Bcd-mediated translational repression involves the HD which binds directly to a *cis*-acting Bcd-binding region ('BBR', Rivera-Pomar *et al*., 1996) within the 3′ untranslated region (3′UTR) of *cad* mRNA. The BBR (also termed Bicoid-response element: 'BRE'; Dubnau and Struhl, 1996) has been shown to mediate Bcd-dependent translational repression both in cell culture and in transgenic *Drosophila* embryos (Dubnau and Struhl, 1996; Rivera-Pomar *et al*., 1996). While the Bcd HD is necessary and sufficient for binding to *cad* mRNA (Rivera-Pomar *et al*., 1996; Chan and Struhl, 1997), the HD alone cannot mediate translational repression *in vivo* indicating the requirement for additional functional sequences.

Here we show that Bcd-dependent translational repression requires the C-terminal portion of the PEST motif, a protein sequence best known as a proteolytic signal to target proteins for degradation by the 26S proteasome (Rogers *et al*., 1986; Rechsteiner and Rogers, 1996). Additionally, at least in cell culture, Bcd-dependent translational repression functions in a cap-dependent manner. We also show that a Bcd mutant protein which contains amino acid replacements in the corresponding region of

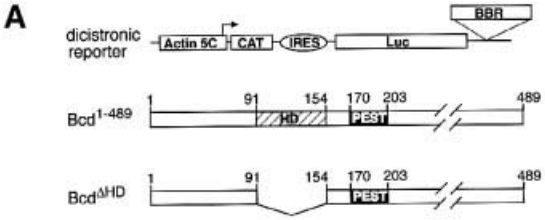
the PEST motif activates zygotic target genes but fails to suppress *cad* mRNA translation in the anterior region of the embryo. Thus, transcriptional activation and translational repression are separable functions of Bcd involving different non-overlapping portions of the protein. *cad* activity impairs proper head morphogenesis when present in the anterior region of the early embryo.

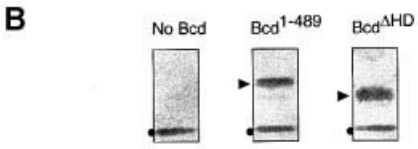
Results

Bicoid represses cap-dependent translation in cell culture

Cell culture cotransfection experiments and transgene studies in embryos have shown that Bcd represses translation of reporter gene mRNAs containing the BBR-element in the 3′UTR (Dubnau and Struhl, 1996; Rivera-Pomar *et al*., 1996). It had been suggested that Bcd functions in a cap-dependent manner (Dubnau and Struhl, 1996). To test this proposal, we designed reporter gene constructs which express a dicistronic transcript under control of the constitutively active actin 5C promotor (Heberlein *et al*., 1985; Heberlein and Tjian, 1988). The first cistron, encoding chloramphenicol acetyltransferase (CAT), was separated from the second cistron by an internal ribosome entry site (IRES) that is derived from the *Antennapedia* leader (Oh *et al*., 1992). The second cistron, encoding luciferase (Luc), was followed by 3′UTR sequences containing the BBR of *cad* mRNA (Figure 1A). Thereby, translation of the first cistron is cap-dependent, whereas the translation of the second cistron is mediated in an IRES-dependent manner (Oh *et al*., 1992; Jackson, 1996).

We cotransfected *Drosophila* Schneider cells (Schneider, 1972) with a plasmid bearing the dicistronic reporter gene and with effector plasmids expressing either Bcd, a Bcd mutant lacking the HD, or with mock DNA (Figure 1A). The expression of the dicistronic reporter transcript in the cells was monitored by RNase protection assays and translation of the resulting mRNA by measuring CAT and Luc activities. The presence of effector protein was monitored by Western blotting (see Materials and methods). In the absence of Bcd, both CAT and Luc are expressed at control levels. The same result was observed in the presence of effector Bcd lacking the HD (‘Bcd$^{\Delta HD}$’; Figure 1B and C) or with dicistronic reporter genes lacking the BBR in the 3′UTR region (Figure 1C). These findings are consistent with recent results showing that the HD of Bcd and the BBR are necessary for the suppression of *cad* mRNA translation in *Drosophila* embryos (Dubnau and Struhl, 1996; Rivera-Pomar *et al*., 1996). However, in cells expressing full-length Bcd (Bcd$^{1–489}$), CAT activity was significantly reduced, both in absolute terms and in comparison with Luc activity (Figure 1C). Thus, Bcd is able to repress translation of dicistronic target mRNA and, at least in tissue culture, operates in a cap-dependent manner.





