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Phosphorylation of Bicoid on MAP-kinase sites: contribution to its interaction with the torso pathway

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SUMMARY

The Torso signal transduction pathway exhibits two opposite effects on the activity of the Bicoid (Bcd) morphogen: (i) Bcd function is repressed by Torso (Tor) at the anterior pole of the embryo leading to a retraction of the expression of many Bcd targets from the most anterior region of the embryo, where the Tor tyrosine kinase receptor is activated, and (ii) Bcd function is strengthened by Tor in a broader anterior region, as indicated by a shift of the posterior border of Bcd targets towards the anterior pole in embryos deprived from Tor activity. Anterior repression of Bcd targets was not observed in embryos lacking maternal contribution of *D-sor*, which acts downstream of Tor and encodes a MAP-kinase kinase. This indicates that the Ras signalling cascade is directly involved in this process, although the known transcriptional effectors of the Tor pathway, *tll* and *hkb*, are not (Ronchi, E., Treisman, J., Dostatni, N., Struhl, G. and Desplan, C. (1993) *Cell* 74, 347-355). Bcd is a good in vitro substrate for phosphorylation by MAP-kinase and phosphorylation of the protein occur in vivo on MAP-kinase sites. In the presence of a Bcd mutant that could no longer be phosphorylated by MAP-kinase, expression of Bcd targets remained repressed by Tor at the pole while strengthening of Bcd activity was reduced. These experiments indicate that phosphorylation of Bcd by MAP-kinase is likely to be required for the Tor pathway to induce its full positive effect on Bcd. This suggests that Tor signalling acts at a distance from the anterior pole by direct modification of the diffusing Bcd morphogen.

Key words: Phosphorylation, MAP-kinase, Bcd, Torso, *Drosophila*

INTRODUCTION

Polarity along the anteroposterior axis of the *Drosophila* embryo is established by the activity of maternal gene products deposited into the egg during oogenesis. These activities direct specialised domains of zygotic gene expression required for the determination of cell fate (St. Johnston and Nüsslein-Volhard, 1992). The anterior system, which is required for the formation of the head and the thorax, is dependent on the activity of the Bicoid (Bcd) maternal morphogen (Frohnhöfer and Nüsslein-Volhard, 1986). Maternal *bcd* mRNA is localised at the anterior tip of the egg (Berleth et al., 1988). Its protein product is translated upon egg laying and diffuses along the anteroposterior axis of the syncytial blastoderm to form a concentration gradient (Driever and Nüsslein-Volhard, 1988a,b). The Bcd protein is a homeodomain (HD)-containing transcription factor that differentially activates target genes in distinct anterior domains. It has been proposed that Bcd zygotic targets respond to different threshold levels of the protein, depending on the affinity of Bcd binding sites in their promoters (Driever and Nüsslein-Volhard, 1989; Struhl et al., 1989; Gao and Finkelstein, 1998). The localised expression of Bcd targets in distinct domains is an example of how a morphogen can control pattern formation at the transcriptional level, depending on its concentration. In addition to activating transcription of its targets, Bcd has also been implicated in the translational suppression of maternal *caudal* (*cad*) mRNA. The Bcd HD that mediates DNA binding to the promoter of Bcd target genes also mediates binding of the Bcd protein to the 3′UTR of *cad* mRNA (Rivera-Pomar et al., 1996; Dubnau and Struhl, 1996; Chan and Struhl, 1997).

Patterning of the terminal regions of the embryo is achieved by transfer of spatial information from the follicle cells to the oocyte and is mediated by activation of the Torso (Tor) signal transduction pathway. The Tor receptor tyrosine kinase (RTK) is evenly distributed at the surface of the embryo. Its restricted activation at the poles depends on factors encoded by the *Nasrat*, *pole hole*, *trunk* and *torso-like* genes. These products act upstream of the Tor RTK and they are likely to play key roles in production and accessibility of the Tor ligand (Furriols et al., 1998). Once the Tor RTK is activated, the signal is transduced by the conserved Ras signalling module (Perrimon

and Desplan, 1994) that includes among others: Drk, a SH3-SH2-SH3 protein that couples the RTK to the product of the *son of sevenless* gene (*sos*); Sos, a guanine nucleotide exchange factor for D-ras1; D-ras1, which plays a role in D-raf activation; D-raf, a serine/threonine kinase; D-sor, the putative D-raf substrate and threonine/tyrosine kinase similar to MEK; and MAP-kinase (*rolled*), which presumably transmits the signal into the nucleus. The Tor signalling pathway culminates with the derepression of the *tailless* (*tll*) and *huckebein* (*hkb*) zygotic genes, which are maintained silent in the remaining part of the embryo by the Groucho maternal co-repressor (Paroush et al., 1997).

In response to the activity of the Tor RTK, the expression of Bcd targets is modified in two opposite ways. First, expression of target genes, such as *hunchback (hb)* and *orthodenticle (otd)*, is repressed at the anterior pole. This repression is dependent on Tor activity and indicates that Tor acts negatively at the pole to counter Bcd function (negative effect) (Driever and Nüsslein-Volhard, 1989; Finkelstein and Perrimon, 1990; Ronchi et al., 1993). We have shown that Bcd transcriptional activity itself is directly repressed at the pole by activation of the Tor pathway and that the HD is dispensable for this effect (Bellaïche et al., 1996). Repression depends on the function of *D-raf* but does not depend on the activity of the two zygotic effectors of the terminal system, *tll* and *hkb* (Ronchi et al., 1993). Second, the posterior border of expression of Bcd targets, such as *hb* and *otd* is also shifted towards the anterior in embryos from *tor* loss-of-function females (Bellaïche et al., 1996; Gao et al., 1996). The magnitude of the anterior shift is fairly mild (2-3% egg length) but it indicates that Tor acts positively on Bcd, by strengthening its morphogenetic activity (positive effect). Consistent with this observation, a posterior shift of the posterior border of expression of Bcd targets has also been observed in embryos from *tor* gain-of-function females (Grossniklaus et al., 1994; Wimmer et al., 1995; Bellaïche et al., 1996). Therefore, the Tor signal transduction pathway exhibits two opposite effects on Bcd activity that are detected in distinct parts of the embryo: Tor-induced anterior repression of Bcd targets (negative effect) is only observed at the pole where the Tor RTK is active whereas the strengthening of Bcd morphogenetic activity by Tor (positive effect) is observed at some distance from the pole in regions where Tor activity is probably very weak.

The Bcd protein is phosphorylated in vivo and at least some of these phosphorylations are dependent on the activity of Tor (Ronchi et al., 1993). In the present study, we analysed the role of Bcd phosphorylations in vivo in order to determine whether they directly account for the Tor-induced repression of Bcd targets at the pole, as we had previously proposed (Ronchi et al., 1993). Genetic analysis indicated that D-sor (MKK) or a kinase acting downstream of D-sor in the Tor pathway acts on Bcd. We mapped the MK phosphorylation sites of Bcd in vitro. Phosphatase treatment combined with site-directed mutagenesis indicated that most of Bcd phosphorylations occur also on these sites in vivo. As indicated by transient transfection experiments in S2 cells and transgenic study, Bcd transcriptional activity was not drastically modified by point mutations at MK sites. In contrast, Bcd sensitivity to Tor was selectively affected by these mutations: Bcd targets were still repressed at the pole by Tor but the posterior shift of *otd* expression observed in embryos from *tor* gain-of-function females was strongly reduced, though not eliminated. These observations indicate that Bcd phosphorylations on MK sites are not required for Tor-induced anterior repression of Bcd activity at the pole, but that they mediate part of Tor-dependent strengthening of the Bcd activity gradient.

