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Bicoid

Morphogen function revisited

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Bicoid (Bcd) functions as a morphogen during Drosophila development. Accordingly, *bcd* mRNA is maternally localized to the anterior pole of the embryo, and Bcd forms an anterior/posterior gradient, which functions in a concentration dependent fashion. Thus, nuclei receiving identical amounts of Bcd should express the same target genes. However, we found that ectopic, uniform expression of Bcd causes anterior gene expression in the posterior with mirror image polarity, indicating that one or several additional factors must provide positional information. Recently, we have shown that one of these factors is Capicua (Cic), a ubiquitous maternal repressor that is downregulated at the embryonic termini by maternal Torso, a key component of the maternal terminal system. Cic acts on Bcd dependent enhancer elements by repression and thereby controls the posterior limit of Bcd target gene expression. Based on these new findings, we propose that spatial control of gene expression in the anterior region of the embryo is not solely the result of Bcd morphogen action. Rather, it relies on a "morphogenic network" that integrates the terminal system and Bcd activities, providing both polarity and spatial information to the prospective head region of the developing embryo.

Key words: bicoid, morphogen, terminal system, pattern formation, transcriptional activation, gene expression

Abbreviations: AP, anterior/posterior

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In Drosophila, anterior development is totally dependent on Bicoid (Bcd). Consequently, in the absence of maternally deposited *bcd*, head and thorax regions are replaced with posterior structures.[1] *bcd* is localized to the anterior of the egg through its 3'UTR.[2] Recently, it was found that the mRNA is released from the anterior pole of the embryo and forms a gradient,[3] which upon translation gives rise to the anterior/posterior (AP) gradient of Bcd protein.[4] Bcd is a homeodomain containing transcriptional activator and regulates target gene transcription in a concentration dependent fashion.[5,6] For example, in embryos that receive only half of the normal maternal *bcd* dose, anterior gene expression is shifted to the anterior. Conversely, in embryos that receive double the amount of *bcd*, anterior target gene expression is shifted toward the center of the embryo.[6] Thus, cells, or in the case of the Drosophila blastoderm embryo, nuclei, can translate the concentration of Bcd into positional information. Taken together, the localization of *bcd* to a source, the formation of a Bcd gradient from that source and the concentration dependent activation of target gene expression are all hallmarks of a morphogen.[7] Accordingly, interest in Bcd function and gradient formation has always been high. Understanding how a factor, even one that is not conserved outside the higher dipterans, can infer positional information on a rather homogenous field of cells (or nuclei) is paramount to understanding basic developmental principles.

Most factors considered to be morphogens are ligands of signal transduction pathways that form gradients in the extracellular space, bind to receptors on target cells, set off a signal transduction cascade and thus, regulate the transcription of a specific set of target genes.[7,8] Bcd as a transcription factor in a cell free

space, bypasses the necessity of intercellular signal transduction cascades. Instead, it has been proposed that the affinity of binding sites for Bcd within target gene enhancers is responsible for specific concentration dependent readouts of the gradient.[2] For example, enhancers of genes expressed only in the anterior most regions of the embryo would contain low affinity binding sites for Bcd, whereas genes expressed in more central regions would contain high affinity binding sites. Also, cooperative binding of Bcd to target genes has been shown.[9,10] In this case, the binding of Bcd to a high affinity site would promote the binding of Bcd to a low affinity site in the vicinity.

Interestingly, no correlation between the affinity of binding sites for Bcd in target gene enhancers and the positioning of the associated gene expression domains could be found. Instead, with the computational input available, Bcd appears to activate targets in a broad anterior domain and furthermore most enhancer modules that bind Bcd appear to be highly dependent on the input from other factors.[11,12] These findings indicate that the model of Bcd function via binding site affinity is highly over-simplified and that other factors must be present in the embryo that regulate target genes in concert with Bcd.

In order to further examine the function of Bcd independent of its graded expression, we ectopically expressed *bcd* without its localizing 3'UTR in the germline of bcd^{E1} females that do not contribute any endogenous *bcd*, resulting in uniform levels of Bcd.[13] We then monitored the expression of Bcd target genes to examine the effects of uniform Bcd. Under the premise that Bcd instructs nuclei in a concentration dependent fashion, we expected that those target genes activated by the level of Bcd present in the embryo would be uniformly expressed in the embryo or rather, that one concentration threshold of Bcd would elicit one response in all nuclei. However, this was not the case. Instead, we observed that anterior expression patterns were duplicated with mirror image polarity in the posterior. For example, the gene *cap'n'collar* (*cnc*) is normally expressed, as it's name quite aptly implies, in an anterior cap and a more central collar domain. In bcd^{E1} embryos *cnc* expression is completely lost. Surprisingly, in embryos with only uniform levels of Bcd, *cnc* was not only restored to a cap domain in the anterior, but was also ectopically expressed in a cap domain in the posterior. Thus, it appears that anterior patterning, i.e., the expression of target genes such as *cnc* in a spatially defined domain, is not dependent on the presence of Bcd in a gradient.