C

effector	BBR	CAT	Luc	CAT/Luc	average CAT/Luc
None	+	60,179	98,122	0.613	0.594±0.030
		57,482	109,479	0.525	
		62,215	99,189	0.627	
		62,061	104,213	0.603	
		59,517	99,771	0.597	
		53,617	88,618	0.605	
		73,920	124,516	0.594	
		75,711	129,131	0.586	
Bcd$^{1-489}$	+	27,633	85,394	0.324	0.312±0.053
		18,817	52,316	0.360	
		30,577	88,943	0.344	
		3,105	12,862	0.241	
Bcd$^{1-489}$	−	58,528	94,744	0.618	0.539±0.065
		42,250	88,560	0.477	
		32,788	57,924	0.566	
		44,227	89,272	0.495	
Bcd$^{\Delta HD}$	+	44,612	82,713	0.539	0.524±0.042
		34,416	68,987	0.499	
		33,914	70,430	0.482	
		49,480	86,143	0.574	
Bcd$^{\Delta HD}$	−	27,438	49,833	0.551	0.607±0.038
		36,382	58,589	0.621	
		46,489	75,103	0.619	
		44,924	70,620	0.636	

Fig. 1. Bcd-dependent regulation of translational repression in *Drosophila* Schneider cells. (**A**) Reporter gene designed to express a dicistronic transcript in which the cistrons chloramphenicol acetyltransferease (CAT) and luciferase (Luc) are separated by an internal ribosome entry site (IRES); the second cistron is followed by the SV40 3′UTR, including or excluding the BBR. The reporter genes are under the control of a constitutive promoter (actin 5C) and result in a dicistronic transcript. Translation of the 5′ transcript (CAT) occurs in a cap-dependent manner, whereas translation of the 3′ transcript (Luc) is IRES-dependent. Effector genes under the control of the actin 5C promoter express full-size Bcd (Bcd$^{1–489}$; sequence according to Berleth *et al*., 1988) or an inactive Bcd (Bcd$^{\Delta HD}$) in which the RNA-binding homeodomain (HD, position 91–154; hatched box) was deleted. (**B**) Western blots tested with polyclonal anti-Bcd antibodies, showing that the Bcd$^{\Delta HD}$ control protein (see panel C) is produced in the cotransfected cells. Note the presence of a crossreacting band (dot) which served as an internal control for the amount of effector protein produced (arrowhead). (**C**) Translation-dependent reporter gene activities in cotransfected cells. ‘None’ refers to the absence of an effector gene; ‘+’ or ‘−’ BBR refers to the presence or absence of the BBR in the 3′UTR of the dicistronic mRNA. CAT and Luc activities are shown from eight independent transfection experiments involving new plasmid DNA preparations and batches of cultured cells. Note that the CAT activities and the ratio of CAT/Luc activities were altered by full-size Bcd (Bcd$^{1–489}$) when co-expressed with the BBR-containing reporter construct. Thus, Bcd$^{1–489}$ interferes primarily with cap-dependent translation of CAT. Mean values and standard deviations (±) are shown.

Bcd-dependent translational repression involves PEST sequences

Previous results have shown that Bcd-dependent translational repression requires the RNA-binding HD (Dubnau and Struhl, 1996; Rivera-Pomar *et al*., 1996; Chan and Struhl, 1997). In order to identify other regions of Bcd requisite for mediating translational control, we used the previously described tissue culture system to assay truncation mutants of Bcd, created by the insertion of stop codons (Figure 2A), for their ability to repress cap-dependent translation of the dicistronic mRNA. Luc activity served as an internal reference. For this, we standardized the cotransfection conditions so that translation of CAT mRNA in response to full-size Bcd was reduced to ~50% of the values obtained in the absence of

