Antagonistic action of Bicoid and the repressor Capicua determines the spatial limits of *Drosophila* head gene expression domains

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Bicoid (Bcd) is the anterior determinant in *Drosophila*. Accordingly, loss of Bcd causes loss of head and thorax and their replacement with posterior structures. *bcd* mRNA is maternally deposited at the anterior pole and Bcd forms an anterior-to-posterior (AP) concentration gradient. The expression of a series of zygotic head genes is thought to be differentially regulated by distinct threshold concentrations of the Bcd gradient. Thereby Bcd functions as a morphogen, instructing fields of cells to take on specific fates. Here, we show that spatial limits of anterior genes are also set in the absence of a Bcd gradient and depend on factors of the maternal terminal system. The receptor tyrosine kinase Torso (Tor), a key component of this system, is active in the pole regions of the embryo. Its activity downregulates the maternally deposited repressor Capicua (Cic), leaving high Cic activity in the central regions and decreasingly lower Cic activities toward the poles. We show that the positions of posterior boundaries of Bcd target genes are dependent not only on Bcd, but also on Tor-mediated Cic activity. The results indicate that Cic can mediate repression through distinct binding sites within a Bcd responsive enhancer and that gene activation by Bcd is antagonized by Cic. The activating and repressive effects of Bcd and Cic, respectively, are integrated by the Bcd target gene enhancer. We conclude that the spatial domains of head gene expression are determined by Bcd in concert with Tor-dependent repressors.

bicoid antagonist | *Drosophila* development | gene regulation | head development | morphogen gradient

Bcd is a homeodomain-containing transcription factor required for head development in *Drosophila* (1, 2). *bcd* mRNA is maternally deposited and localized to the anterior egg pole by its 3′-UTR (3, 4). From there both the mRNA and ultimately the protein form a concentration gradient along the anterior–posterior (AP) axis (5, 6). Interestingly, a change in *bcd* dosage leads to shifts in target gene expression. Reduction by one *bcd* copy leads to an anterior shift of target gene expression boundaries, whereas an additional copy results in a posterior shift (7). Thus, it has been suggested that Bcd functions as a morphogen (8, 9) and that target gene expression depends directly on distinct concentrations of Bcd along the AP axis (7). This concentration-dependent gene activation is thought to be mediated by the affinity of binding sites for Bcd within target genes (10–12). Targets expressed close to the source would contain low affinity binding sites, whereas targets far from the source would contain high affinity sites. Bcd also can activate enhancers via cooperative binding (13, 14).

In addition to Bcd, the terminal system has been shown to affect gene expression in the head region of the embryo. Torso (Tor), a receptor tyrosine kinase, is activated only at the poles (15) from where it signals through the MAP kinase pathway and regulates terminal gene expression by relief of repression (15–18). *capicua* (*cic*) mRNA is maternally deposited in the embryo, resulting in ubiquitous Cic expression. It has been shown that Tor downregulates the DNA binding repressor Cic at the termini by phosphorylation via the activated MAP kinase Rolled (19, 20), resulting in low Cic activity at the termini and high activity in the center of the embryo. Loss of Cic leads to the derepression of head and tail genes and the expansion of these regions at the cost of the trunk and abdomen (19). Also, it has been suggested that Tor activity results in the phosphorylation of Bcd (21) strengthening its morphogenic nature along the AP axis (22, 23). At the anterior pole Tor has been proposed to have an opposite function, i.e., to downregulate Bcd activity, and that this effect causes repression of *hunchback* and *orthodenticle* at the anterior tip (21, 24).

Here we show that uniform expression of Bcd leads to ectopic head gene expression with mirror image polarity at the posterior pole. This effect is dependent on Tor-regulated Cic activity, confirming a major role of the terminal system in the spatial control of Bcd-dependent head gene expression. We found that Cic activity is necessary to determine the spatial limits of head gene expression by repression. These results suggest that this effect of the terminal system is mediated by binding sites located in Bcd responsive enhancers. We conclude that anterior patterning is dependent on the interpretation of activation by Bcd relative to repression by Cic by the enhancers.

Results

Uniform Expression of Bcd Causes the Mirror Image Duplications of Target Gene Expression. To assess the ability of Bcd to activate gene expression independent of its gradient, we used the UASp/Gal4 system (25, 26) to ectopically express *bcd* without its localizing 3′-UTR in the female germline. In embryos that derive from such females, the endogenous Bcd gradient was superimposed with transgene-derived Bcd, resulting in uniform Bcd levels in the posterior half of the embryo (*SI Materials and Methods* and Fig. S1 *A*, *B*, *D*, and *E*). In such embryos, the Bcd target gene *hunchback* (*hb*) (27), which is normally detected in the anterior 50% of the embryo, is ubiquitously expressed (Fig. S2 *A* and *B*). However, Bcd target genes that are normally confined to more anterior regions, such as *cap-n-collar* (*cnc*) (28), *tailless* (*tll*) (29), and *giant* (*gt*) (30) are expressed in distinct but ectopic domains (Fig. 1; see also Figs. S2 *C*, *D*, *G*, and *H* and S3 *A*–*C*). *cnc*, normally expressed only in the anterior region (28) (Fig. 1 *A* and *B*), was also expressed in the posterior of embryos, which contain uniform high levels of Bcd (Fig. 1 *E* and *F*). Similarly, the anterior tip domain of Gt expression (Fig. 1 *A* and *C*) was duplicated in the posterior (Fig. 1 *E* and *G*). Gt expression was also detected in a central, ventrolaterally repressed domain, which resembled the anterior portion of the anterior stripe in wild-type embryos. Finally, the dorsolateral anterior domain of *tll* (Fig. 1 *A* and *D*) was broadly expanded along