We found that the expected phenotype of ectopic uniform Bcd, namely that all nuclei receiving the same positional information from Bcd also expressed the same target genes, was only observed in the absence of two major terminal system components, Torso (Tor) and Capicua (Cic). The terminal system is required for the formation of the embryonic termini, which fail to form in the absence of the Tor receptor tyrosine kinase. Tor is maternally deposited in the embryo membrane, but only active at the poles, where its ligand is present in its active form.[14] Thus, upon Tor signaling, a steep gradient of the activated Drosophila MAPkinase is formed from the termini, decreasing toward the center.[15] Tor activates anterior and posterior target gene expression indirectly by downregulating ubiquitous repressors, such as the DNA-binding repressor Cic.[14,16,17] *cic* is maternally deposited in the embryo, but the interaction of Cic with the activated MAPkinase (MAPK), leads to its phosphorylation and most likely to its degradation, resulting in an activity gradient of Cic, that is highest in the central regions and decreases toward the poles.[18-20] Furthermore, it has been suggested Bcd activity is directly regulated through Tor mediated phosphoylation and that this phosphorylation of Bcd increases its ability to activate target genes at a distance from the anterior pole.[21,22] However, it is unclear how relevant this effect is, as a non-phosphorylatable version of Bcd can rescue the bcd^{E1} phenotype.[23]

In the absence of maternal *cic*, the trunk regions of the embryo fail to form. This indicates that a major function of *cic* is to repress the expression of head and tail specific genes in the central regions of the embryo, which encompass the prospective thoracic and abdominal segments.[18] Cic mediates the repression of Bcd target genes in the anterior 35% of the embryo, encompassing the procephalic and most of the gnathal regions. Thus, in the absence of maternal *cic*, the expression domains of the Bcd targets expressed in that region are expanded toward the posterior. Considering that Cic is a DNA binding protein and that Cic has a negative effect on the expression of Bcd target genes, we asked if Bcd and Cic might act on the same enhancers. We tested the ability of Cic to mediate repression of a well-characterized artificial Bcd reporter (bcd3T)[21] by adding one or two potential Cic binding sites.[16,24] We found that the addition of the Cic dependent sites into the bcd3T did indeed cause a contraction of reporter gene expression toward the anterior, and that the effect was stronger with increased number of sites added. Expression of the bcd3T itself was independent of Cic, indicating that this element is activated by Bcd alone and that Bcd is not directly affected by Cic. Thus, without manipulating the Bcd gradient, we were able to modify the expression of a Bcd dependent enhancer.

In our model of the "morphogenic network" (**Fig. 1A**),[13] the relative levels of the activating input from Bcd and the repressive input from Cic are integrated on the level of the target gene enhancers to pattern the anterior. While Bcd levels are highest in the anterior and decrease toward the center, a reciprocal gradient of Cic activity is formed in the embryo by the action of Tor. Thus, genes containing no Cic responsive sites are only dependent on Bcd and expressed up to the point on the AP axis at which Bcd levels can no longer activate the enhancer (**Fig. 1B**). In turn, enhancers containing for example one Cic dependent site, can only be expressed in the area in which activating functions of Bcd outweigh the repressive activity of Cic. The effect is stronger the more repressor binding sites are added. Thus, in this way a field of cells can be instructed to be patterned very specifically by one concentration of Bcd, only by changing the number and probably also the quality of repressor binding sites.

It has long been known that Bcd responsive enhancers receive input from other factors.[11] For example, it has been suggested that Hunchback (Hb) and/or Caudal (Cad) together with Bcd, may define a broad domain in which enhancer

activation can occur and that this broad domain is then further refined by repressive inputs from factors such as Krüppel (Kr).[8] However, all of these factors are under the direct control of Bcd itself: Bcd activates zygotic Hb in the anterior half of the embryo, both Hb and Bcd regulate *Kr* and Bcd translationally represses *cad* in the anterior.[25-27] Thus, the effect of these factors on Bcd regulated targets has been set up by Bcd itself and is equal to cross regulation. In contrast, the terminal system is independent of Bcd and the regulatory effects of the terminal system on Bcd are rather unclear. The members of the "morphogenic network" are a set of functionally independent maternal factors, which are necessary to initiate spatially limited gene expression in the embryo.