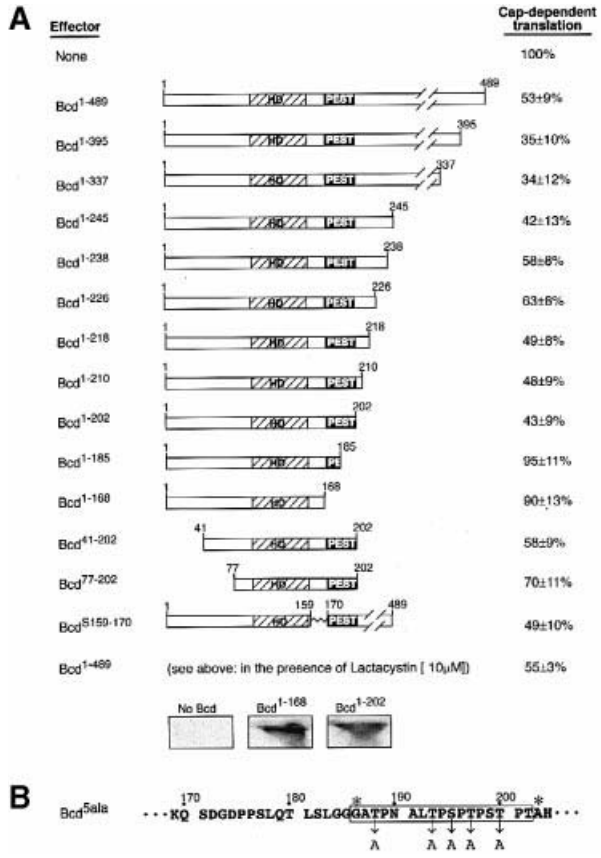


Fig. 2. The Bcd PEST motif is required for translational repression in *Drosophila* Schneider cells. (**A**) Effector plasmids (left side) were co-transfected with the dicistronic reporter gene encoding cap-dependent CAT and IRES-dependent Luc transcripts (details in Figure 1A). Using the assay system described in Figure 1, the results show that C-terminal Bcd truncations up to the PEST motif are able to exert translational repression ($Bcd^{1–489}$ to $Bcd^{1–202}$), whereas Bcd truncations affecting the PEST motif ($Bcd^{1–185}$, $Bcd^{1–168}$) failed to repress. Deletion of N-terminal sequences of Bcd ($Bcd^{41–202}$) did not significantly interfere with repression, whereas the deletion of the N-terminal sequences up to near the homeodomain ($Bcd^{77–202}$) resulted in reduced repression. We do not know whether this is due to a less stable protein or to structural changes close to or affecting the HD required for RNA-binding. Replacement of the amino acid stretch between the HD and the PEST motif (159-EGMPLSPGMKQ-171 by 159-SRGDASLAGYET-171; $Bcd^{S159–170}$) did not affect translational repression. Also, a specific inhibitor of ubiquitin-mediated protein degradation, Lactacystin, did not interfere with translational repression (see Materials and methods and text). Note that $Bcd^{1–202}$, which causes translational repression, and non-repressing $Bcd^{1–168}$ are produced in co-transfected Schneider cells as shown by Western blot analysis with polyclonal anti-Bcd antibodies (for details see Figure 1 and Materials and methods). (**B**) Sequence of the Bcd PEST motif and replacements of tyrosine and serine residues by alanines (Bcd^{5ala}; see Figure 3). Position of stop codons causing truncated versions of Bcd ($Bcd^{1–185}$, $Bcd^{1–202}$; see panel A) are indicated by asterisks above the sequence.

Bcd, in the presence of Bcd lacking the HD, and the values obtained with reporter genes lacking the BBR element (Figures 1A and 2A; for details see Materials and methods).

Deletion of the C-terminal half of Bcd up to position 245 ($Bcd^{1–245}$; position refers to the sequence of Berleth *et al*., 1988) eliminating two transactivation domains previously shown to be required for Bcd-dependent transcriptional activation (Driever *et al*., 1989a; Sauer *et al*., 1995) had no effect on translational repression (Figure 2A). Furthermore, successive removal of additional C-terminal sequences up to the C-terminal end of the PEST motif (as represented by $Bcd^{1–202}$) did not interfere with the ability of the protein to repress translation, although the degree of repression with different truncations was variable (Figure 2A). These results establish that mutants containing an intact N-terminal portion of Bcd including the PEST motif were able to repress translation, whereas C-terminal deletions which lack part or all of the PEST motif ($Bcd^{1–185}$, $Bcd^{1–168}$) lose this ability (Figure 2A). This indicates that a stretch of 17 amino acids at the C-terminus of the PEST motif (see Figure 2B) includes sequences necessary to mediate Bcd-dependent translational repression (see also below).

We next asked whether the N-terminal region of Bcd contains sequences that are necessary for translational repression. We therefore generated N-terminal deletion mutants of $Bcd^{1–202}$. Deletion of the N-terminal most 40 amino acids ($Bcd^{41–202}$), which includes 'paired repeats' (Berleth *et al*., 1988), did not affect the ability of the protein to repress translation. Also, a truncated protein that contains the HD (position 91–154), the PEST motif and a stretch of 14 amino acids between the two domains ($Bcd^{77–202}$) was able to repress translation, albeit more weakly than $Bcd^{41–202}$ (Figure 2A). We do not know whether the reduced repressor activity of $Bcd^{77–202}$ protein is due to structural changes, the requirement of sequences between amino acid residues 41 and 77, or reduced stability of the truncated protein. Finally we asked whether the stretch of amino acids connecting the HD and the PEST motif contributes to repression. To address this question, we altered the sequence of the joining region (position 159 to 170) by inserting two complementing frame-shift mutations after the HD and in front of the PEST motif, respectively (see altered sequence in legend of Figure 2). The resulting $Bcd^{S159–170}$ mediated translational repression (Figure 2A). These results establish that the C-terminal 17 amino acids of the PEST motif are necessary for Bcd-dependent translational repression (Figure 2A and B). Furthermore, a minimal Bcd protein, composed of little more than the HD and the PEST motif and lacking the regions required for Bcd-dependent transcriptional control, is sufficient to provide translational repression.

The finding that the region of Bcd necessary for repression includes sequences of the PEST motif could mean that translational repression and ubiquitin-dependent degradation are functionally linked. Ubiquitin-dependent degradation has been shown to act *in trans* on associated proteins (Johnson *et al*., 1990). We asked whether the mechanism of translational repression by Bcd involves PEST motif-dependent *in trans* protein degradation of, for example, translation initiating factors which associate with Bcd. To test this, we added Lactacystin, a specific and irreversible inhibitor of ubiquitin-dependent protein degradation (Fenteany *et al*., 1995), to the tissue culture medium and monitored Bcd-dependent translation repression. Lactacystin was found to be active in the co-transfected cells (see Materials and methods), but did not prevent Bcd-dependent translational repression (Figure 2A). Thus, translational repression by Bcd is not likely to involve ubiquitin-dependent protein degradation (see Discussion).