MATERIALS AND METHODS

Drosophila stocks and transgenic lines

The fly stock *wy* LF^{133} FRT^{101}, which carries a *D-sor* mutant on a FRT chromosome, was generously provided by N. Perrimon. Germline clones homozygous for *D-sor* were obtained by heat-shock-induced site-specific mitotic recombination using the FLP/DFS technique (Chou and Perrimon, 1992) in females hemizygous for *wy* LF^{133} FRT^{101} and a dominant female-sterile ovo^{D1} FRT^{101} chromosome. These females also carried a transgene (hsFLP38) allowing expression of the yeast FLP recombinase under heat shock. Mutant alleles used were *bcd* ^{E1} (Frohnhöfer and Nüsslein-Volhard, 1986), tor^{PM} and tor^{4021} (Klingler et al., 1988). The *Bcd3-lacZ* reporter transgene is described in Ronchi et al. (1993). Injection for transgenics were performed as described in Bellaïche et al. (1996).

Plasmids

Sequences coding for different portions of the Bcd protein were amplified by PCR from the Bcd genomic sequence and cloned into the *Bam*HI and *Eco*RI unique restriction sites of the pGEX2T plasmid (Pharmacia). Oligonucleotides used for amplification allow in frame cloning between the Bcd sequences (underlined) and the GST protein. Sequences of oligonucleotides used were:

5′-AATT**GGATCC**GCGCAACCGCCGCCAGATC-3′ (oligo I, Fig. 2),

5′-GTAT**GGATCC**CATATGCCACGTCGCACCCGCACCAC-3′ (oligo II, Fig. 2),

5′-CTAT**GAATTC**TCACGATTGGATCTTGTGACGAC-3′ (oligo III, Fig. 2),

5′-CTAT**GAATTC**TGAAGAACTGGCCGCCATTG-3′ (oligo IV, Fig. 2),

5′-**GGATCC**CATATGGCCAGCGCCTGTCGCGTC-3′ (oligo V, Fig. 2),

5′-CTAT**GAATTC**CTACTGTAGCGTCGTCTTC-3′ (oligo VI, Fig. 2),

5′-AATT**GGATCC**ATCTTGGAGCCTTTG-3′ (oligo VII, Fig. 2),

5′-CTAT**GAATTC**CTAATTGAAGCAGTAGGCAAA-3′ (oligo VIII, Fig. 2).

Sequences coding for GST fusion proteins were obtained with oligonucleotides indicated in parenthesis: GST-Nterm protein (oligo I and oligo III), GST-HD protein (oligo II and oligo III), GST-HD/ST protein (oligo II and oligo IV), GST-Cterm protein (oligo V and oligo VIII), GST-CTermA protein (oligo V and oligo VI) and GST-CTermB (oligo VII and oligo VIII).

Site-directed mutagenesis was performed on single-stranded DNA using a pKS derivative containing the Bcd genomic sequence (Bellaïche et al., 1996). Oligonucleotides used to transform MK phospho acceptor S or T into A were:

5′CTGTTTCATACCCGGCGCCAGAGGCATCCCCTC-3′ (S165A),

5′-GTGTGCAGTGGGCGCCGAGGGCGTGGGTGACGG-3′ (T200A),

5′-CGTCATTCTTGGGTGCTAATGGCTCGTAGACC-3′ (T353A),

5′GCAGTGGGCGCCGAGGGCGCGGGTGCCGGAGCCAAAG-CGTTGGGCGCGGCTCCTCCACCCAAGCTAAG-3′(T188A/T193A/S195A/T197A/T200A).

Once mutated, Bcd sequences were further analysed by sequencing, PCR amplified with the required oligonucleotides to be cloned into

pGEX2T for expression of GST fusion proteins bearing given point mutations, isolated as a *Bam*HI-*Bam*HI cassette to be either introduced into the unique *Bam*HI restriction site of pPAC, the protein producer plasmid used in transfection experiments, or introduced in the unique *Bgl*II site of the pCaSpeRBcdBglII, which was used to express the mutated forms of Bcd in the embryo after P-element transformation (Bellaïche et al., 1996). In transgenics, the sequence of a HA epitope TAG (YDVPDYASLPG) was inserted by site-directed mutagenesis after the second amino acid of the Bcd protein. Proteins expressed in transgenics were referred to as Bcdwt NTAG or Bcd-[S/T→A]$_7$ NTAG when the presence of the HA-TAG was of interest (western blot analysis). In other cases, these proteins were referred to as Bcdwt or Bcd-[S/T→A]$_7$. Truncated wild-type and mutated forms of Bcd (Bcdwt ΔQAC and Bcd ΔQAC-[S/T→A]$_6$) were expressed from pPAC derivatives which contain PCR amplified fragments cloned at the unique *Bam*HI restriction site. Oligonucleotides used for amplifications were 5′-AATTGGATCC-ATGGCGCAACCGCCGCCAG-3′ and 5′-TTAAGGATCCCTAGA-AGAACTGGCCGCC-3′.

GST fusion proteins and in vitro MK assay

GST fusion proteins were expressed and purified as described in Ausubel et al. (1991), except that proteins were not eluted from the beads. In vitro MK assay was directly performed on 20 μl of beads which were previously washed four times in MK reaction buffer (12.5 mM MOPS pH 7.2, 12.5 mM β-glycerophosphate, 7.5 mM $MgCl_2$, 0.05 mM NaF, 2 mM DTT). Reactions were carried out at room temperature for 1 hour by addition of 1 μl of a ATP dilution containing 1 pmole of ^{32}P-γATP (3000 Ci/mmole), 2000 pmoles of cold ATP and either 4 μl of buffer or 20 ng (4 μl) of enzyme (purified from the sea star *Pisaster ochraceus*) commercialised by UBI. Beads were washed four times with kinase buffer, proteins were denatured in SDS loading buffer and separated on 10% SDS-PAGE. Gels were stained with Coomassie or silver staining, dried and exposed for autoradiography.

Protein extraction, immunoprecipitation and western blot

Isolation of nuclei from embryos and extraction of nuclear proteins from transfected cells were performed as described in Bellaïche et al. (1996) and immunoprecipitation and alkaline phosphatase treatment were performed as described in Ronchi et al. (1993). Proteins were separated in 10% SDS-PAGE and wet electrotransfers of gels to immobilon-P membranes (Millipore) were performed for 1 hour at 1 ampere. Blots were saturated overnight at 4°C in PBT (0.8% Tween 80 in PBS) containing 10% low-fat milk and incubated for 1 hour at room temperature with 1/500 dilution in PBT of an anti-Bcd monoclonal antibody (Driever and Nüsslein-Volhard, 1988b). Ascites were prepared using the hybridoma cell line (Bcd MAB 23) available at the ATCC and standard procedures (Harlow and Lane, 1988). Blots were washed four times, incubated for 1 hour with a 1/10000 dilution of a monoclonal anti-mouse HRP conjugated (Jackson). Blots were revealed using ECL photoluminescent procedure (Amersham) after four washes in PBT.

Tissue culture and transactivation assay

Drosophila S2 cells were grown in M3 medium supplemented with 10% fetal calf serum. Transfections were performed at 50-70% confluence by the calcium phosphate procedure (Wigler et al., 1979). For western blots analysis, 10 μg of producer plasmids were used per plate in addition to 1 μg of hsp28/*lacZ* used as control for transfection efficiency. For transactivation assay, each plate was transfected with 1 μg of producer plasmid (pPAC and derivatives), 1 μg of hsp28/*lacZ* and 1 μg of Bcd responder plasmid (*Bcd3-CAT*), which contained three Bcd DNA-binding sites upstream of a hsp70 promoter driving the *CAT* gene. CAT assays were performed as described in Bellaïche et al. (1996) on 20 μl or normalised quantities of extract (relative to β-galactosidase activities). Radiolabeled and acetylated forms of chloramphenicol were detected using a Fuji BioImaging Analyser and quantification was made using Mac Bas V2.2 computer software.