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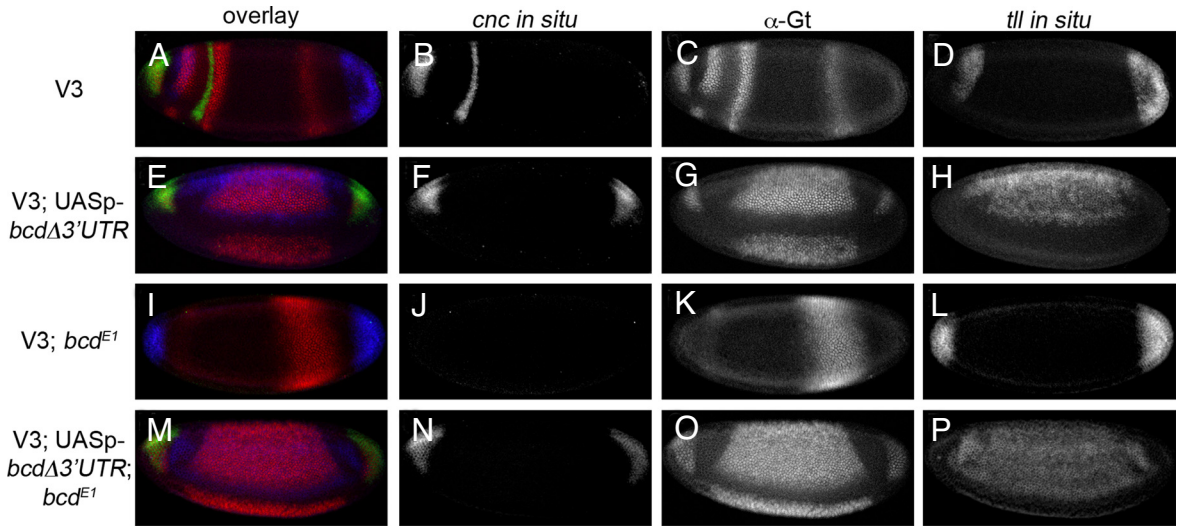


Fig. 1. Uniform expression of transgene-derived Bcd in wild-type and bcd^{E1} embryos causes mirror image duplications of anterior expression domains in the posterior region. *cnc* and *tll* mRNAs were detected by fluorescent *in situ* hybridization (green and blue, respectively); Gt was detected by immunohistochemistry (red); *A*, *E*, *I*, and *M* are overlays; *B–D*, *F–H*, *J–L*, and *N–P* are single channel gray scale images; anterior is to the *Left*; dorsal is *Up*. (*A–D*) Expression of *cnc*, Gt, and *tll* in a control embryo (V3). (*A* and *B*) *cnc* is expressed in an anterior cap and a more central collar. (*A* and *C*) Gt is expressed in an anterior tip domain, an anterior double stripe domain, consisting of a discontinuous and a continuous stripe, and a posterior stripe domain. (*A* and *D*) *tll* is expressed in an anterior dorsal-ventral and a posterior cap domain. (*E–H*) Transgene-derived ubiquitous Bcd causes mirror image duplications of anterior patterns in the posterior. (*E* and *F*) The *cnc* cap domain is duplicated at the posterior pole. (*E* and *G*) The tip domain of Gt is also duplicated at the posterior and Gt is expressed centrally as a ventrolaterally repressed stripe. This expression corresponds to the discontinuous anterior stripe. (*E* and *H*) *tll* is expressed in a broad, ventrally repressed domain in the center of the embryo. (*I–L*) Expression of *cnc*, Gt, and *tll* in bcd^{E1} embryos. (*I* and *J*) *cnc* expression is completely absent. (*I* and *K*) Gt is only detected as a broadened posterior stripe. (*I* and *L*) In the anterior *tll* is expressed in a cap, resembling posterior expression. (*M–P*) Uniform Bcd in bcd^{E1} embryos causes mirror image duplications. (*M* and *N*) The anterior cap of *cnc* expression is restored and duplicated in the posterior. (*M* and *O*) Gt tip expression is also restored and duplicated, while a ventrolaterally repressed stripe is expressed in the central regions. (*M* and *P*) *tll* is expressed in a central ventrally repressed domain. Note that the expression patterns observed in *M–P* strongly resemble those observed in *E–H*, showing that the mirror image duplications do not depend on the endogenous Bcd gradient.