Repression of maternal cad mRNA involves PEST motif sequences

In order to establish the biological significance of the results obtained in tissue-culture cells, we performed transgene-dependent rescue experiments with *bcd*-deficient embryos. The transgenes were designed to express Bcd or Bcd mutants under the control of the *nos* promoter in homozygous *bcd* mutant females (see Materials and methods). Due to the presence of the *bcd* mRNA localization element in the 3′UTR, the transgene-derived mRNAs were localized in the anterior pole region of the embryo as described for *bcd* mRNA.

Embryos from *bcd* mutant females lack head and thoracic pattern elements which are replaced by a duplication of the posterior abdominal and terminal regions (Frohnhofer and Nusslein-Volhard, 1986). In molecular terms, such embryos fail to express the zygotic Bcd target gene *hunchback* (*hb*) in the anterior half of the blastoderm embryo. Instead, the posterior domain of *hb* expression, which is activated in a Bcd-independent fashion, is duplicated anteriorly (Tautz *et al*., 1987; Tautz, 1988). Furthermore, the Cad gradient does not form, i.e. Cad accumulates in all nuclei of the precellular blastoderm (Mlodzik and Gehring, 1987). In contrast, mutant embryos which express transgene-derived wild-type Bcd show a normal *hb* expression pattern and Cad forms a posterior-to-anterior concentration gradient.

We first asked whether expression of transgene-derived $Bcd^{1–202}$, which contains an intact PEST motif, can rescue the molecular and functional aspects of the *bcd* mutants. $Bcd^{1–202}$ expression produced a normal Cad gradient (Figure 3A and B) but failed to activate anterior expression of *hb* (Tautz, 1988). The embryos showed a duplicated Bcd-independent posterior *hb* expression domain in the anterior region, indistinguishable from *bcd* lack-of-function mutants (Figure 3C; compare with Figure 3E). $Bcd^{1–185}$, which lacks the C-terminal 17 amino acids of the PEST motif, also failed to activate the anterior *hb* expression domain (not shown), and additionally, no Cad gradient was formed (Figure 3D). This indicates that $Bcd^{1–185}$ has lost the ability to both control translation and transcription in the anterior half of the embryo. Thus, as observed in cell culture, translational repression by Bcd requires a stretch of 17 amino acids at the C-terminus of the PEST motif of Bcd (position 186–202; see Figure 2B).

The transactivation domains of Bcd necessary for target gene activation *in vivo* were previously shown to be localized C-terminal to the PEST sequences (Driever *et al*., 1989a). This suggests that transcriptional activation and translational repression are mediated by different and separable modules that are required in addition to the DNA/RNA binding HD of Bcd. In order to show that the C-terminal region of Bcd's PEST motif is indeed only necessary for translational repression, we generated a Bcd mutant that contains five alanine residues replacing the serine/threonines in position 188, 193, 195, 197 and 200 (Bcd^{5ala}; Figure 2B). Transgene-dependent expression of Bcd^{5ala} in embryos of *bcd* mutant females activated zygotic *hb* expression in the anterior half of the embryo (Figure 3E). However, translational repression of *cad* mRNA occurred not in embryos, i.e. Cad is present in the anterior region of the embryo (Figure 3F). Bcd^{5ala} expression was able to rescue the head and thorax development of the otherwise Bcd-deficient embryos (Figure 3G and H). This result demonstrates the importance of the 17 amino acid PEST sequence interval for Bcd-dependent translational repression of *cad* mRNA. In addition, it confirms that Bcd-dependent regulation of translation and transcription require distinct and non-overlapping regions of the protein with exception of the common DNA/RNA-binding HD (Dubnau and Struhl, 1996; Rivera-Pomar *et al*., 1996; Chan and Struhl, 1997). However, it is not clear whether serine/threonine phosphorylation, a given structure or a specific amino acid identity within the C-terminal part of the PEST motif is necessary for the Bcd-dependent repression of *cad* mRNA translation. It is important to note that Bcd-dependent repression also occurs in unfertilized eggs (not shown), implying that nuclear Bcd-protein in the early embryo is unlikely to participate in this process. Conversely, translational repression does not occur in unfertilized eggs expressing the Bcd^{5ala} mutant protein.

Ectopic cad activity interferes with head morphogenesis

The differential effect of the Bcd^{5ala} mutant on Bcd-dependent translational and transcriptional control allowed us to examine whether and how the presence of Cad in the anterior region of the embryo interferes with embryonic development. Approximately one third of the offspring of Bcd^{5ala} transgene-bearing *bcd* mutant females gave rise to fertile adults at 25°C. This demonstrates that in principle, embryos which received maternal *cad* activity in the anterior region are able to develop normally. On the other hand, the majority of the embryos that expressed the Bcd^{5ala} mutant died and developed a variable but strong head defect (Figure 3I). At 29°C, nearly all embryos (98%) died. All of them developed the same head mutant phenotype as observed with embryos at 25°C.