In situ hybridization and data anlaysis

Digoxigenin-labelled RNA probes for in situ hybridisation were prepared as described in (Bellaïche et al., 1996). In situ hybridisation on whole-mount embryos were performed as originally described by Tautz and Pfeifle (1989) with adaptation from M. Klinger: prehybrisation and hybridisation were performed at 70°C at pH 5. The anti-digoxigenin antibody (Boehringer) was coupled to AP. Since expression of *otd* is extremely dynamic during cellularisation process only embryos beginning cellularisation and not more than 1/3 cellularised were chosen for measurements. Quantifications were made by drawing a tangent to the posterior limit of *otd* expression perpendicular to the anteroposterior axis. The position of the posterior border of *otd* expression was calculated as the % of egg length (0 at the posterior) using the point of intersection of this line with the anteroposterior axis.

RESULTS

Requirement of D-sor (MEK) maternal function for anterior repression

Tor-induced anterior repression of Bcd targets depends on Raf function but does not requires the zygotic effectors of the pathway, *tll* and *hkb* (Ronchi et al., 1993). The function of genes encoding kinases acting downstream of Tor is provided maternally. We used the DFS/FLP method (Chou and Perrimon, 1992) to generate female germline mosaics and remove the maternal contribution of a downstream component of Raf, MAP-kinase kinase (MKK), which is the product of the *D-sor* gene. Bcd transcriptional activity was monitored through the expression of *hb*, *otd* and the artificial reporter construct (*Bcd3-lacZ*) with three Bcd binding sites upstream of a naive promoter driving expression of the *lacZ* reporter gene (Bellaïche et al., 1996; Ronchi et al., 1993). Fig. 1 shows that, while the three Bcd target genes were repressed at the anterior pole during cellularisation in wild-type embryos (Fig. 1A,C,E), they remained expressed as an anterior cap in embryos deprived from the maternal component of *D-sor* (Fig. 1B,D,F). This indicates that MKK is involved in Tor-induced anterior repression of Bcd targets. It also suggests that if Bcd phosphorylation is involved in this process, the kinase should be either MKK, or a kinase acting downstream of it, such as the product of the *rolled* gene, which encodes a MAP kinase (MK) (Brunner et al., 1994). For technical reasons, it was not possible to determine whether *rolled* was required for Bcd repression at the pole.

MK phospho acceptor sites in the Bcd protein

Several arguments suggest that Bcd could be phosphorylated by MK. First, MKs are localised both in the nucleus and in the cytoplasm (Gabay et al., 1997; Lenormand et al., 1993) whereas MKKs are only found in the cytoplasm (Fukuda et al., 1997). Second, phosphorylations by MKs have been shown to be involved in the regulation of activity of transcription factors (Mantrova and Hsu, 1998; Rebay and Rubin, 1995). Finally, examination of Bcd sequence revealed three optimal (S165, T200 and T353) and seven weak (T188, T193, S195, T197, S343, S359 and S439) consensus sites for phosphorylation by MK (Fig. 2, red boxes). Six of these sites are concentrated in

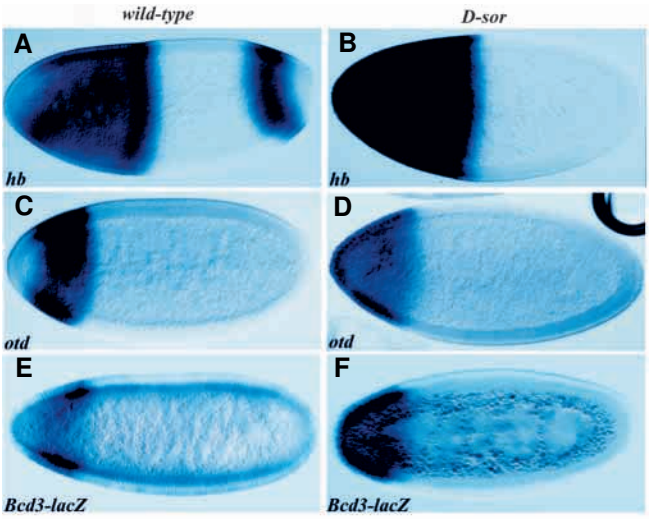

Fig. 1. Involvement of D-sor in anterior repression of Bcd targets. In situ hybridisations were performed with anti-sense RNA probe for *hb* (A,B), *otd* (C,D) and *lacZ* (E,F) on whole-mount embryos. Embryos were from wild-type females (A,C,E) and from females lacking *D-sor* activity in the germline (B,D,F). Embryos carried one copy of a paternally inherited *Bcd3-lacZ* reporter transgene (E,F). Anterior is at the left.

the serine/threonine (S/T)-rich domain (Fig. 2) that follows the HD. This domain also contains a PEST sequence (Fig. 2) that has been implicated in the degradation of proteins with short half-lives (Rechsteiner and Rogers, 1996; Rogers et al., 1986). The four remaining MK sites are located within the activation domain in the C-terminal part of the protein (Fig. 2).

In vitro phosphorylation of Bcd by MK

To analyse phosphorylation of Bcd by MK in vitro, GST fusion proteins containing different parts of Bcd were expressed in *E. coli*. The proteins were purified using glutathione agarose beads, subjected to phosphorylation by MK in the presence of labelled ^{32}P-γATP and analysed on SDS-PAGE for change in mobility and incorporation of ^{32}P. As shown in Fig. 3B, ^{32}P incorporation only occurred on the GST fusion proteins containing either the internal region spanning the HD and the S/T-rich domain (GST-HD/ST, lane 8) or the C-terminal domain (lane 10) but not the HD alone (lane 4). In addition, the mobility of the GST-HD/ST protein was significantly modified (Fig. 3A, compare lane 7 and 8, arrowhead) probably because of multiple phosphorylations within the S/T-rich domain. This experiment indicates that in vitro phosphorylations of Bcd by MK only occur in the S/T-rich and the C-terminal domains, which are the sole parts of the protein containing consensus sites for MK phosphorylation.

To determine whether phosphorylation occurred on the consensus sites, the putative phospho-acceptor serines (S) or threonines (T) were changed into alanines (A). As shown in Fig. 3C, the S165A and T200A mutations reduced the mobility shift of the GST-HD/ST protein after treatment with MK (compare lane 2 with lanes 4 and 6). The double mutant (S165A/T200A) was still phosphorylated by MK as indicated by incorporation of ^{32}P (Fig. 3D, lane 8) and a small change in mobility (Fig. 3C, compare lanes 7 and 8). Further mutagenesis of S195, T188, T193 and T197 as well as S165 and T200 to A, led to the complete loss of ^{32}P incorporation and confirmed that the remaining phosphorylations occur on these residues (data not shown). A similar analysis was performed with the C-terminal part of the Bcd protein using the two GST-CTermA and GST-CTermB proteins which contain different portions of the C-terminal domain of Bcd (respectively from S300 to Q395 and from I396 to N490, Fig. 2). Mutation T353A led to the loss of ^{32}P incorporation after MK treatment (Fig. 3F, lanes 4 and 6) while the GST-CTermB protein was not phosphorylated by MK (Fig. 3F, lane 8), indicating that S343, S359 and S439, located within a weak potential sites (Fig. 2), are not good substrates for MK.