the AP axis of the embryo (Fig. 1 *E* and *H*). These findings indicate that the anterior patterns of these three genes were mirrored along an axis, which runs vertically through the anterior *tll* domain, in an area in which it overlaps with the anterior Gt stripe. We confirmed that the ectopic expression domains observed for *cnc* and Gt were indeed mirror image duplications by the use of *lacZ* reporter constructs. The cnc_(+5)-*lacZ* construct recapitulates *cnc* in the wild-type embryos (31) (Fig. S2*E*), while gt_(−6)-*lacZ* recapitulates the *gt* tip expression (31) (Fig. S2*I*). In the presence of unlocalized Bcd both the cnc_(+5) and the gt_(−6) drive *lacZ* expression in distinct domains in the posterior (Fig. S2 *F* and *J*) as was observed for *cnc* and Gt in the presence of unlocalized Bcd. Thus, uniformly expressed Bcd causes an expansion and mirroring of anterior expression domains at the posterior pole (Fig. 1*E*). In fact, the Hox gene *labial* (*lab*) (32), normally expressed in a stripe anterior to the cephalic furrow (33) (Fig. S2*K*), was duplicated in a mirror image fashion in the posterior of embryos (Fig. S2*L*), indicating that these cells have indeed taken on specific anterior identities. Additionaly, we observed that the expression domains of other potential Bcd target genes in the head region, such as those of *knirps* (*kni*) (34), *orthodenticle* (*otd*) (12), *buttonhead* (*btd*) (35), *empty spiracles* (*ems*) (36), and *sloppy-paired* (*slp2*) (37), are duplicated with mirror image polarity in the posterior region in the presence of uniform Bcd (Fig. S3).

To exclude that the endogenous Bcd gradient caused the observed mirror image duplications, we examined the effects of uniform Bcd in embryos lacking endogenous Bcd. In the absence of endogenous Bcd no gradient is detectable in the presence of ectopic unlocalized Bcd, indicating that all nuclei in the embryo receive a similar amount of Bcd (Fig. S1 *C* and *F*). In embryos from females homozygous for the bcd^{E1} loss-of-function allele (2), the anterior domains of *cnc* and Gt are lost and anterior *tll* expression strongly resembles its posterior expression (28–30, 38) (Fig. 1 *I–L*). In such embryos, uniform Bcd caused mirror image expression of head genes indistinguishable from its effect in the presence of endogenous Bcd (compare *E–H* and *M–P* in Fig. 1). *cnc* expression was restored at both poles of the embryos (Fig. 1 *M* and *N*). Thus, endogenous Bcd gradient information cannot be responsible for residual anterior gene expression and their mirror image expression patterns in the posterior. This result shows that although Bcd is necessary to activate head genes such as *cnc*, differential activation of target genes and their spatial order is not dependent on Bcd gradient information. Similar effects have been observed in embryos that express low levels of Bcd uniformly along the entire axis (11, 22, 39).

Terminal System Activity Is Required for the Mirror Image Duplications of Head Gene Expression Domains. It has been shown that the maternal terminal system activates anterior target genes by relief of repression (16, 17, 19). One of the main effectors of Tor signaling is Cic, a ubiquitous repressor of anterior and posterior gene expression that is downregulated at the embryonic termini by activated Tor signaling (19). Consequently, Cic and/or other Tor-dependent repressors could antagonize Bcd-dependent gene activation and thereby position posterior boundaries (PBs) of Bcd target genes in the head. As Tor signals at both termini, Cic could also repress Bcd-dependent target genes in embryos which have received uniform levels of Bcd, causing the observed mirror image duplications of anterior expression domains in the posterior region.

In embryos from females homozygous for the cic^{1} loss-of-function allele, the head and tail regions are expanded at the expense of the trunk (19). Consequently, *cnc*, Gt, and *tll* expression is expanded toward the center (19) (Fig. 2 *A–D*), indicating that although their anterior domains were delimited, their PBs were not properly positioned (see below). In cic^{1} embryos uniformly expressing Bcd, *cnc* expression domains appeared at both termini and were connected via a ventrolateral stripe (Fig. 2 *E* and *F*). Gt was also expressed in both pole regions (Fig. 2 *E* and *G*) and *tll* expression