To exclude the possibility that increased stability of Bcd^{5ala} as compared with wild-type Bcd (N.Dostatni, manuscript in preparation) is the cause of abnormal head development, we examined the phenotypic consequences in embryos laid at 25°C that were derived from Bcd^{5ala} transgene-bearing wild-type females. Such embryos did not display head defects and developed into normal adults. Thus, Bcd^{5ala} did not interfere with wild-type development by acting in a gain-of-function mutant manner. The head defects observed in response to Bcd^{5ala} expression in embryos from *bcd* mutant females must therefore be due to the presence of *cad* activity in the anterior region. To demonstrate that non-repressed Cad activity is the cause of defective head morphogenesis, we used the GAL4/UAS system (Brand and Perrimon, 1993) to express *cad* mRNA lacking the BBR in the 3′UTR directly in wild-type embryos (see Materials and methods). Since the *cad* transgene lacks the BBR, Bcd can not repress *cad* mRNA translation and thus, Cad accumulates throughout the embryo, irrespective of the presence of Bcd (not shown). Such embryos develop a head mutant phenotype that closely resembles the phenotype observed with embryos expressing Bcd^{5ala} (compare Figure 3I and J), whereas embryos bearing only the GAL4-driving transgene or the UAS–*cad*ΔBBR transgene show no head phenotype.

Examination of Bcd^{5ala}-dependent and $cad^{\Delta BBR}$-dependent development showed that the cuticular head pattern