Bcd is phosphorylated on MK sites in vivo

Bcd expressed in S2 cells is a phosphoprotein (Driever and Nüsslein-Volhard, 1989). To determine whether phosphorylation of Bcd occurs on MK sites in vivo, S2 cells were transfected with expression constructs for Bcd variants carrying simple or multiple point mutations at these positions. The phosphorylation state of these proteins was determined by assaying their mobility on western blots of nuclear extracts using antibodies directed against Bcd (Driever and Nüsslein-Volhard, 1988b). As shown in Fig. 4A, mutations in the cluster of MK sites located in the S/T-rich domain dramatically modified the migration of the protein (compare lanes 2 and 10). A mutant Bcd protein (Bcd-[S/T→A]$_7$) carrying seven point mutations (S165A, T188A, T193A, S195A, T197A, T200A, S353A) at the MK sites that were good substrates for phosphorylation in vitro, migrated almost at the position of the non-phosphorylated protein produced in *E. coli* (Fig. 4A, compare lanes 11 and 13) indicating that the Bcd-[S/T→A]$_7$ protein might still be slightly modified in S2 cells. As indicated in Fig. 4B, when wild-type Bcd was treated with alkaline phosphatase its migration was also modified (compare lanes 3 and 4) whereas the same treatment did not affect the migration of the Bcd-[S/T→A]$_7$ mutant protein (compare lanes 5 and 6). Similar results were obtained with C-terminal truncations of Bcd (Fig. 4C) carrying either wild-type MK sites (Bcd ΔQAC) or six point mutations transforming the MK phospho acceptor S/T into A (Bcd ΔQAC-[S/T→A]$_6$). This indicates that most of Bcd phosphorylation occur in the S/T region. Since the unphosphorylated form of Bcd (Fig. 4B, lane 4) migrated at the position of the mutant Bcd-[S/T→A]$_7$ (Fig. 4B, lane 5), we concluded from these experiments that phosphorylations of Bcd in S2 cells occur on MK sites. Although the MAP kinase is activated in S2 cells (Biggs and Zipursky, 1992), these experiments did not determined the nature of the kinase that is phosphorylating Bcd in these cells.

To determine whether similar phosphorylation occurs in the embryo, and to assay their function, transgenic lines expressing the Bcd-[S/T→A]$_7$, were generated using P-element transformation and were placed in a *bcd*$^-$ background. As described in Fig. 4D, the mutant protein expressed from three independent lines migrated as a unique sharp band (lanes 5-7), at the same position as Bcd-[S/T→A]$_7$ expressed in S2 cells (lane 2). In contrast, the pattern of migration of wild-type or wild-type tagged Bcd appeared as a broader and more diffuse band, indicating several slower migrating bands (Fig. 4D, lanes 3 and 4, arrowheads). Together with a previous study indicating that phosphorylations of Bcd in the embryo slow down the migration of the protein in a SDS-PAGE (Ronchi et al., 1993),

these results indicates that most of the phosphorylations of the Bcd protein occur on MK sites in the embryo.

Phosphorylations on MK sites does not modify Bcd transcriptional activity in vivo

To determine whether phosphorylation plays a role in Bcd transcriptional activity, the Bcd-[S/T→A]$_7$ mutant protein was analysed in co-transfection experiments for its ability to activate expression of a Bcd reporter gene. As shown in Fig. 5A, the transcriptional activity of the mutant protein was almost two times stronger than the activity of the wild-type protein, suggesting that phosphorylations do not drastically impair the activity of the protein (compare lane 2 and 3). The C-terminal portion of Bcd (from aa 150 to aa 489) carries the activation domain of Bcd (Driever and Nüsslein-Volhard, 1988a). This region contains the S/T-rich domain as well as a glutamine-rich domain (Q) and an alanine-rich (A) domain which bind, respectively, TAF_{110} and TAF_{60} in vitro (Sauer et al., 1995; Schaeffer et al., 1999). A C-terminal acidic domain (C) also contributes to Bcd transcriptional activity (Driever and Nüsslein-Volhard, 1988a) (Fig. 2). Since the presence of several potential activation domains may mask the putative modulation of transcriptional activity by phosphorylations, we also analysed the activity of C-terminal truncations that lack the Q, A and C domains (Bcd ΔQAC). This deleted protein has conserved a large part of Bcd function in vivo as it is able to rescue a *bcd* mutant to viability (Schaeffer et al., 1999). As shown in Fig. 5A, Bcd ΔQAC still exhibited transcriptional activity indicating that the remaining N-terminal part of the molecule contains an additional activation domain. As observed for the full-length proteins, there was only a two times difference in the activities of Bcd ΔQAC-[S/T→A]$_6$ and Bcd ΔQAC (Fig. 5A, compare lane 4 and 5). This indicates that phosphorylations on MK sites do not drastically impair the transcriptional activity of the Bcd protein in S2 cells.

Expression of Bcd targets was then analysed in embryos from transgenic lines expressing the Bcd-[S/T→A]$_7$ transgene in a *bcd*$^-$ background. In this situation, Bcd target genes were solely expressed under the control of the mutant Bcd-[S/T→A]$_7$. We analysed expression of *hb* (compare Fig. 5B and C), *otd* (compare Fig. 5D and E) and the *Bcd3-lacZ* reporter transgene (compare Fig. 5F and G). Anterior repression of Bcd targets occurred normally (Fig. 5C,E,G) and late expression of the *Bcd3-lacZ* reporter was modified in the mutant during gastrulation with a domain of expression in the cephalic region that appeared to be maintained longer (compare Fig. 5H and I). Similar late aberrant expression of *hb* in the head was also observed (not shown). As also shown in Fig. 5B-I, the position of the posterior border of Bcd target genes in the Bcd-[S/T→A]$_7$ transgenic line analysed here was expanded towards the posterior (compare Fig. 5B,D,F with C,E,G), and was similar to a situation with four copies of wild-type *bcd* (not shown). This increased morphogenetic activity in the mutant is likely due to the site of insertion of the transgene since it was correlated with an increase in protein expression, when analysed on western blot (Fig. 4D, lane 7). As shown in Fig. 4D, different transgenic lines expressed variable amount of mutant protein (lanes 5-7) and exhibited variable Bcd morphogenetic activity as judged by the position of posterior border of Bcd target gene expression (not shown). This particular line was chosen for molecular analysis because the high expression of the transgene correlates with a phenotype of maternal lethality (see details below). However, normal downregulation of Bcd activity was also observed with the other independent lines with weaker morphogenetic activity (not shown). This experiment indicates that Bcd phosphorylations on MK sites do not drastically modify Bcd transcriptional activity in vivo and that they are not required for Tor-induced anterior repression of Bcd targets.

We had previously shown that the *Bcd3-lacZ* reporter gene containing only three Bcd binding sites was repressed during the cellularisation process in embryos from *tor* gain-of-function females (*tor*^{4021}), which express a constitutively activated form of the Tor RTK (Ronchi et al., 1993). This observation suggested that the intrinsic transcriptional activity

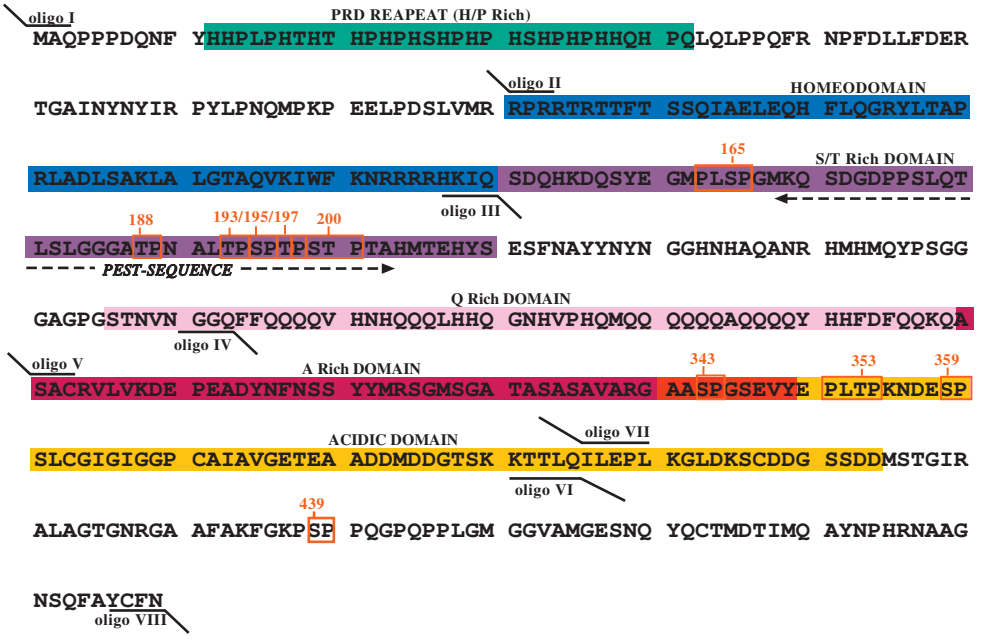

Fig. 2. Amino acid sequence and structural motifs of the Bcd protein. Several domains are recognisable in the Bcd protein: the PRD repeat which is rich in histidines and prolines (H/P rich, green box), the DNA binding homeodomain (HD, blue box), the S/T-rich domain (purple box) which contains multiple potential sites for MK phosphorylation (red boxes) and a potential PEST sequence (underlined). The C-terminal part contains the activation domain which is composed of a glutamine-rich domain (Q rich domain, light pink), an alanine-rich domain (A rich domain, dark pink) and an acidic domain (yellow). Positions of the MK phospho acceptor S and T as well as position of primers used to construct GST fusion protein is indicated.