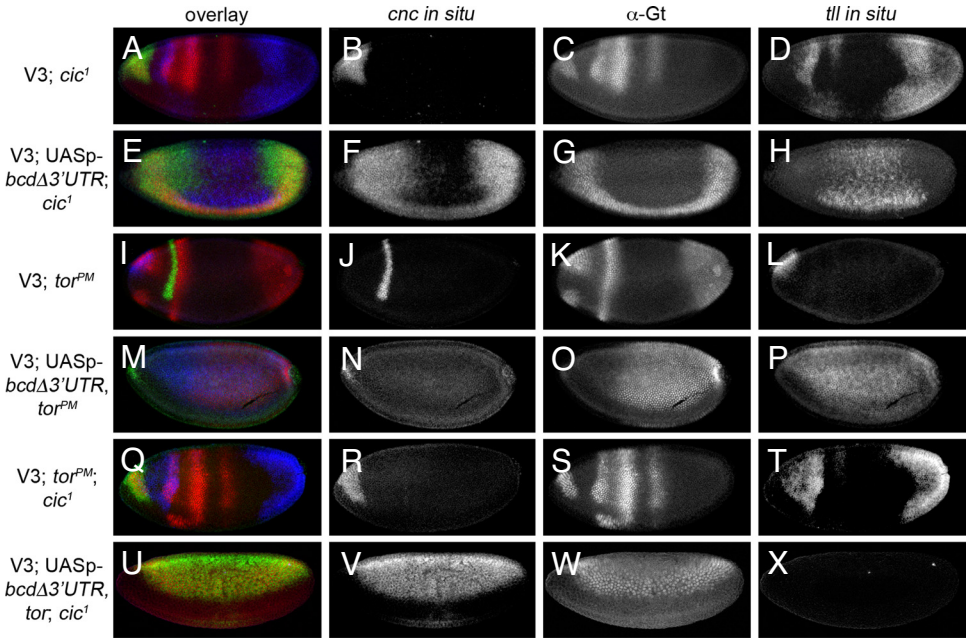


Fig. 2. The terminal system components Cic and Tor are required for mirror image duplications in response to uniformly expressed Bcd. Gene expression was visualized as described in Fig. 1. *A*, *E*, *I*, *M*, *Q*, and *U* are overlays of all three channels; *B–D*, *F–H*, *J–L*, *N–P*, *R–T*, and *V–X* are single channel gray scale images; anterior is to the *Left*; dorsal is *Up*. (*A–D*) Expression of *cnc*, Gt, and *tll* in cic^1 embryos. (*A* and *B*) The cap domain of *cnc* is expanded toward the center and the collar is missing; compare to Fig. 1*B*. (*A* and *C*) Main domains of Gt expression are formed, but both stripe domains are ventrally repressed. The posterior Gt stripe is shifted toward the center. (*A* and *D*) The anterior and posterior expression domains of *tll* are expanded toward the center. (*E–H*) Ubiquitous Bcd in cic^1 embryos causes duplications with minimal patterning information. (*E* and *F*) The cap domain of *cnc* is expanded and duplicated at the posterior, and both caps are connected by a ventrolateral stripe of *cnc* expression. (*E* and *G*) Anterior tip expression of Gt is expanded and duplicated. Both domains are connected by a ventrolateral stripe. (*E* and *H*) *tll* is expressed in the central region but not on the ventral side. This amounts to a duplication of only the anteriormost region with very limited positional information. (*I–L*) Expression of *cnc*, Gt, and *tll* in tor^{PM} embryos. (*I* and *J*) The *cnc* cap domain is absent; the collar is shifted toward the anterior. (*I* and *K*) The anterior tip domain of Gt is absent, the anterior Gt stripe domain is shifted to the anterior pole region and the expanded posterior Gt stripe is shifted to the posterior pole. (*I* and *L*) *tll* expression is absent in the posterior and the anterior dorsal-ventral wedge of expression is shifted to the anterior pole. (*M–P*) Uniformly expressed Bcd in tor^{PM} embryos does not cause duplications, but is unable to activate *cnc*. (*M* and *N*) *cnc* expression is only detected in a few anterior cells, indicating that it is strongly repressed even in the presence of excess Bcd. (*M* and *O*) Anterior tip expression of Gt is not recovered, but a broad ventrolaterally repressed, continuous Gt domain is observed, which most likely corresponds to the anterior discontinuous stripe. (*M* and *P*) *tll* is expressed throughout the embryo and overlaps with Gt expression. Note that *tll* expression is not excluded from the posterior pole. (*Q–T*) Expression of *cnc*, Gt, and *tll* in *tor*; cic^1 embryos resembles expression in cic^1 embryos (*A–D*). (*Q* and *R*) *cnc* expression at the anterior is restored in these embryos (compare to *I* and *J*) and the collar is missing. (*Q* and *S*) Gt expression strongly resembles Gt expression in cic^1 embryos (*A* and *C*) as does *tll* expression (compare *Q* and *T* to *A* and *D*). (*U–X*) Ubiquitous Bcd in *tor*; cic^1 embryos does not cause duplications, but continuous expression of the anteriormost targets. (*U* and *V*) *cnc* is expressed from the anterior to the posterior tip in a continuous, ventrally repressed domain. (*U* and *W*) Gt is detected in a ventrolateral stripe spanning the entire embryo, most likely corresponding to an extremely elongated tip domain. (*U* and *X*) *tll* is absent in these embryos. Thus, all cells resemble the anterior tip of a wild-type embryo.

