Establishing the primary axes (anterior-posterior and dorsal-ventral) is one of the first steps in patterning bilateral animals. In Drosophila, this process is well understood at the molecular level. One of the molecules that have been shown to be absolutely critical in patterning wild type embryos is the homeoprotein, Bicoid (Bcd) (St Johnston et al., 1989). Loss of bicoid (bcd) function results in embryos that lack all anterior structures, including the head, thorax, and some anterior abdominal segments (Frohnhofer and Nusslein-Volhard, 1986; St Johnston, 1995). However, the overall polarity of the remaining abdominal segments is retained. The bcd message is deposited into the egg by the mother, and factors binding to its 3'UTR localize it to the anterior pole of the egg (Berleth et al., 1988), via a migration along a polarized array of microtubules (Gonzalez-Reyes et al., 1995; Schnorrer et al., 2000). Translation of the bcd mRNA generates an anterior-to-posterior (A-P) concentration gradient of the Bcd homeodomain protein (Driever and Nusslein-Volhard, 1988). The resulting Bcd morphogenetic gradient differentially activates specific effector genes. The highest concentrations activate the head gap genes (e.g. orthodenticle) (Gao et al., 1996), while lower levels activate the thoracic gene hunchback (hb) (Tautz, 1988; Driever and Nusslein-Volhard, 1989; Struhl et al., 1989), and even lower levels activate the abdominal genes Krüppel (Kr) (Hulskamp et al., 1990; Hoch et al., 1991; Struhl et al., 1992) and activate knirps (kni) (Rivera-Pomar et al., 1995). The ability of Bcd to differentially target genes provided the first molecular explanation as to how a transcription factor functions as a morphogen. Further, Bcd is not only a transcriptional regulator, but also represses the translation of the ubiquitously distributed maternal caudal mRNA (Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996; Chan and Struhl, 1997), thus generating an opposite posterior to anterior gradient of the Caudal protein. Bcd performs this function by directly binding to the 3'UTR of the caudal mRNA with its homeodomain (Niessing et al., 1999; Niessing et al., 2000). Therefore, bcd has multiple roles in activating the head (otd), thoracic (hb) and abdominal (Kr and kni) gap genes, as well as in blocking the function of the posterior determinant cad. This places bcd in a central position for patterning the A-P axis of the fly embryo (Fig. 1).

Bicoid as a unique patterning system of higher Diptera

Despite the absolutely critical role for bicoid in patterning the Drosophila embryo, evidence has mounted indicating that the Bicoid gradient is a relatively recent addition to the developmental toolkit of insects, and that it may be unique to higher flies.

Considering first a comparison to more basal insects, the morphology of the Drosophila embryo, occupying the entire egg, allows for the formation of an anterior morphogenetic center where spatial determinants can be localized (e.g. bcd mRNA and tor activity) and can diffuse posteriorly. However, an anterior
patterning system would be unfeasible in insect species that develop with the head anlagen located at the posterior end of the egg, as in the more ancestral *Schistocerca* (Fig. 2). Since the *Schistocerca* embryo is at a considerable distance from the anterior pole of the egg, it is unlikely that an anterior morphogenetic center could pattern the head of the embryo through such a distance (Sander, 1975). However, a posterior morphogenetic center acting via degradation of a uniform factor could function in these conditions, like for example the regulation of maternal *hb* (\(hb^{ma}\)) mRNA translation by *nanos* (Curtis et al., 1995).

