High Bicoid levels render the terminal system dispensable for Drosophila head development

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Accepted 26 June; published on WWW 22 August 2000

SUMMARY

In Drosophila, the gradient of the Bicoid (Bcd) morphogen organizes the anteroposterior axis while the ends of the embryo are patterned by the maternal terminal system. At the posterior pole, expression of terminal gap genes is mediated by the local activation of the Torso receptor tyrosine kinase (Tor). At the anterior, terminal gap genes are also activated by the Tor pathway but Bcd contributes to their activation. Here we present evidence that Tor and Bcd act independently on common target genes in an additive manner. Furthermore, we show that the terminal maternal system is not required for proper head development, since high levels of Bcd activity can functionally rescue the lack of terminal system activity at the anterior pole. This observation is consistent with a recent evolution of an anterior morphogenetic center consisting of Bcd and anterior Tor function.

Key words: bicoid, Morphogen, torso, Terminal system, Patterning, Drosophila

INTRODUCTION

The establishment of the Drosophila basic body plan is directed along the anteroposterior axis by three sets of maternal genes: the anterior, posterior and terminal systems (reviewed in St Johnston and Nüsslein-Volhard, 1992). The anterior and posterior systems depend on localized mRNAs, such as bicoid (bcd) and nanos, whereas the terminal system requires the localized generation of an extracellular ligand that activates the receptor tyrosine kinase Torso (Tor) (Klingler et al., 1988; Sprenger and Nüsslein-Volhard, 1992; Casanova and Struhl, 1993). The torsolike (tsl) gene is expressed and required in somatic follicle cells at each end of the maturing oocyte, and is involved in generating the localized terminal signal (Stevens et al., 1990; Savant-Bhonsale and Montell, 1993; Martin et al., 1994). The trunk gene is required in the female germline and is likely to encode the extracellular Tor ligand (Casanova et al., 1993). Mutations in any of these three genes cause identical phenotypes that affect both ends of the embryo: lack of the most anterior structures (labrum, dorsal bridge) and of all structures posterior to the seventh abdominal segment (A7) (Schüpbach and Wieschaus, 1986a; Klingler et al., 1988; Stevens et al., 1990). Ligand-bound Tor acts through the Ras/Raf pathway to activate the Rolled MAP kinase (MAPK) (reviewed in Perrimon and Desplan, 1994), and results in the expression of zygotic target genes, such as huckebein (hkb) and tailless (tll), at both poles of the embryo (Pignoni et al., 1990; Brönnner et al., 1994). However, the genetic analysis of these target genes (Weigel et al., 1990), the analysis of the regulatory region of tll (Rudolph et al., 1997), and the isolation of posteriorly restricted, terminal system-dependent genes (Kispert et al., 1994; Vorbrüggen et al., 1997) all indicate major differences in the response to the terminal system between the anterior and the posterior poles. Such differences in the patterning function of the terminal system are expected as, in many short-germband insects, the anteriormost parts of the oocyte give rise to extraembryonic membranes and are probably dispensable for pattern formation (Sander, 1994). At the anterior of the Drosophila embryo, the terminal system acts in the presence of the morphogens Bcd and Hunchback (Hb), which are absent in the posterior half of the early embryo. While no interactions between Hb and the terminal system have been described so far, several influences of the terminal system on the function of Bcd have been identified.

bcd mRNA is localized to the anterior pole (Berleth et al., 1988) and a gradient of Bcd forms by diffusion of the translated protein (Driever and Nüsslein-Volhard, 1988). The homeodomain-containing transcription factor Bcd (Berleth et al., 1988) then regulates target genes like the gap genes hunchback (hb) and orthodenticle (otd) in a concentration-dependent manner (Driever et al., 1989; Struhl et al., 1989) and acts as a translational repressor of the posterior gene caudal (Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996; Chan and Struhl, 1997). Complete lack of bcd activity results in the loss of head and thorax, which are replaced by posterior terminal structures, whereas hypomorphic bcd alleles, similar to terminal mutants, only lack labrum and dorsal bridge (Frohnhöfer and Nüsslein-Volhard, 1986).
Identical phenotypes are also caused by mutations in the genes *exuperantia* (*exu*) and *swallow*, which lead to a failure of proper anterior localization of the *bcd* mRNA, resulting in reduction of *bcd* activity (Frohnhöfer and Nüsslein-Volhard, 1987). Thus, lack of terminal system activity and hypomorphic *bcd* situations cause very similar anterior phenotypes, which suggests that the anterior and terminal maternal systems are linked or share common targets.