was confined to dorsolateral regions, excluding *cnc*- or Gt-expressing cells (Fig. 2 *E* and *H*). These patterns were distinctly different from those observed upon ubiquitous Bcd expression in wild-type or bcd^{E1} embryos (compare Fig. 2 *E–H* with Fig. 1 *E–H* and *M–P*), indicating that the removal of Cic activity resulted in the expansion of Bcd target gene expression toward the center. However, as minimal anterior patterning was observed at both poles in cic^1 embryos expressing uniform Bcd (i.e., *cnc* and Gt expression at both poles separated by *tll* expression), positional information must be provided by additional factors.

To test whether such additional factors are also under the control of the terminal system, we examined the effects of ubiquitous Bcd in embryos lacking Tor activity. Such embryos, derived from tor^{PM} homozygous females, fail to develop head and tail structures (15). Because of ectopic Cic activity in the terminal regions of tor^{PM} embryos (19), the more central expression domains are shifted toward the termini, i.e., *cnc* and Gt expression are lost from the anterior tip and both anterior Gt stripe and *tll* are shifted to the anterior pole (28–30) (Fig. 2 *I–L*). Uniform Bcd expression in tor^{PM} embryos caused no duplications (Fig. 2 *M–P*), but embryos were continuously patterned, i.e., Gt and *tll* were expressed in overlapping, ventrally repressed domains throughout the embryo. However, *cnc*, a marker of anteriormost gene expression, was only weakly restored in some embryos. Thus, in embryos lacking Tor activity, genes normally expressed at the anterior tip of the embryo were repressed despite the presence of Bcd. Thus the entire embryo was continuously patterned, but the anteriormost information (i.e., *cnc* expression) was missing. Corresponding results were reported from embryos containing low uniform levels of Bcd in the absence of *tor* (22).

We next expressed Bcd in embryos devoid of both *tor* and *cic* to observe possible Cic-independent effects of Tor. Overall, expression of target genes in embryos devoid of both *tor* and *cic* (Fig. 2 *Q–T*) was very similar to that in cic^1 embryos (19) (Fig. 2 *A–D*). Upon uniform Bcd expression, *cnc* was expressed in a dorsolateral domain along the entire length of the embryo (Fig. 2 *U* and *V*), Gt was expressed in a horizontal stripe of cells (Fig. 2 *U* and *W*), and *tll* expression was undetected (Fig. 2 *U* and *X*). Thus, the anterior pattern was not duplicated, but instead the entire embryo resembled the anterior tip region. In summary, uniformly expressed Bcd can cause anterior gene expression throughout the entire embryo only in the absence of two key components of the terminal system. These results indicate that (*i*) terminal Tor signaling is necessary to establish mirror image duplications in the presence of Bcd in the posterior pole region and (*ii*) Tor does not act in this process through Cic alone. In the presence of uniform Bcd, positional

information can then be provided via Tor-dependent factors, such as Cic, which antagonize activation of Bcd target genes and thus, cause the mirror image duplications.

Cic Is Required for the Determination of Spatial Limits of Bcd Target Genes. To assess the Cic requirement for the positioning of PBs of potential Bcd target genes expressed in the embryonic head region, we measured their positions in wild-type and *cic*^{1} mutant embryos. We found that the PBs of the *gt* anterior tip domain as well as the *huckebein* (*hkb*), *kni*, *otd*, *ems*, *slp2*, and *cnc* anterior domains were strongly dependent on Cic activity (summarized in Fig. 3*A*; for RNA expression see Fig. S4). Target genes with PBs posterior to 60% egg length (i.e., the anterior *gt* stripe and *hb*; Fig. S4 *Q–T*, anterior pole is 100%, posterior 0%) were not notably affected by the loss of *cic*. These findings suggest that Cic limits Bcd target gene expression strongest, or exclusively, in the presumptive head region. Interestingly, the expression pattern of *gt* appears to contain both a *cic* sensitive (*gt* anterior tip) and insensitive domain (*gt* anterior stripe). Although anterior *gt* stripe expression is not affected in *cic*^{1} embryos, it is clearly shifted to the anterior tip in embryos lacking *tor* (30) (see Fig. 2 *I* and *K*). This observation indicates that Tor-dependent factors other than Cic participate in establishing the PBs of Bcd-responsive genes posterior to 60% egg length. Additionally, our results suggest that repression by Cic antagonizes Bcd activity in the anterior regions of the embryo where Bcd levels are much higher than necessary for target gene activation (39). Conversely, the distinct PBs observed in *cic*^{1} mutants suggest that the Bcd gradient provides some information for the spatial activation of target genes which, however, is not accurate enough to properly pattern the head region of the embryo. It is also possible that additional Tor-dependent factors participate in repression of anterior Bcd targets when Cic is absent.