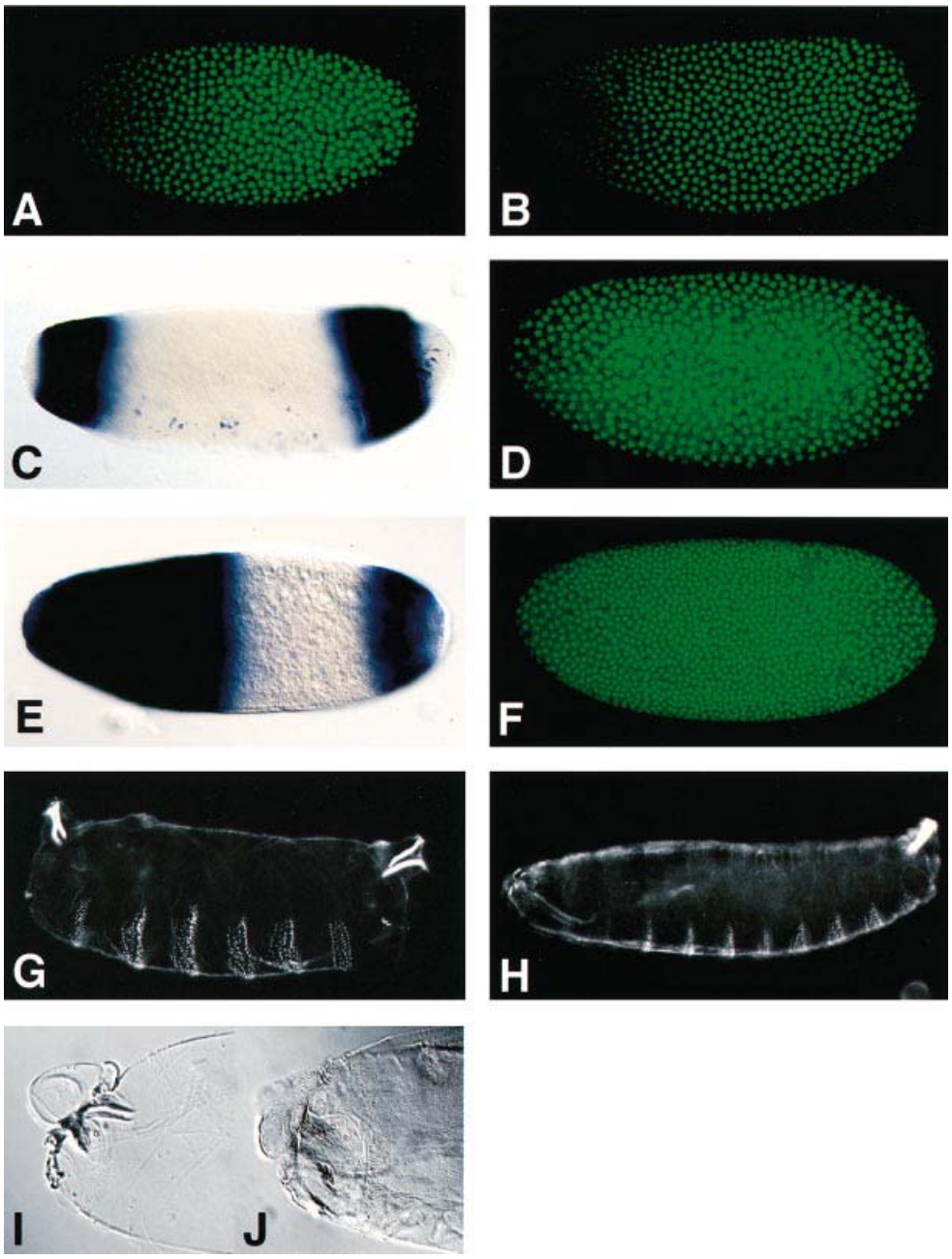


Fig. 3. Translational repression of *cad* mRNA in the anterior region of the embryo requires an intact PEST motif of Bcd. (**A**) Cad gradient formed in early *Drosophila* embryos (stage 11). (**B**) A transgene-expressed truncated version of Bcd ($Bcd^{1–202}$, see Figure 2A) which removes several distinct C-terminal domains including most of the serine- and threonine-rich sequences required for transcriptional activation and causes a Cad gradient in embryos derived from *bcd* mutant females. Such embryos develop a *bcd* mutant phenotype (see cuticle preparation in G). (**C**) *In situ* hybridization showing that the posterior expression domain of *hb* is duplicated, whereas the anterior, Bcd-dependent expression domain of *hb* (see panel E for a wild-type-like expression pattern) is absent in such embryos. Thus, $Bcd^{1–202}$ has lost the ability to activate transcription but maintains translational suppressor activity on *cad* mRNA. (**D**) Lack of Cad gradient formation in embryos derived from *bcd* mutant females expressing the $Bcd^{1–185}$ transgene. (E–I) Alanine replacements within the C-terminal portion of the Bcd PEST motif interfere with translational repression of *cad* mRNA. *In situ* hybridization of an embryo from a *bcd* mutant female bearing the Bcd^{5ala} transgene (see Figure 2B and text) showing a wild-type like expression pattern of *hb* expression (**E**), indicating that Bcd^{5ala} causes activation of transcription. Anti-Cad antibody staining showing that Cad is present in the anterior region of a corresponding embryo (**F**). Thus, Bcd^{5ala} functions as a transcriptional activator but fails to suppress the translation of *cad* mRNA. However, it rescues the *bcd* mutant phenotype (**G**). Rescued embryos develop a normal larval segment pattern (**H**) associated with a severe but variable head defect (**I**) due to an aberrant head involution process (**J**). A corresponding head phenotype was observed when Cad was expressed throughout the embryo using the GAL4/UAS system (for details see text). Orientation of embryos is anterior to the left, dorsal side up. Cad protein distribution was monitored by fluorescent anti-Cad antibodies viewed by confocal microscopy (see Materials and methods).

elements (Jürgens and Hartenstein, 1993; Schmidt-Ott *et al*., 1994) were formed in the larvae, whereas head morphogenesis was disrupted. The results suggest that ectopic *cad* activity did not interfere with head segmentation but rather with head involution movements. A detailed analysis of the Cad-dependent head phenotype will be presented elsewhere (D.Niessing, manuscript in preparation).

Discussion

Recent studies have shown that the Bcd-dependent regulation of target gene activities in the early *Drosophila* embryo, i.e. control of zygotic transcription (Driever *et al*., 1989a,b) as well as translational repression of maternal *cad* mRNA (Dubnau and Struhl, 1996; Rivera-Pomar *et al*., 1996), involves the HD which has DNA- and RNA-binding properties. The HD of Bcd has been found to be necessary and sufficient for an interaction with DNA sites of transcriptional target genes (e.g. Treisman and Desplan, 1989; Burz *et al*., 1998) and for the binding to *cad* mRNA (Rivera-Pomar *et al*., 1996; Chan and Struhl, 1997). Our results establish that the HD is the only region of Bcd involved in both regulatory functions of Bcd. The N-terminal half of Bcd (amino acids 1–202), which includes the HD and the PEST sequences, is sufficient to function as a translational repressor but not for Bcd-dependent transcriptional activation of zygotic target genes.

Transcriptional control by Bcd requires transactivation domains within the C-terminal half of Bcd (amino acids 203–489) which are necessary to mediate activation of transcription both *in vivo* (Driever *et al*., 1989a) and *in vitro* (Driever *et al*., 1989a; Sauer *et al*., 1995). Embryos which express a Bcd mutant lacking the C-terminal half, such as $Bcd^{1–202}$, consistently develop the *bcd* mutant phenotype, i.e. head and thoracic structures are absent and replaced by duplicated abdominal pattern elements including posterior terminal structures (see Frohnhofer and Nusslein-Volhard, 1986). However, $Bcd^{1–202}$ represses translation of BBR-containing mRNA in cotransfected cells and suppresses *cad* mRNA translation in the anterior region of the embryo. Translational repression by Bcd is impaired if the C-terminal region of the PEST motif of Bcd is affected. Thus, a sequence interval of 17 amino acids is necessary for translational repression by Bcd both in cell culture and *in vivo*. Mutations affecting the 17 amino acid interval do not interfere with Bcd-dependent transcriptional activation. Thus, with the exception of the HD, the sequences required for translational repression are not necessary for transcriptional activation and vice versa.