There are several interactions between the terminal system and Bcd. First, most Bcd target genes that are expressed as an anterior cap in the early syncytial embryo later retract from the tip of the embryo (Ronchi et al., 1993). This retraction depends on the terminal system and appears to be important for the correct development of the anterior regions of the embryo (Janody et al., 2000b). In this respect, it is interesting to note that Bcd is phosphorylated in a *tor*-dependent manner, possibly indicating a direct modification of Bcd activity by the *tor* pathway (Ronchi et al., 1993). However, this modification does not appear to be involved in the downregulation of Bcd activity at the pole (Janody et al., 2000a). Although this observation suggests a negative interaction between Tor and Bcd, analysis of the regulation of several *bcd*-dependent head gap genes reveals that *tor* activity can also act positively in potentiating the activator function of Bcd (Grossniklaus et al., 1994; Wimmer et al., 1995; Gao et al., 1996).

As mentioned above, *tor*, weak *bcd* and *exu* mutants exhibit very similar phenotypes. However, *tor exu* double mutants exhibit a stronger enhancement of the anterior phenotype with almost no head structures left, arguing that the terminal and the anterior systems function by additive pathways (Schüpbach and Wieschaus, 1986b). These additive but independent activities of the terminal and anterior systems are also revealed by the analysis of *hkb* regulation, as either *tor* or *bcd* activity alone can activate *hkb*, while both activities together enhance *hkb* expression at the anterior pole (Brönnner and Jäckle, 1996). *hkb* is not expressed in *tor, bcd* double mutants. Therefore, *hkb* behaves as a common target gene that can be activated independently by either the terminal or the anterior maternal system.

Here, we present further evidence that *tor* and *bcd* act on common target genes independently in an additive manner. Moreover, we show that the terminal maternal system is not required for proper head development, as high levels of Bcd can functionally rescue the lack of terminal system activity at the anterior pole.

**MATERIALS AND METHODS**

**Bcd deletion constructs and transgenic fly lines**

BcdΔΔA and BcdΔΔAC transgenic lines were described previously (Schaeffer et al., 2000). The lines with the strongest rescue ability were chosen for further analysis. We used an X-chromosome carrying two wild-type *bcd* rescue constructs (bcd*5*bcd*8*, gift from W. Driever) for the rescue experiment with the tsl* and trk alleles. The rescue experiment with the *tor*ΔX allele used a line carrying two wild-type *bcd* rescue constructs on the third chromosome (*tor*ΔX; BB610 line, gift from G. Struhl).

**Whole-mount in situ hybridization, immunohistochemistry and cuticular preparations**

Digoxigenin-labeled RNA probes for in situ hybridization were prepared as described in Schaeffer et al. (1999). The *hb* probe was prepared from a 2 kb fragment of the *hb* gene (gift from Steve Small) linearized by *Bam*HI and transcribed by T7. The *lacZ* probe was prepared from a 2 kb fragment of the *lacZ* gene (gift from Jessica Treisman) linearized by *Pst* and transcribed by T3. The *hkb* probe was prepared from a 1.8 kb fragment of the *hkb* gene (gift from Ronald Kühnlein) linearized by EcoRI and transcribed by T7. In situ hybridization on whole-mount embryos were performed as originally described (Tautz and Pfeifle, 1989) with adaptation from M. Klingler. Prehybridization and hybridization were performed at 70°C at pH 5. Embryos were mounted either in methyl salicilate: Canada balsam (1:2) or Aqua-Mount (Lerner Laboratories) and photographed using Nomarski optics. Cuticular preparations were performed as previously described in Nüsslein-Volhard et al. (1984). The rabbit-anti-Bcd antibody (a gift from Gary Struhl) was used 1:1000 and revealed by goat-anti-rabbit biotinylated antibody (Vectorstain Elite ABC kit; Vector Laboratories) used 1:500 followed by a diaminobenzidine revelation.