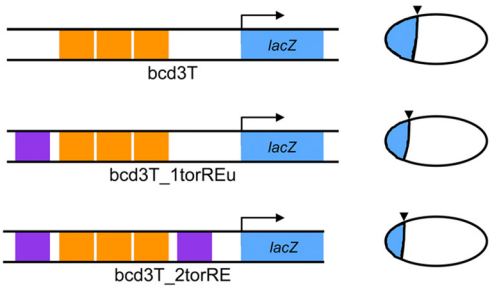
Repression by Cic Is Mediated Through the torRE. Our results show that Tor-dependent repressors such as Cic position the PBs of Bcd targets in the presumptive head region. Does this happen on Bcd-dependent enhancers themselves? It has been shown that human Cic binds to the sequence motif TGAATGAA (40), which is remarkably similar to the Torso-response element (torRE; TCGTCAATGAA) that mediates repression in the *tll* enhancer (16). To identify enhancers that contain both Bcd binding sites and torREs, we screened DNA fragments previously identified as Bcd targets in a chromatin immunoprecipitation assay with subsequent microarray analysis (41) for torREs (Table S1). We identified fragments that bound Bcd and contained torREs in the vicinity of known Bcd targets such as *tll*, *otd*, *ems*, *btd*, and overlapping enhancer modules previously identified in a computational screen as containing both Bcd binding sites and torREs [cnc_+5, btd_head, kni_(−5) and slp2_(−3)] (31). Additionally, we identified fragments overlapping with the enhancer module for the anterior tip expression of *gt* (gt_(−6) (31) and in the vicinity of a number of genes of known [e.g., *homeobrain* (42), *goosecoid* (43) and *Dichaete* (44)] and unknown function (e.g., *CG31670*) with anterior expression domains (Table S1). These observations support our hypothesis that Bcd and Tor-dependent DNA-binding factors share common enhancers and together regulate the spatial limitations of anterior expression domains.

To test whether Cic can indeed repress Bcd-dependent target genes through torREs, we used a minimal Bcd-responsive enhancer containing three Bcd sites (21) ("bcd3T"), and added one ("bcd3T-1torREu") or two ("bcd3T_2torRE") torREs upstream of a *lacZ* reporter gene (Fig. 3*B*). If the posterior expansion of gene expression is dependent on repression by Cic, the addition of torREs should shift the PB of the reporter gene toward the anterior in wild-type embryos and this effect should be lost in *cic*^{1} embryos. As summarized in Fig. 3*C* (Fig. S5 for RNA expression), torRE-dependent shifts were indeed observed, and the extent of the shift was dependent on the number of torREs. Furthermore, the PBs of all *lacZ* reporters were found at around 70% egg length in the absence of Cic, indicating that the repressive effect mediated by the torREs is dependent on Cic. Also, these results indicate that Cic

A

	wt			*cic*^{1}		
gene	PB average position	stdv	n	PB average position	stdv	n
gt tip	88%	1%	71	83%	2%	42
cnc	86%	2%	62	80%	2%	121
hkb	86%	2%	96	78%	2%	140
kni	72%	2%	100	64%	3%	97
otd	69%	2%	71	66%	2%	63
ems	69%	2%	116	63%	2%	122
slp2	68%	2%	115	61%	2%	78
btd	65%	2%	82	60%	2%	107
gt stripe	60%	2%	225	58%	2%	112
hb	50%	2%	165	49%	2%	33

B



C

	wt			*cic*^{1}		
lacZ reporter	PB average position	stdv	n	PB average position	stdv	n
bcd3T	68%	3%	489	69%	3%	146
bcd3T_1toREu	72%	3%	184	70%	3%	126
bcd3T_2torRE	77%	3%	464	70%	2%	255

Fig. 3. Cic establishes the proper posterior boundaries (PBs) of head genes and can act upon a Bcd-responsive enhancer through torREs. PBs of anterior Bcd-dependent gene expression and reporter genes under control of Bcd-dependent enhancers were analyzed in wild-type (wt) and *cic*^{1} embryos (PB position in % of egg length (EL); anterior tip is 100%, the posterior 0%). (*A*) In *cic*^{1} embryos, the PBs of gene expression domains anterior to 60% EL in wt embryos (i.e., *gt* anterior tip domain, *cnc*, *hkb*, *kni*, *otd*, *ems*, *slp2*, and *btd*) are shifted by around 3–8% toward the posterior, whereas no effect was observed on Bcd target genes whose PBs were located posterior to 60% EL (the *gt* anterior stripe and anterior *hb* expression; see Fig. S4 for RNA expression). Note that the strength of the Cic repression effect declines toward the center of the embryo. (*B*) Schematic representations of minimal enhancers (for sequences see *Materials and Methods*) and expected positions of PBs in a wt background. bcd3T: a minimal *bcd* responsive enhancer containing three Bcd binding sites (orange boxes) was placed upstream of *lacZ* (blue box). bcd3T_1torREu: one torRE (purple box) upstream of the bcd3T. bcd3T_2torRE: torREs on either side of the bcd3T. The most central PB is expected for bcd3T, whereas bcd3t_2torRE should show the most anterior PB (PBs indicated by arrowheads in schematic). The position of the bcd3T_1torREu PB was expected to lie between the two. (*C*) Addition of torREs shifts the PB of the bcd3T toward the anterior. The PB of the bcd3T is located most centrally of the three constructs at 68% EL, whereas that of bcd3T_1torREu is more anterior at 72% EL and that of bcd3T_2torRE is observed at the most anterior position, 77% EL. Unlike the torRE-containing enhancers, bcd3T is not dependent on *cic*. In the absence of *cic* the PBs of all enhancers were measured at around 70% EL (see Fig. S5 for representative stainings). Values are average position in % EL; stdv: standard deviation.