Morphogen vs. "Morphogenic Network"

Traditionally, the model has been that Bcd conveys pattern information onto the anterior in a concentration dependent manner. Thus, specific nuclei express a specific set of target genes according to the absolute amount of Bcd they receive. In contrast, our model of the "morphogenic network" proposes, that nuclei are not only dependent on the concentration of Bcd, but also need the input from the maternal terminal system, through repressors such as Cic, to judge their position along the AP axis and express target genes accordingly.

Previously, it was suggested that Bcd together with Hb activates target genes in a broad anterior domain and the terminal system regulates repressor activity to refine anterior gene expression into actual patterns. Outside of the terminal system influence, Bcd and Hb only establish the boundaries of zygotic *hb* and *Kr* expression.[17,26] We have now been able to support this model with experimental data and have shown that the repressor Cic indeed does function to refine the patterns of anterior target genes. Additionally, it has been shown that other repressors, such as Grainyhead,[16] Tramtrack[28] and Female Sterile (1) Homeotic[29] are downregulated at the embryonic termini by Tor and thus may also be part of the "morphogenic network". Consequently, a number of partially redundant repressors may spatially restrict the expression domains of Bcd targets in the anterior.

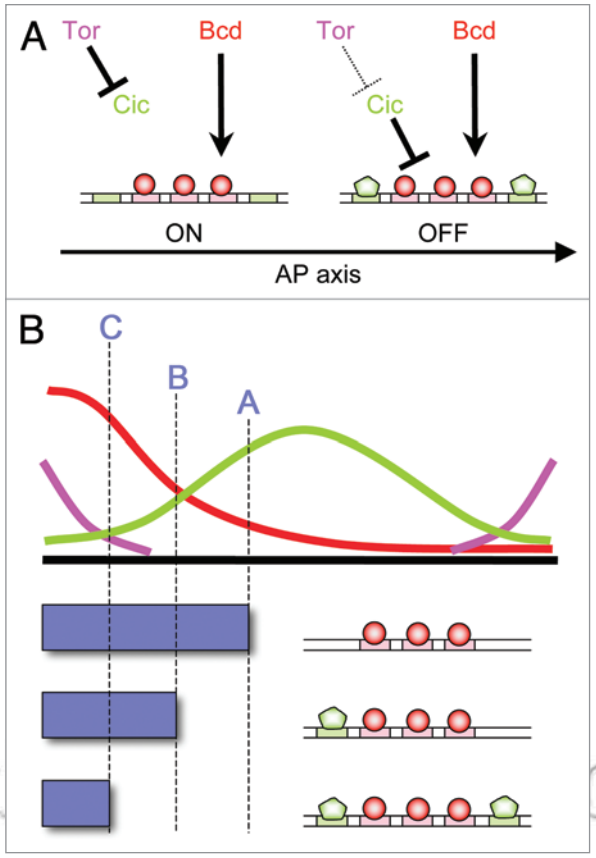

Figure 1. A "morphogenic network" patterns the anterior region of Drosophila. (A) The inputs from the "morphogenic network" are integrated on the level of target gene enhancers. An enhancer that contains both binding sites for Bcd (red boxes) and Cic (green boxes) is in the "on" state at the anterior of the embryo. Here Tor downregulates Cic (thick T-bar) and Bcd (red circles) binds to its sites, activating the enhancer (thick arrow). Further along the anterior/poster (AP) axis (black horizontal arrow) Tor signaling becomes weaker and Cic is no longer downregulated (dotted T-bar). Thus, Cic (green pentagon) can interact with its binding sites to repress the enhancer (thick T-bar), even in the presence of an activating Bcd input (black arrow). In these areas the enhancer is in the "off" state. (B) Posterior boundaries of anterior genes depend on the activating functions of Bcd and the repressive functions of Cic on the enhancer. A schematic representation of Bcd distribution (red), Cic activity (green) and Tor signaling (pink) along the AP axis is shown in the top half. The enhancer of gene A contains only Bcd binding sites (red boxes) and A is expressed in a broad anterior domain (blue rectangle). In addition to the Bcd binding sites, the enhancers for gene B and C contain one or two Cic binding sites respectively. Gene B is expressed in an area in which the activating input form Bcd outweighs the repressive input from Cic. The expression domain of gene C is more confined to the anterior than gene A or B, as its enhancer contains two Cic binding sites and is more susceptible to Cic mediated repression. Please note that the positioning of the posterior boundaries of expression (dashed lines) are not dependent on the concentration of Bcd alone, but on the relative levels of Bcd and Cic input on the enhancers.