**RESULTS AND DISCUSSION**

*tor* mutants do not affect the rescue potential of a truncated Bcd protein

The terminal maternal system directly modifies Bcd by phosphorylation at several MAPK sites in a Ser/Thr (S/T)-rich region located between the homeodomain and the identified transcriptional activation domains (Ronchi et al., 1993; Janody et al., 2000a). A deletion variant of Bcd that lacks all these activation domains but still contains the S/T-rich region (BcdΔQAC) is able to rescue to viability *bcd* loss-of-function mutants (Schaeffer et al., 1999). Hence, it is conceivable that the ability of the *tor* pathway to create negative charges through phosphorylation of this region of Bcd might result in an acidic-rich transcriptional activation domain that compensates for the loss of all the other activation domains. If this were the case, then the transcriptional activity of the BcdΔQAC deletion variant should be highly dependent on *tor* function. To test this hypothesis, we assayed the ability of a BcdΔQAC transgene to rescue the *bcd* phenotype in embryos derived from *bcd tsl* double mutant mothers. BcdΔQAC rescues the *bcd* phenotype of the *bcd tsl* double mutant similarly to a wild-type *bcd* transgene, resulting in a *tsl* only phenotype (Fig. 1A–C). Since BcdΔQAC is functionally independent of the *tor* pathway, we concluded that the terminal system is not responsible for its activation potential. This result is also consistent with the notion that, in transient transfection experiments and transgenic studies, Bcd transcriptional activity is not significantly modified by mutations of the putative MAPK consensus sites (Janody et al., 2000a; Niessing et al., 1999). Thus, the described direct modification of Bcd by the *tor* pathway does not appear to be necessary for its function.

**Bcd is not a direct target of the terminal system**

*tor* function is necessary to allow a normal expression pattern of most Bcd target genes: many Bcd target genes such as *otd* are expressed in a reduced anterior domain in *tor* mutants (Gao et al., 1996). Furthermore, the expression domain of these genes is expanded in *tor* gain-of-function backgrounds, again suggesting that the *tor* pathway potentiates Bcd function. This effect could be direct, as Bcd transcriptional activity might be enhanced by direct modification of the protein (for instance by
phosphorylation). Alternatively, the effect might be indirect as most Bcd target promoters might also be responsive to Tor through distinct elements.

A direct effect should be detectable with simply organized Bcd target promoters that only contain Bcd-response elements and no Tor-response elements. The proximal hb promoter (P2) resembles such a simple Bcd-response element, which uses activators to set an expression border without the assistance of repressors (Driever and Nüsslein-Volhard, 1989; Struhl et al., 1989; Simpson-Brose et al., 1994; Wimmer et al., 2000). We could show that the hb P2 promoter is not directly responsive to the terminal pathway as, in the absence of Tor activity, the posterior border of the reporter gene expression domain does not move in response to a tor gain-of-function allele. However, the expression pattern does not change in a tor4021 background (Fig. 2E). This argues for a Bcd activator function that is not under direct control of the terminal system. Thus, Bcd and Tor seem to be part of two independent pathways, which share common target genes.

**Rescue of the anterior terminal phenotype by a bcd transgene**

When we assayed a complete series of Bcd deletion variants (Schaeffer et al., 1999) for their ability to rescue the bcd loss-of-function phenotype in the absence of terminal system activity, we found one transgenic line that not only rescued the bcd phenotype but also the anterior part of the tsl phenotype (labrum and dorsal bridge), resulting in a posterior terminal mutant phenotype only (Fig. 3A). This particular transgenic
line carried a bcd variant that deletes an alanine-rich domain (BcdΔA) and had previously been shown to activate the bcd target gene hkb in a widely enlarged expression domain (Schaeffer et al., 1999). Using Bcd immunostaining, we could show that this transgenic line exhibits levels of Bcd that are approximately 2- to 3-fold higher than wild type (Fig. 3B,C). Since other BcdΔA lines did not exhibit the same ability to rescue the tsl phenotype, we concluded that the higher expression level of this particular line rather than the lack of a specific negative protein element (alanine-rich domain) is responsible for overcoming the requirement for the terminal pathway at the anterior.