does not act directly on Bcd, as the PB of bcd3T-*lacZ* was independent of Cic. Hence, the spatial limits of Bcd target gene expression can be modified at the level of the enhancer without manipulating the Bcd gradient or Bcd binding sites.

Discussion

A morphogen should be able to induce the expression of the same target gene in all cells in which it is expressed at the same level (9). When Bcd was uniformly expressed, we found that different Bcd target genes were expressed in spatially distinct domains with mirror image polarity. Thus, Bcd gradient information alone does not delimit the expression domains of target genes in the head region. Removal of *cic* from such embryos caused the duplication of only the anteriormost target gene expression. This residual patterning of the embryo could be the result of Tor functioning independently of Cic. Removal of *tor* from embryos uniformly expressing Bcd in the posterior caused the continuous patterning of the embryo. However, in the absence of Tor, Cic is also active at the anterior pole and thus able to repress for example *cnc* and *gt*. Therefore, to obtain the expected response of target genes to uniform levels of Bcd, both *tor* and *cic* must be removed from the embryo.

It has been shown previously that *tor* is required for the duplication of *otd* at the posterior of embryos that express low levels of ubiquitous Bcd (22). This effect was attributed to the phosphorylation of Bcd by the Tor pathway (21). However, nonphosphorylatable Bcd can rescue the bcd^{E1} phenotype (23), showing that its phosphorylation is not essential for its function. In addition, we observed that Cic activity affects the mirror image duplications in embryos that express uniform levels of Bcd. This result and the observation that Tor mediates the relief of repression of anterior gene expression (16–18) indicate that the posterior duplications are not caused by a direct effect of Tor signaling on Bcd. The appearance of Tor-dependent upregulation of Bcd activity toward the center of the embryo (22, 23) and its downregulation at the anterior tip (21, 24) can be explained by the local downregulation of repressors, such as Cic. Consistently, both the cuticular phenotype and the expression patterns of Bcd target genes in embryos lacking only Cic activity are indistinguishable from those also lacking both Cic and Tor activity (19) (compare *A* and *Q* in Fig. 2). Notably, *cnc* expression, which is lost in tor^{PM} mutants (Fig. 2*I*), is recovered in embryos devoid of both *tor* and *cic* (Fig. 2*Q*). Thus, the anteriormost Bcd target genes are not dependent on Tor-mediated phosphorylation of Bcd. It is likely that Tor downregulates a set of partially redundant repressors at the termini, including Grainyhead, Trithorax-like (16), Tramtrack69 (45), and Female Sterile (1) Homeotic (46), gradually restricting their activities to the central region, which results in Tor-dependent relief of repression (16). The available evidence supports the argument that mirror image duplications that are caused by uniform expression of Bcd are the result of the spatial restriction of repressors by Tor activity as exemplified here for Cic.

It has been previously suggested that repressors regulated by the terminal system are necessary for the proper patterning of the head region of the embryo (17, 39, 47). Our results provide evidence that the Tor-dependent repressor Cic can position PBs of genes that are activated by Bcd. The effect of Cic is strongest on genes expressed at the very anterior and weakens toward the center. This observation is not surprising, as genes whose PBs are found in regions with high Cic activity (such as *hb*) should be less susceptible to repression by Cic or regulated in a different manner. Recently, it has been reported that Bcd levels in the anterior are much higher than needed for target gene activation (39). In fact, we observed that the expression domains of genes, such as *cnc*, indeed expand toward posterior when Cic is absent. Thus, Bcd activates such genes in a broad domain, which becomes restricted by repression through Cic and possibly other Tor-dependent repressors.