It is well established that PEST motifs serve as proteolytic signals to target the proteins for degradation by the 26S proteasome (Rogers *et al*., 1986; Rechsteiner and Rogers, 1996). This process is mediated by phosphorylation-dependent ubiquitination and provides a widespread regulatory mechanism to control the rapid turnover of cellular regulators such as cell-cycle proteins, transcription factors and metabolic enzymes (Hochstrasser, 1996; Varshavsky, 1997). In fact, the PEST motif of Bcd is important for controlling Bcd stability (N.Dostatni, manuscript in preparation) which in turn may contribute to the shape of the Bcd concentration gradient along the anterior–posterior axis of the embryo. Since inhibition of the proteasome pathway by Lactacystin did not interfere with Bcd-dependent translational repression in cell culture, we suspect that ubiquitin-dependent protein degradation is not part of the PEST-dependent translational repression mechanism.

Our data do not exclude the possibility that ubiquitination or the binding of an ubiquitin-like protein mediated by the PEST motif (Johnson and Hochstrasser, 1997) is necessary to initiate or mediate Bcd-dependent translational repression. We suspect, however, that PEST-dependent control of degradation and translational repression by Bcd are separate and unlinked functions which both involve the PEST motif of Bcd. In this context, it is noteworthy to mention that sequence comparison with a Bcd homolog of a lower dipteran fly, *Megaselia abdita*, revealed a stretch of conserved amino acids included in the C-terminal 17 amino acid interval of Bcd's PEST motif. However, the conserved amino acids, 191-LTPSxTPS-200, reside in different portions of the PEST motifs, i.e. C-terminal in *Drosophila* Bcd and N-terminal in *Megaselia* Bcd (Stauber *et al*., 1999). Since PEST motifs are not highly conserved in terms of amino acid identity (reviewed in Hochstrasser, 1996), the sequence identity of PEST sequences between the two Bcd homologs, which is affected by the Bcd^{5ala} mutation (see Figure 2B), may indicate a functional domain necessary to mediate translational repression. We are currently addressing this question by swapping the PEST motifs between the two Bcd homologs.

The recent finding that Bcd exerts a cap-dependent translational suppressor function on mRNA in the embryo (Dubnau and Struhl, 1996) could not be reproduced (Dubnau and Struhl, 1997). However, our results showing cap-dependent translational repression by Bcd in cell culture are consistent with such a mechanism. They imply that the 3′UTR-bound Bcd is able to act directly or indirectly with components at the 5′ end of the mRNA. Other examples for 3′ UTR-mediated repression of translation are the *nanos*-dependent translational control of *hb* mRNA (Murata and Wharton, 1995; Wharton *et al*., 1998) and the regulation of 15-lipoxygenase mRNA translation (Ostareck-Lederer *et al*., 1994; Ostareck *et al*., 1997). *nanos*-dependent translational suppression involves Pumilio, which binds to the *nanos*-response element. Pumilio may serve as a tether to recruit additional factors that either prevent docking of the ribosome or the ribosome assembly at the 5′ end of the mRNA (Wharton *et al*., 1998). Repression of 15-lipoxygenase translation involves the proteins hnRNP-K and hnRNP-E1 ('KH-proteins'). These proteins act at the last step of the translational initiation process by preventing the assembly of the 80S ribosome (Ostareck-Lederer *et al*., 1994; Ostareck *et al*., 1997). Our results argue more in favor of a Bcd-dependent mechanism that involves, for example, an interference of Bcd with components that recognize or assemble at the cap structure of the mRNA or with the assembly of the initiation complex itself (reviewed in Thach, 1992).

The mutant Bcd^{5ala} protein, which affects Bcd-dependent translational repression but not transcriptional activation, allowed us to examine the biological consequences of unsuppressed *cad* mRNA in the anterior region of the embryo. The results show that the ectopic activity of Cad results in a variable, temperature-sensitive head phenotype. The defective heads of such embryos show all known pattern elements (Jurgens and Hartenstein, 1993; Schmidt-Ott *et al*., 1994), but they were not properly assembled. Previous studies involving heat shock-induced overexpression of *cad* throughout the blastoderm embryo were shown to cause transformations of head segments in addition to head involution defects (Mlodzik *et al*., 1990). We have not observed transformations when maternal Cad

was present in the anterior region of the embryo. Thus, *cad* activity in the anterior region of the embryo appears not to interfere notably with the formation of head pattern elements but rather affects processes underlying head morphogenesis exclusively (reviewed in Jurgens and Hartenstein, 1993). The molecular and cellular mechanisms underlying the complex cell movements during head morphogenesis are not yet established. However, the required gene activities are likely to represent potential targets of unsuppressed Cad activity in the anterior region of the embryo, a proposal that requires further investigation.