**High expression levels of Bcd are able to rescue the anterior terminal phenotype**

To further address whether high levels of bcd activity are sufficient to rescue the anterior terminal system phenotype or, if only a particular Bcd deletion variant is capable thereof, we tested the ability of increased doses of wild-type bcd transgenes to rescue several terminal mutant backgrounds. Since the previous experiments were performed with the tsl1 allele, which might only represent a strong hypomorphic allele rather than a null, we included another tsl mutant, tsl4, that is among the strongest in the allelic series, as well as null mutant alleles of the terminal genes trk and tor. To increase the Bcd expression level, we used flies containing an X chromosome or a third chromosome each carrying two wild-type bcd rescue constructs (bcd+5bcd+8, W. Driever, personal communication and BB+10, Struhl et al., 1989), which allowed us to obtain flies carrying up to six copies of bcd. The phenotypes of all terminal mutants (tsl, trk or tor) are similar: lack of labrum and dorsal bridge in the anterior and deletion of all structures posterior to A7 (Fig. 4A,B; data not shown). Four copies of the bcd gene were able to rescue anterior structures including labrum and dorsal bridge in about 40% of all (80% of analyzable) embryos derived from a tsl4 mutant background, while the posterior terminal phenotype was unaffected (Fig. 4C,D). Six copies of bcd were necessary to obtain the same anterior rescue in about 15% of all (55% of analyzable) embryos derived from trk mutants (Fig. 4E,F) and in about 5% of all (45% of analyzable) embryos derived from tor mutants (Fig. 4G,H). However, not all embryos with rescued labrum and dorsal bridge had a perfectly aligned head skeleton. This might be due to incomplete rescue, but it could also be due to Bcd-mediated overexpression of hkb at the anterior pole, which results in terminal-like phenotypes (Janody et al., 2000b). Actually 50%, 70% or 85% of the head cuticles of tsl, trk or tor, respectively, could not be analyzed for rescue due to severe anterior defects, which seemed more severe than normal terminal phenotypes. Nonetheless, some of the rescued embryos (less than 2%) were able to hatch and move around, which suggests complete anterior rescue. These probably represent embryos where just enough Bcd was present to overcome the lack of the terminal system but not too much to induce the phenotype due to high ectopic expression of hkb. All larvae died within 2 hours, likely due to the posterior terminal defects. It should be noted that very few embryos exhibited the type of abdominal segment fusions that have been described for embryos derived from mothers carrying excess copies of the bcd gene (Busturia and Lawrence, 1994). This might be due to the lack of terminal system function at the posterior pole in our experiments. Since no tail is made, there is probably more space for fate-map shifts towards the posterior, resulting in the correct establishment of abdominal segments A1 to A6. The rescue of the anterior terminal phenotype by high levels of bcd further indicates that the major role of the anterior terminal system is the potentiation of Bcd activity.

**High-level Bcd activity restores hkb expression levels**

In the posterior region of the embryo, the tor pathway activates the zygotic effectors tll and hkb, which are sufficient to specify the most posterior anlagen and the gut of the larva (Weigel et al., 1990). At the anterior, the function of the terminal system is more difficult to interpret and, in tor mutants, hkb expression is only reduced. It actually requires bcd tsl double mutants to lose all anterior hkb expression (Brönner and Jäckle, 1996), which indicates additive functions of the anterior and terminal systems on this common target gene. hkb seems particularly interesting in this context, as its function is required for the formation of the labrum (Schmidt-Ott et al., 1994): Reduction of hkb expression, as observed in terminal mutant background (Fig. 5A,B; Brönner and Jäckle, 1996) leads to the deletion of this particular structure.