of Bcd was also downregulated by the Torso cascade when the RTK was activated on the whole surface of the embryo. The expression of the *Bcd3-lacZ* transgene was also found to be repressed during the cellularisation process in embryos expressing the Bcd-[S/T→A]$_7$ protein in *tor*^{4021} mutant background in the absence of endogenous Bcd (not shown). This observation confirmed that phosphorylations of Bcd on MK sites do not modify the intrinsic transcriptional activity of Bcd and that they are not involved Tor-induced repression.

Tor-induced strengthening of Bcd activity is reduced by S/T→A mutations

In embryos from *tor* loss-of-function mutant females, the position of the posterior border of endogenous Bcd target genes such as *otd* is slightly shifted towards the anterior (2% egg length (EL), compare Fig. 6A and B). Conversely, in embryos from *tor* gain-of-function females (*tor*^{4021}), the posterior border of *otd* expression is slightly weaker but it is also significantly shifted posteriorly (14% EL, compare Fig. 6B and C) (Gao et al., 1996). In order to determine whether phosphorylations of Bcd could be involved in Tor-induced strengthening of Bcd morphogenetic activity, expression of *otd* was analysed in embryos from *tor*^{4021} females expressing either the wild-type Bcd or the Bcd-[S/T→A]$_7$ protein in a *bcd* – background. In the presence of one or two copies of a Bcd-[S/T→A]$_7$ transgene, *tor*^{4021} still induced a posterior expansion of *otd* (compare Fig. 6E and F or G and H). However this shift was significantly reduced as compared to wild-type (7% EL versus 14% EL, compare Fig. 6B and C with E and F or G and H). These observations show that the S/T→A mutation at MK sites in Bcd reduces the Torso induced positive effect on *otd* expression and suggests that the phosphorylations of Bcd on these sites are partially involved in this effect.

Consequences of preventing phosphorylations of Bcd on MK sites

Two independent transgenic lines (out of six) expressing Bcd-[S/T→A]$_7$ in a *bcd*$^-$ mutant background exhibited a maternal lethal phenotype, with complete absence of progeny. However, the phenotype varied with different lines, ranging from full rescue of the *bcd* mutant phenotype by the Bcd-[S/T→A]$_7$ transgene to lines for which two copies of Bcd-[S/T→A]$_7$ transgene gave rise to 100% of maternally induced lethality and to a specific phenotype of the embryos. Analysis of transgenes expression by western blots indicated that the strength of the phenotype correlated with the amount of mutant protein produced i.e. the rescued lines expressed low amounts of Bcd-[S/T→A]$_7$ (Fig. 4D, lane 5) whereas lines that gave 100% lethality expressed higher amounts (Fig. 4D, lane 7). In these latter lines, two copies of the transgene were as potent as four copies of wild-type Bcd, as indicated by expression of Bcd targets (Fig. 5C,E,G,I). As expected, a single copy of the same transgene was as potent as two copies of wild-type Bcd (compare Fig. 6B with E). Furthermore, a single copy of this transgene allowed rescue of 90% of the embryos, most of which reached adulthood. This indicates that normal levels of expression of

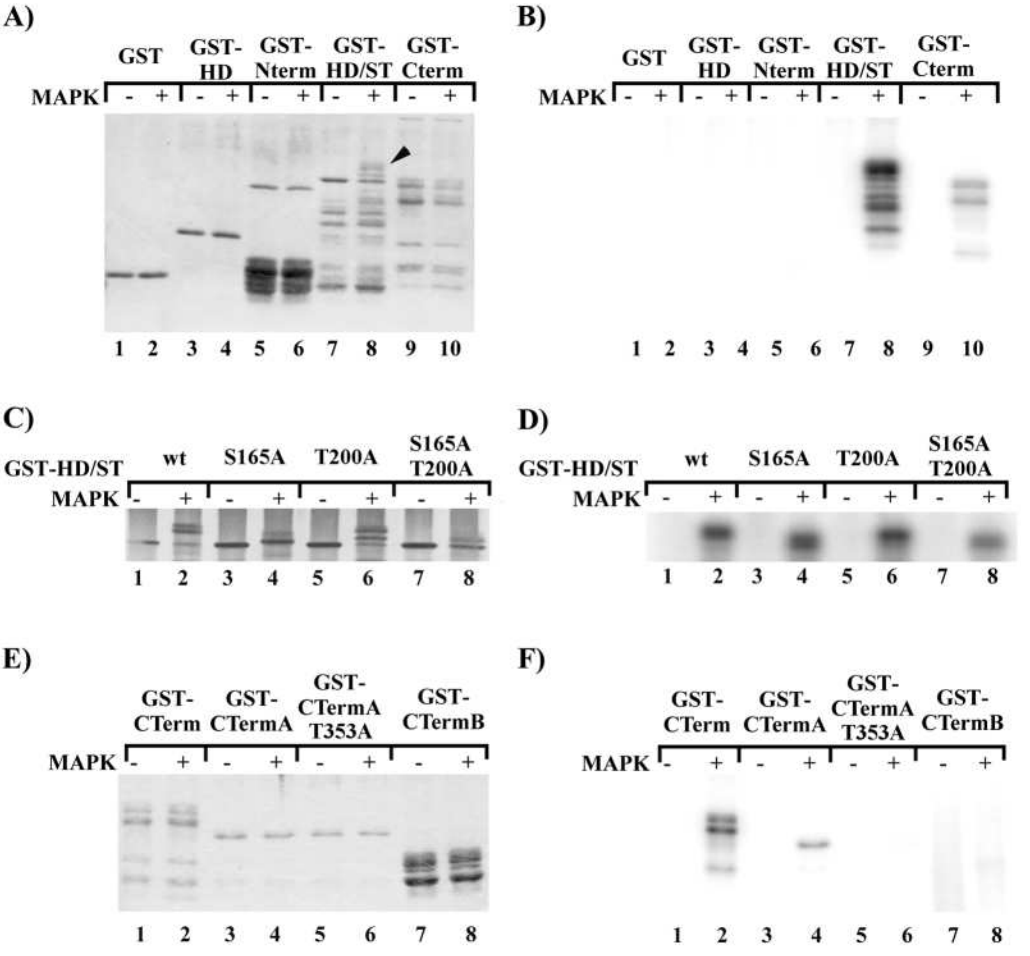

Fig. 3. In vitro phosphorylation of Bcd by MK. GST fusion proteins bearing different parts of Bcd were subjected to phosphorylation by MK in vitro using P^{32}-γATP and separated on SDS-PAGE. Gels were either analysed directly by Coomassie staining (A,E) and silver staining (C) or exposed for autoradiography (B,D,F). Proteins treated by MK in vitro are indicated (+). (A,B) GST (lanes 1 and 2), GST-HD (lanes 3 and 4), GST-Nterm (lanes 5 and 6), GST-HD/ST (lanes 7 and 8), GST-Cterm (lanes 9 and 10). Arrowhead indicates changes in the migration of phosphorylated proteins. (C,D) Wild-type GST-HD/ST (lanes 1 and 2), GST-HD/ST(S165A) (lanes 3 and 4), GST-HD/ST(T200A) (lanes 5 and 6), GST-HD/ST(S165A/T200A) (lanes 7 and 8). (E,F) GST-Cterm (lanes 1 and 2), GST-CtermA wild-type (lanes 3 and 4), the GST-CtermA(T353A) (lanes 5 and 6) and GST-CtermB (lanes 7 and 8).