The ability of target genes to react to different thresholds of Bcd has been proposed to depend on the affinity of Bcd binding sites within the enhancer (10–12). However, a correlation between binding site affinity and posterior expression boundaries could not be observed in recent computational studies (48, 49). We and others (31) suggest that Bcd responsive enhancer modules such as those of *tll*, *cnc*, *knirps*, *gt*, *otd*, *btd*, and *slp2* contain torREs, originally identified in the *tll* enhancer (16). These sites are similar to the binding sites reported for human Cic (40). Our results demonstrate that the addition of torREs to the bcd3T enhancer caused a Cic-dependent anterior shift of the boundary that corresponds to about one parasegment per added binding site. Thus, such Cic responsive repressor sites have the potential to be important for the precise positioning of the PBs of Bcd activated genes by mediating repression in response to Tor activity.

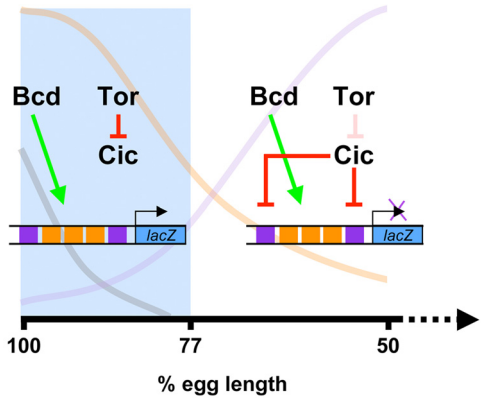


Fig. 4. Model of anterior patterning with respect to Bcd and the terminal system. Interactions of the terminal system and Bcd on shared enhancers, exemplified with the bcd3T_2torRE, result in proper positioning of posterior boundaries (PBs). The schematic in the background represents the approximate distribution of Bcd (light orange), Cic (light purple), and Tor (gray) along the anterior half of the embryo. The expression domain of the bcd3T_2torRE is observed from 100 to 77% egg length (EL; blue box). At 100% EL, Tor strongly inhibits activity of Cic and possibly other factors (broad red T-bar), leading to relief of repression of the enhancer. At the same time, Bcd has a strong activating effect on the enhancer (green arrow). At 77% EL the inhibitory effect of Tor is very weak (pink T-bar), so that Cic exerts a strong negative effect on the enhancer (red T-bar), and *lacZ* is no longer expressed at this position. However, we know that Bcd has an activating effect at this position, as it is able to activate the bcd3T construct to 68% EL. Thus, the enhancer is repressed at the position at which the repressive action of Cic outweighs the activating effect of Bcd. We propose that both Bcd activator function and Cic-dependent repression are integrated via the enhancer elements of the target genes, resulting in distinct domains of gene expression. Orange boxes: Bcd binding sites; purple boxes: torRE; blue square: *lacZ* gene. For details on the enhancers see Fig. 3 legend.

Our results suggest a model in which the input from the Bcd gradient alone is not sufficient to determine the spatial limits of target gene expression. Bcd is necessary and sufficient to activate head genes, however, an antagonizing Cic activity gradient regulated by Tor determines their PBs. We propose a mechanism, which integrates information provided by activating Bcd and repressing Cic activities via corresponding binding sites in the target gene enhancers (Fig. 4). At the anterior pole, where Bcd levels are highest, Cic is downregulated by Tor, allowing the activation of anterior genes by Bcd. Further along the AP axis, Tor activity fades (50) and thus, the antagonizing repressor activity of Cic increases. Cic and possibly other Tor-dependent factors can now repress genes controlled by torRE-containing enhancers. Hence, the positions of PBs of anterior genes are determined by activation by Bcd relative to antagonizing repression by Cic on the target gene enhancers.

The dependence of the precise determination of the spatial domains of Bcd target genes on specific Bcd threshold levels has been called into question by this and recent other studies (39). Whereas Ochoa-Espinosa et al. (39) found that Bcd is present at

much higher levels in the anterior of the embryo than necessary for proper gene activation, we have found that Bcd is not able to precisely position the PBs of head genes in the absence of Cic. In summary, we conclude that Bcd is not a "classical morphogen" as initially defined by Wolpert (8), but rather represents the activating component of a maternal "morphogenic network" that includes the terminal system. This network is required to set up both anterior–posterior polarity and to determine the spatial limits of gene expression in the head region of *Drosophila* embryos.

Materials and Methods

The following mutant alleles were used: w^{1118}, for *P*-element transformation and as wild-type reference strain; bcd^{E1} (bcd^{6}), tor^{WK} (tor^{1}), tor^{PM} (tor^{4}), and cic^{1}. *bcd* cDNA was cloned into UASp without its 3′-UTR (UASp-*bcdΔ3′UTR*) and expressed with V3-Gal4 (in figures and figure legends referred to as V3) (51) in the female germline. The bcd3T enhancer was cloned with or without torREs into pCaSpeR-hs43-*lacZ* containing *attB* sites. *attB* vectors were injected into embryos from females carrying the *ϕC31*-integrase on chromosome IV and an *attP* landing site on chromosome III at 86Fb (line ZH-*attP*-86Fb) (52). Immunohistochemistry and *in situ* hybridizations were conducted using standard methods. See *SI Materials and Methods* for additional information.

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