Materials and methods

Transfections of cultured cells

The dicistronic reporter gene containing the first cistron (CAT), the *Antennapedia* exon D and the second cistron (Luc) was removed from the plasmid pSV_2CAT/D/LUC (Oh *et al*., 1992). It was inserted into pBluescript-KS(+) after fusing its 5′ end to the actin 5C-promoter (Heberlein *et al*., 1985; Heberlein and Tjian, 1988) and the 3′ end to the SV40 late T-antigen 3′UTR containing the BBR (for details see Rivera-Pomar *et al*., 1996). Mutant Bcd-effector plasmids (see Figure 2A and B) were generated by site-directed mutagenesis (Quik Change™; Stratagene) or by PCR-based deletion using pAct Bcd plasmid (Sauer and Jackle, 1991). The resulting mutations in *bcd* were confirmed by sequencing; the modified proteins are schematically shown in Figure 2A.

Cotransfections of *Drosophila* Schneider cells (line 2, Schneider, 1972) and control experiments were performed as described (Sauer and Jackle, 1991). To monitor the translational repression by Bcd mutant proteins, the cotransfection experiments were standardized to yield ~50% repression of CAT activity in response to full-size Bcd (see below). In all experiments 1 µg dicistronic reporter plasmid and 5 µg Bcd-effector plasmid were used for cotransfection (Sauer and Jackle, 1991). Cells were harvested 40 h after transfection. Expression of the dicistronic transcripts was monitored by RNase-protection assay (Haines and Gillespie, 1992), Bcd or truncated Bcd proteins were detected by Western blotting ($Bcd^{1–489}$, $Bcd^{\Delta HD}$, $Bcd^{1–168}$, $Bcd^{1–202}$). CAT activity and Luc activity were determined as described (Rivera-Pomar *et al*., 1996). As controls for CAT and Luc translational efficiency in response to various Bcd mutants, we determined the ratio between CAT and Luc activities in the absence of Bcd and in the absence of the BBR from the 3′UTR of the reporter mRNA, respectively. Since the ratio of CAT/Luc activities was invariant in the control experiments, we set this ratio to an arbitrary unit (100%; corresponding to unrepressed translation of CAT and Luc) and compared it to the ratio obtained in response to the Bcd mutant proteins (see Figure 1B). Luc activity was not significantly altered in the response to Bcd when experiments were carried out in parallel. However, CAT activity was decreased. This allowed us to use CAT activity as a direct measure to determine the cap-dependent translation (%CAT activity as compared with CAT activity in the absense of Bcd or $Bcd^{\Delta HD}$) in response to each of the Bcd mutants shown in Figure 2A, using Luc activity as an internal control. Lactacystin (Biomol, Hamburg) was applied by replacing tissue culture medium 18 and 36 h after transfection by Lactacystin-containing (10 µM final concentration) medium (Fenteany *et al*., 1995). In these cells, expression of the reporter genes were significantly increased, indicating that Lactacystin is active. Higher Lactacystin concentrations than applied in the experiments described led to loss of reporter gene activity, suggesting that such cells are not healthy. Thus, we have used the maximum possible concentration for our analysis. The data presented in Figure 2A were obtained from at least four independent transfection experiments using full-size Bcd ($Bcd^{1–489}$) as control in each cotransfection series. Other controls were performed as described in Rivera-Pomar *et al*. (1996).

Antibody staining and in situ hybridization of embryos

Fixation of embryos and antibody staining (rabbit anti-Cad 1:500; goat anti-rabbit Cy3-labeled 1:2000 after two rounds of preabsorption on wild-type embryos) was carried out as described (Driever *et al*., 1989a). Stained embryos were embedded in Mowiol mounting medium and photographed with a Zeiss laser-scanning microscope. Whole mount *in situ* hybridizations of staged embryos were performed as described (Tautz and Pfeifle, 1989; Klingler and Gergen, 1993).

Transgenic flies

In vitro mutagenized *bcd* cDNAs (see above and scheme in Figure 2) were cloned into the P-element-based pCaSpeR vector DNA to be framed by the *nanos* 5′UTR and *bcd* 3′UTR sequences (E.Wimmer, unpublished data). Cloning and DNA analysis were performed according to standard protocols. Several transgenic lines were established by P-element-mediated germline transformation (Rubin and Spradling, 1983). Transformants were crossed to bcd^{E1} mutants. The GAL4/UAS system to ectopically express Cad throughout the preblastoderm embryo involved a maternally expressing GAL4-driver line (gift of P.Gergen) and a UAS-responder line that contains the *cad* cDNA lacking the BBR in the 3′UTR (*cad*ΔBBR; gift from G.Morata) was used as described by Brand and Perrimon (1993). Transgene-dependent zygotic *hb* expression and Cad gradient formation were monitored by antibody staining and whole mount *in situ* hybridization, respectively. Rescue of the bcd^{E1} mutant cuticle phenotype was scored as described in the text using head markers outlined in Jurgens and Hartenstein (1993).

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