the Bcd-[S/T→A]$_7$ protein are able to replace the lack of endogenous Bcd whereas higher levels (as potent as four copies of wild type) induce lethality. Since embryos expressing four copies of wild-type *bcd* develop normally, it is likely that the S/T→A mutations modify the normal properties of the Bcd protein but that the effect of these mutations is only revealed when a certain threshold level of protein is present in the embryo. Analysis of a line that gave 100% lethality indicated that 76% of the embryos did not hatch while the remaining 24% rarely reached second instar larvae. 17% of the unhatched embryos did not show any obvious morphological defects, whereas 31% developed no anterior structures with a hole at the anterior and 52% developed normal tail, abdomen and thorax but exhibited head involution defects (Fig. 7C). Detailed analysis of the head phenotype indicated that all recognisable head structures were present but not assembled properly, with a collapse of the head skeleton at the position where it normally involutes (Fig. 7F). This head involution phenotype was observed both in the presence (Fig. 7B,E) or in the absence (Fig. 7C,F) of the wild-type *bcd* gene, indicating a dominant function of Bcd-[S/T→A]$_7$. Therefore, the Bcd-[S/T→A]$_7$ behaves as a weak neomorphic allele of the *bcd* gene. However, since it can rescue to viability the lack of endogenous *bcd* when present at low levels, this indicates that phosphorylations of Bcd on MK sites are not essential for development.

DISCUSSION

Selective modulation of Bcd activity by Tor-induced phosphorylations on MK sites

Analysis of expression of Bcd target genes in wild-type and *tor* mutants indicates that activation of the Tor signal transduction cascade is able to both repress Bcd activity at the most anterior pole and to strengthen Bcd morphogenetic activity in anterior region of the embryo. In order to understand the molecular mechanism leading to the interaction between the Tor pathway and Bcd, we have analysed Bcd phosphorylations both in vitro and in vivo. We have shown that most Bcd phosphorylations in the embryo occur on MK consensus sites. Repression of Bcd targets by Tor is still occurring when Bcd cannot be phosphorylated on these sites. However, preventing phosphorylations of Bcd reduces the expansion of Bcd targets expression (e.g. *otd)* in *tor* gain-of-function alleles. This observation indicates that direct phosphorylations of Bcd by MK upon activation of the Tor pathway may be involved in increasing the strength of the Bcd morphogenetic gradient in vivo. Tor-dependent anterior repression of Bcd targets directly involves Bcd transcriptional activity (Bellaïche et al., 1996) and it is probably essential for head development since expression of a mutant form of Bcd insensitive to Tor-dependent repression induces head defects in the embryo (Ronchi et al., 1993). The experiments described

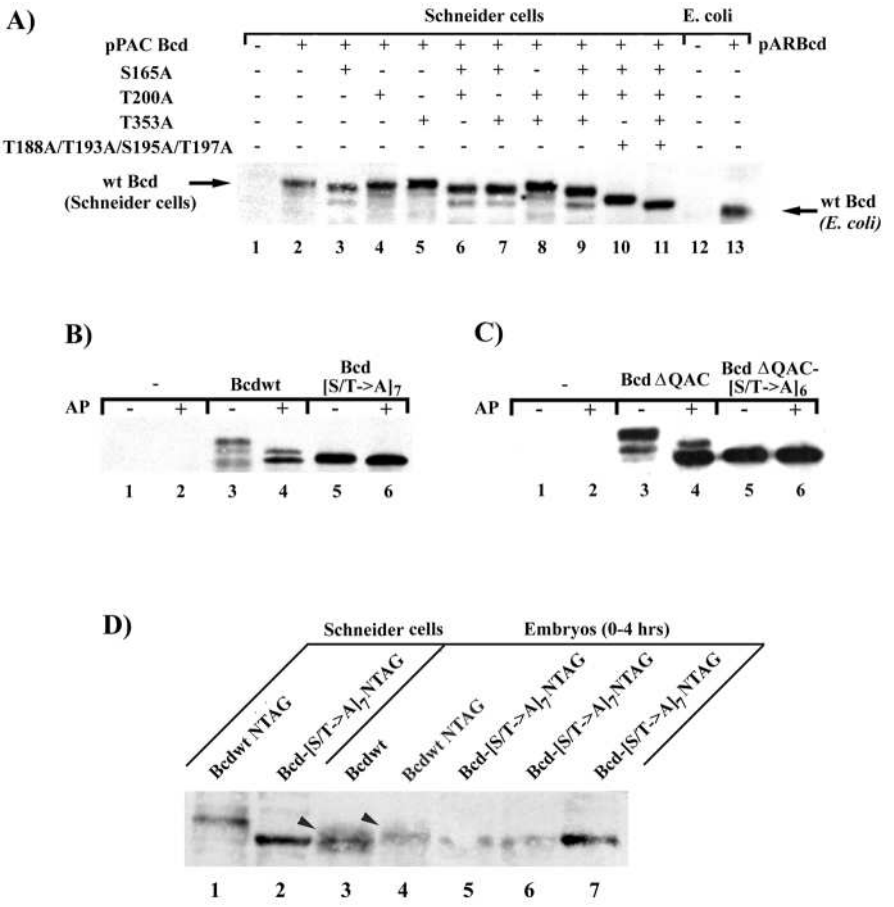

Fig. 4. Phosphorylation of Bcd in vivo. (A) Wild-type and mutant proteins were expressed transiently in S2 cells under the control of the *Drosophila actin* gene promoter (pPAC). Nuclear extracts were analysed on Western blots using the anti-Bcd antibody. Cells were transfected with pPAC alone (lane 1) or with pPAC expression plasmids for mutant forms of Bcd bearing single or multiple point mutations at the position of MK sites (lanes 2 to 11). Lanes 12 and 13 were loaded with 2 μl of extracts of *E. coli* strains bearing respectively the control (pAR) or the Bcd producer (pARBcd) plasmids. Positions of the wild-type Bcd protein expressed in S2 cells (highly phosphorylated form) and in *E.coli* (non phosphorylated form) are indicated (arrowheads). (B,C) S2 cells were transfected with expression plasmids and protein were extracted, immunoprecipitated with the monoclonal anti-Bcd antibody, treated with alkaline phosphatase (+) and analysed by Western blot using the monoclonal anti-Bcd antibody: pPAC alone (B,C, lanes 1 and 2), pPAC expression plasmids for Bcdwt (B, lanes 2 and 3), Bcd-[S/T→A]$_7$ (B, lanes 5 and 6), Bcd ΔQAC (C, lanes 3 and 4), Bcd ΔQAC-[S/T→A]$_6$ (C, lanes 5 and 6). (D) Nuclear proteins, from transgenic lines expressing the Bcd-[S/T→A]$_7$ mutant, were analysed by western blot using the monoclonal anti-Bcd antibody. 30 μl (120 μg) of nuclear extracts of S2 cells producing the wild-type tagged protein (Bcdwt NTAG, lane 1) or the mutant tagged form (Bcd-[S/T→A]$_7$ NTAG, lane 2); nuclear proteins from wild-type embryos (50 μl) (lane 3); nuclear proteins from embryos (50 μl) from *bcd^{E1}* females carrying two copies of a transgene expressing the Bcdwt NTAG protein (lane 4) or the Bcd-[S/T→A]$_7$ NTAG protein (lanes 5, 6 and 7). Lanes 5, 6 and 7 correspond to three independent transgenic lines.