Therefore, we asked whether the rescue of anterior structures (e.g. the labrum) mediated by high levels of Bcd in terminal system mutants was correlated with the restoration of the hkb expression pattern. Expression of hkb is first detected in the terminal regions (anterior and posterior) of the syncytial blastoderm (Fig. 5A). In terminal mutant embryos, the posterior domain is absent, whereas the anterior domain is

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**Fig. 3.** Highly expressed Bcd deletion variant rescues terminal system phenotype. (A) A transgenic BcdΔA line promotes the rescue of the anterior terminal phenotype: all structures posterior to the seventh abdominal segment (A7) are missing while the labrum and the dorsal bridge (arrowhead) are recovered. Immunostaining for Bcd in (B) a wild-type embryo, and (C) an embryo from a female homozygous for bcdΔ/ with two copies of the rescuing BcdΔA line, showing that this line mediates two to three times higher Bcd levels than wild type.
High levels of Bcd rescue anterior Tor function

Reduced (Fig. 5B). In a tsl background with four or six copies of bcd, however, hkb expression extends further towards the posterior (Fig. 5C,D). Hence, the level of hkb expression could be regained by increasing the levels of Bcd in a terminal system mutant, even though its exact expression domain could not be restored (compare Fig. 5A with D). It is likely that fate-map shifts are able to absorb the slightly changed expression domain of hkb. This suggests that the lack of terminal system activity at the anterior can simply be overcome by another system through enhancement of transcriptional activation of common target genes.

Tor has been shown to antagonize Groucho-mediated repression of genes such as hkb and tll (Paroush et al., 1997), probably by acting on the HMG-box transcription factor Capicua (Jimenez et al., 2000). Therefore, it is likely that Tor enhances Bcd activity by derepression, i.e. the inactivation of potential repressors of Bcd target genes, and thereby rendering any transcriptional activator more potent. As the cis-regulatory control regions of most developmental genes comprise both repressor and activator sites, the inactivation of potential repressors should lead to enhanced expression, or enlarged expression domains, as observed for several bcd target genes in a tor gain-of-function background (Grossniklaus et al., 1994; Wimmer et al., 1995; Gao et al., 1996).

No need for evolutionary conservation of an anterior morphogenetic center

As bcd and tor appear to function independently of each other, it is conceivable that anterior tor activity could also enhance the function of other transcriptional activators through derepression. Therefore, in long-germband insects that might lack a true bcd homolog, the anterior terminal system could also assist other activators, like homologs of Otd or Hb. Moreover, the fact that, in certain situations in the Drosophila embryo, anterior Tor activity can be dispensable for proper head development, is consistent with the observation that a posterior morphogenetic center is more frequently found in insects than an anterior center. Although flies accumulate bcd mRNA and tor activity at the anterior pole of the egg, this...
might not be useful for most short-germband insects as their embryos develop in the posterior part of the egg. In this case, the anterior of the embryo is far away from the anterior of the egg and the morphogenetic role of bcd and tor would not be effective in patterning the head. In Tribolium castaneum, an intermediate-germband beetle, the activity of the terminal system is conserved at the anterior of the embryo, but it does not appear to have a specific function for pattern formation (Schröder et al., 2000). Correspondingly, the tll homolog of Tribolium is only expressed at the posterior pole of the embryo (Schröder et al., 2000). This is in contrast to Drosophila, where tll is expressed at both poles of the early embryo (Pignoni et al., 1990). Moreover, in spite of arguments supporting the presence of a bcd-like function in Tribolium (Wolff et al., 1998), no bcd homologous gene has been identified outside higher diptera (Schröder and Sander, 1993; Stauber et al., 1999) notwithstanding its homeodomain and its location in the Hox cluster (Berleth et al., 1988). It is therefore reasonable to assume that an anterior morphogenetic center consisting of Bcd and anterior Tor activity is not a general feature of insects.

We acknowledge the members of the Desplan laboratory for their constant support during this work, in particular, Terry Turner for her technical assistance. We thank W. Driever, R. Kühnelein, S. Small, G. Struhl and J. Treisman for fly stocks and plasmids. We also thank Nathalie Destatni for important comments and discussion on the experiments and Matthieu Schaeffer for his help as a summer student. V. S. thanks Christine Dambly-Chaudière for her constant support and encouragement. V. S. was supported by a fellowship from the French ‘Ministère de la Recherche’ and E. A. W. by a fellowship from HFSP. This work was initiated with support from the Howard Hughes Medical Institute at the Rockefeller University. It was then supported by a grant from the National Science Foundation NSF IBN-9817981.

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