Our model can also explain the shifts in gene expression boundaries observed when copies of the *bcd* gene are added to or subtracted from the maternal genome.[6] In embryos from mothers lacking one copy of *bcd*, posterior expression boundaries of target genes are shifted towards the anterior. In these embryos, less Bcd is available along the AP axis for target gene activation and thus, Cic can repress and thereby shift target gene expression domains more to the anterior. Conversely, when more *bcd* copies are added, the posterior boundaries are shifted toward the posterior. In these embryos, the posterior boundary of expression would be determined at a position where there is enough Cic activity present to overcome activation by Bcd. It has also been shown, that injection of *bcd* mRNA at around 50% EL can

lead to the formation of anterior structures that recede from the point of injection.[30] Here our model would predict that high levels of Bcd could overcome high repressor activity of Cic allowing the activation of anterior genes. Further from the site of injection, the activating influence of Bcd would decline while the repressive influence of Cic would increase, leading to anterior patterning around the site of injection.

Recently, it has been suggested that Bcd competes with Cic for interaction with MAPK and that as a result of this competition Cic is less efficiently downregulated at the anterior than at the posterior pole.[20] This competition between Bcd and Cic for MAPK interaction would then ensure that when an excess of Bcd is deposited in the embryo, higher levels of Cic would be present to repress Bcd target genes. Conversely, when less Bcd is present in the anterior, Cic would be more efficiently downregulated by MAPK. Consequently, the relative levels of Bcd and Cic remain similar even in the event that more or less Bcd is deposited in the embryo by the mother. Taken together with our model of the "morphogenic system" this competition model would indicate that anterior pattern formation is "buffered" against subtle changes in the levels of Bcd in the embryo.

Is Bcd a bona fide morphogen? Just like the biological systems in which morphogens are proposed to operate, the term morphogen itself has evolved over the years to fit the molecules it classifies. Nonetheless, there are several criteria which morphogens should fulfill.[7] The three most common are the ones stated at the beginning of this article: (1) localization to a source; (2) gradient formation from that source; and (3) concentration dependent instruction of a field of cells (or nuclei) within the gradient. Bcd does fulfill the first and second criteria. The *bcd* mRNA is localized to a source and though it has been recently shown that it is probably the mRNA that forms the actual gradient,[3] Bcd is undeniably detected in a surprisingly precise AP gradient.[31] But does Bcd also fulfill the third criterion? A morphogen should be able to elicit several, or in the minimalist version at least two different developmental responses in a field of cells[7,8] and it is possible that Bcd functions in this way. High levels of Bcd may activate genes in the procephalic and gnathal regions, while lower concentrations of Bcd activate the expression of more central target genes such as *hb* and *Kr*. Alternatively, Bcd could function like any other transcription factor and broadly activate the expression of target genes in the procephalic/gnathal and the central regions, independent of its distribution along the AP axis. Differences in gene expression between procephalic/gnathal and central region would then be due to the inability of the enhancers of centrally expressed genes to respond to Tor dependent repressors. Thus, genes activated by Bcd that lack binding sites for repressors of the terminal system in their enhancers could be expressed in the central region, where the activity of these repressors, as for example Cic, is high.

In conclusion, we cannot rule out that Bcd may act as a morphogen with two thresholds that can instruct nuclei if they are in the procephalic/gnathal or central regions. However, for a nucleus to judge its position within the procephalic/gnathal region it requires the input from the terminal system, as we have shown that the Bcd concentration gradient does not sub-pattern this region. Whether Bcd target gene expression in the central regions of the embryo depends only on Bcd activity, or also on maternal co-regulators, remains to be shown. We favor a model, which challenges the view that Bcd functions as a bona fide morphogen. In order to understand how patterning and polarity is conferred onto a field of cells, it is not enough to observe the concentration of Bcd at a given AP coordinate. Instead, one must take into account the activity of terminal system repressors such as Cic, and the presence of repressor binding sites in target gene enhancers. Thus, the combination of Bcd and Tor dependent repressors into a "morphogenic network" more accurately represents the mechanism by which spatial domains can be set up so precisely and ultimately how the different body parts are distinguished in response to Bcd.

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