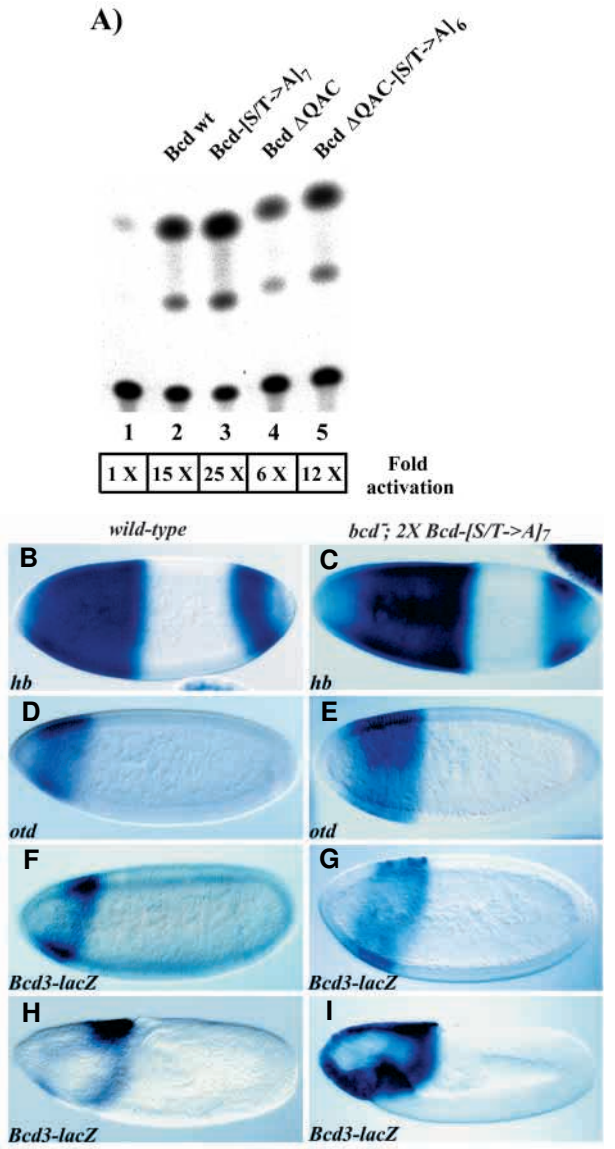

Fig. 5. Transcriptional activity of mutant forms of Bcd. (A) Cells were co-transfected with the *Bcd3-CAT* reporter gene and with pPAC alone (lane 1) or with pPAC expression plasmids for Bcdwt (lane 2), Bcd-[S/T→A]$_7$ (lane 3), Bcd ΔQAC (lane 4) and Bcd ΔQAC-[S/T→A]$_6$ (lane 5). Fold activation is the ratio of CAT activity observed in a given lane to CAT activity obtained with pPAC alone (lane 1). (B-I) Early expression of *hb* (B,C), *otd* (D,E), the *Bcd3-LacZ* transgene (F,G) and expression during gastrulation of the *Bcd3-LacZ* transgene (H,I) in embryos from wild-type females (B,D,F,H) or from *bcd^{E1}* females carrying two copies of the Bcd-[S/T→A]$_7$ transgene (C,E,G,I). Anterior is at the left.

here demonstrate that phosphorylations of the Bcd protein on MK sites are not involved in this process. Since most phosphorylations of Bcd occur on MK sites in the embryo, they also suggest that phosphorylations of Bcd in general are not involved but we can not exclude the possibility that direct modifications of Bcd, other than phosphorylations, are involved. However, the anterior repression of Bcd targets remains localised to the pole and this effect does not diffuse towards the posterior. It is thus more likely that the molecular mechanism leading to anterior repression of Bcd targets is not due to direct modification of the Bcd protein (which diffuses away from the pole) but rather involves modification of a Bcd partner which itself does not diffuse in the embryo at blastoderm stage.

The positive regulation of Bcd targets by Torso

As described previously (Gao et al., 1996, 1998) and in this study, the expression of *otd* is detected and expanded towards the posterior in embryos expressing the *torso* gain of function mutant, *tor^{4021}*. The same observation has been done for other targets of Bcd (Wimmer et al., 1995; Grossniklaus et al., 1994) and indicates that the Torso cascade acts also positively on several Bcd target genes. Surprisingly, the expression of the *Bcd3-lacZ* reporter transgene is silent during the process of cellularisation in the same mutant (Ronchi et al., 1993; this study). These observations indicate that the *Bcd3-lacZ* artificial transgene and several Bcd endogenous targets respond differently to the ubiquitous activation of the Torso cascade in embryos from *tor^{4021}* females. Our interpretation of this paradox is that the *Bcd3-lacZ* reporter only reveals the intrinsic activity of the Bcd protein in vivo whereas *otd* and other Bcd target genes are probably regulated by other transcription factors in conjunction with Bcd. One of these factors might be Hb, which has been shown to synergise with Bcd for the expression of most Bcd targets. Thus the intrinsic transcriptional activity of Bcd is downregulated by Torso in the *tor^{4021}* mutant without possibly interfering with the synergy between Hb and Bcd. The difference in the amount of posterior expansion of *otd* caused by *tor^{4021}* in an otherwise wild-type versus Bcd-[S/T→A]$_7$ mutant indicates that direct modifications of Bcd at MK sites antagonise Tor positive effect. The most obvious interpretation of this observation is that phosphorylation induced by Torso at MK sites of Bcd are involved in increasing the strength of the Bcd gradient. However, we can not exclude the possibility that direct modifications of Bcd at these sites might antagonise the synergy between Bcd and Hb or another factor required for the expression of *otd*. Finally, since the Cad protein was shown to be maintained during blastoderm stages in the anterior half of embryos expressing Bcd-[S/T→A]$_7$ in a *bcd^{-}* background (Niessing et al., 1999), we can neither exclude the possibility that the presence of Cad protein at the anterior is implicated in this effect. However, since Cad has not been implicated in modulating Bcd function, we believe that this is unlikely.

Strengthening of Bcd morphogenetic activity is revealed at a distance

Although a graded activation of MK from the pole (Gabay et al., 1997) has been correlated with a graded activity of the Tor cascade, which induces differential expression of the zygotic genes *tll* and *hkb* (Furriols et al., 1996), the domain of activity of Tor remains restricted to the poles of the embryo. A striking aspect of Tor-induced strengthening of the Bcd gradient is that it expands well beyond this domain: *otd* expression is shifted from 72% EL in wild-type embryos to 74% EL in embryos from *tor* loss-of-function females and a shift of the same magnitude is observed for *hb* whose posterior border of expression is located at 54% EL in wild-type embryos. Direct phosphorylations of Bcd induced by Tor may occur at the anterior pole where the Tor RTK pathway is activated and

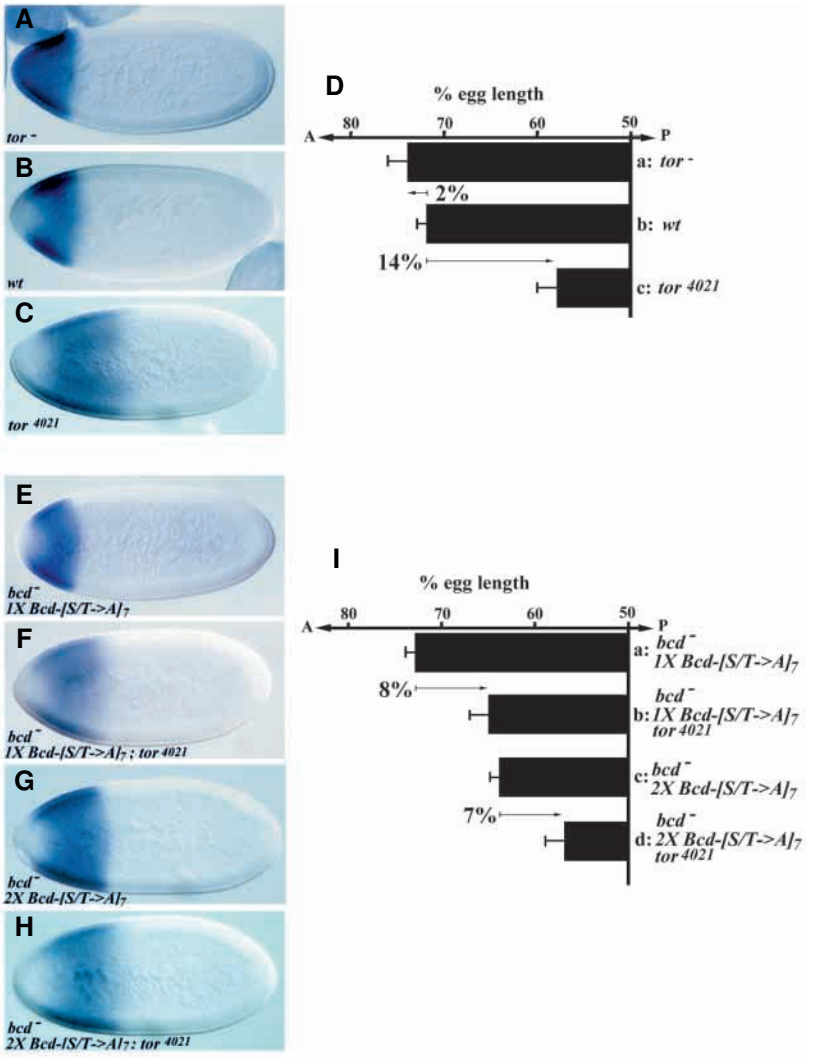

Fig. 6. Sensitivity of otd to Tor. (A-C,E-H) In situ hybridisation with antisense *otd* RNA probe on blastoderm stage embryos (beginning of cellularisation). (D,I) The position of the posterior border of *otd* expression is indicated as a percentage of egg length. Average values were calculated from measurements of 10 embryos of the same stage. s. d. is indicated. Maternal genotype is indicated. (A-D) Embryos were from tor^{PM} (A), wild type (B), tor^{4021} (C) females. (E-I) Mothers carried one copy (E,F) or two copies (G,H) of the Bcd-[S/T→A]$_7$ transgene in a bcd^{E1} (E,G) or tor^{4021}; bcd^{E1} background (F,H).

where the Bcd protein is synthesised. These modifications could affect the activity or the steady-state level of the Bcd protein inducing a shift of the gradient of Bcd morphogenetic activity. Tor signalling might act at a distance through the diffusion of the Bcd morphogen. Measuring the position of the posterior border of Bcd targets remains the most accurate way to assess Bcd morphogenetic activity: doubling the dose of Bcd in the embryos, from $bcd^{-/+}$ heterozygotes to wild-type females, induces a posterior shift of 8% EL. Since embryos from females carrying one or two copies of the wild-type Bcd develop normally, it is likely that Tor-induced strengthening of the Bcd morphogen gradient, whose magnitude is only 2% EL, is not essential for development. Although, the increase in Bcd activity induced by Tor is weak, our analysis provides a possible mechanism to explain how the strength of the morphogenetic gradient is modified by the signal transduction pathway at a distance from the pole where Tor is normally activated.

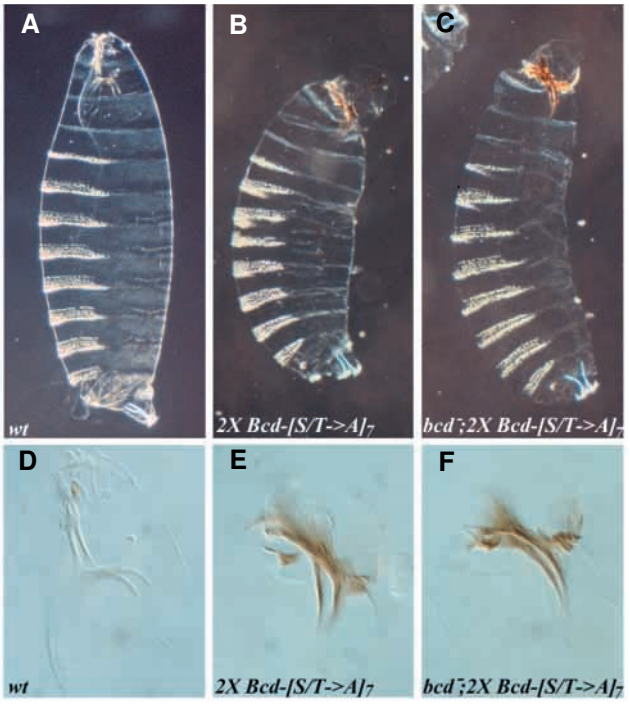

Fig. 7. Phenotype induced by the S/T→A mutations. Cuticle preparations of wild-type embryos (A,D) and of embryos from flies carrying two copies of the transgene expressing the Bcd-[S/T→A]$_7$ protein in wild-type (B,E) or in bcd^{E1} background (C,F). Anterior is at the top.

The possible roles of phosphorylations of Bcd by MK

MK phosphorylations of Bcd could modify the activation potential of the protein by creating negative charges in the S/T-rich domain and generating an acidic activation domain (Cress and Triezenberg, 1991). Since it has been proposed that the position of the posterior border of expression of Bcd targets depends on Bcd concentration and not on its transcriptional activity (Driever and Nüsslein-Volhard, 1988a; Struhl et al., 1989), a change in Bcd transcriptional activity should rather lead to an increase in the level of expression of Bcd targets rather than to a shift of expression boundary. Alternatively, phosphorylations of Bcd could affect the nuclear localisation of the protein. Selective nucleocytoplasmic traffic induced by MK phosphorylation has been proposed for the inactivation of transcription factors such as Yan in the Sevenless signalling pathway (Rebay and Rubin, 1995) or CF2 in the EGF signalling during dorsoventral patterning in oogenesis (Mantrova and Hsu, 1998). In addition, the *semushi* gene product (UBC9) has recently been involved in nuclear transport of Bcd (Epps and Tanda, 1998) suggesting a role for a ubiquitin-conjugating enzyme (E2 enzyme) in this process. Since PEST sequences have been shown to bear proteolytic signals involving phosphorylation-dependent ubiquitination, it is possible that phosphorylations of Bcd on MK sites modify subcellular localisation of Bcd. Finally, it is also possible that phosphorylations of Bcd on MK sites modify the Bcd PEST signal for degradation and increases Bcd half life, leading to an increase of Bcd concentration and a shift of the Bcd morphogenetic gradient.

Phenotype induced by the Bcd-[S/T→A]$_7$ mutation

Expression of the Bcd-[S/T→A]$_7$ mutant protein induces lethality and a head phenotype. However, some transgenic lines are able to rescue completely the *bcd* mutant phenotype. A good correlation was observed between the severity of the phenotype and the amount of Bcd-[S/T→A]$_7$ mutant protein expressed in the embryo. Most phosphorylations occur in the S/T-rich domain that also contains the PEST sequence. Since, the point mutations in Bcd-[S/T→A]$_7$ completely destroy the PEST sequence (Rechsteiner and Rogers, 1996), it is possible that the mutant protein has a longer half-life than wild-type Bcd and persists in the cellularised embryo, inducing lethality and a head phenotype. This hypothesis will be consistent with the transfection data showing a two times stronger transcriptional activity of the mutant proteins which might be slightly more active or more stable than wild-type Bcd. Alternatively, a recent study has shown that part of the Bcd PEST sequence is also required for translational suppression of *cad* mRNA in tissue culture and that Cad protein gradient does not form in embryos expressing the Bcd-[S/T→A]$_7$ protein instead of wild-type protein (Niessing et al., 1999). The phenotype induced by the S/T→A mutations might be due to the absence of Cad gradient formation and the persistence of the Cad protein at the anterior. However, since the phenotype observed with the S/T→A mutation varied with the amount of Bcd-[S/T→A]$_7$ protein expression whereas the persistence of Cad protein at the anterior does not, we believe that it is unlikely.

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