For the beetle *Tribolium canstaneum* (*Tc*) (Fig. 2), recent contradictory studies have provided arguments both for and against the existence of a *bcd*-like function: when the *Tc-cad*mRNA, whose translation is repressed at the anterior of the *Tc* embryo, is placed in *Drosophila*, translation of its mRNA is blocked at the anterior of the embryo in a *bcd*-dependent manner. On the other hand, sequencing of the genomic region encompassing the *Tc-Hox* region indicates that there is no *bcd* gene in the *Hox3/zen* region where it is found in *Drosophila* (Brown et al., 2001). To reconcile these observations with the model that *bcd* is absent from *Tribolium*, it is possible that the regulation of *Tc-cad* mRNA translation is performed by another factor. For example, a homologue to the *C. elegans mex-3* gene (sharing no homology with *Bcd*) which regulates translation of the *C. elegans cad* homologue, *pal-1* (Hunter and Kenyon, 1996) could play a similar role in *Tribolium*, and *Bcd* might recognize the same regulatory element when placed in *Drosophila*. *Tc-cad* mRNA translation is also repressed in anterior regions of the *Tribolium* embryo (Wolff et al., 1998).

Finally, no genes homologous to *bcd* have been identified outside higher Diptera (Schroder and Sander, 1993; Bonneton et al., 1997; Stauber et al., 1999), despite its homeobox and genomic position in the *Hox* cluster (Berleth et al., 1988). Moreover, *bcd* shows an unusually high divergence for a homeobox gene (Somer and Tautz, 1991; Stauber et al., 1999), and its function is not even conserved within higher Diptera (Schroder and Sander, 1993; Bonneton et al., 1997). The most distant species in which *bcd* has been found is the basal cyclorrhaphan (a monophyletic clade of highly derived dipterans that includes *Drosophila* and houssellies, among others) fly *Megaselia* (Fig. 2). In *Megaselia*, *bcd* and *hb* appear to play similar roles as in *Drosophila*. Surprisingly, the phenotype of RNAi experiments with *Megaselia* *bcd* is significantly more severe than that of *Drosophila* *bcd*, suggesting that, in this species, *bcd* has taken even more roles than in the *Drosophila* embryo. Alternatively, *hb*\(^{ma}\), whose function patterns some of the axis of the embryo in *bcd* mutants, might have a lower contribution in *Megaselia* (Stauber et al., 2000).

**The origin of Bicoid**

The combined molecular and embryological data strongly indicate that the *Bcd* morphogen gradient is not universally employed among insects. How, then, could an anterior patterning system based on an anteriorly centered *Bcd* morphogen gradient evolve? *bcd* is present in the *Hox* complex (Antp-C), next to the genes *zerknuellt* (*zen*) and *z2*, a recent duplication of the *zen* gene. Genes at this *Hox3* paralogous position have a tendency to duplicate and diverge (Falciani et al., 1996), and *bcd* is likely to have arisen through an earlier duplication of *zen*. *bcd* has diverged extensively
Hunchback as an A-P patterning gradient

Hb encodes a zinc finger transcription factor that specifies anterior development while preventing posterior development (Hulskamp et al., 1990). As a zygotic gap gene, hbp99 is expressed in response to bcd in the anterior half of the early embryo. The binding of Bcd to high affinity sites in the hbP2 promoter (Driever and Nusslein-Volhard, 1989; Driever et al., 1989; Struhl et al., 1989) directs expression of the anterior hbp99 domain, and has been documented in much detail. This system serves as a paradigm for the functioning of a transcriptional morphogen (Driever and Nusslein-Volhard, 1989; Struhl et al., 1989). Hb is also provided maternally (hbmat) as a ubiquitously distributed mRNA whose translation is blocked by the posterior gene nanos (Sander and Lehmann, 1988; Lehmann and Nusslein-Volhard, 1991), thereby generating another A-P Hb protein.

Fig. 3. Model for the evolution of Bicoid dependent patterning. (See text for details).
near bicaudal phenotype as well, in spite of the presence of \textit{bcd} (Simpson-Brose et al., 1994). This phenotype is reminiscent to that of embryos lacking both \textit{bcd} and \textit{hb}, which are completely bicaudal with a duplicated telson replacing the labrum. This phenotype indicates that \textit{bcd} is not able to create correct long-range polarity in the absence of \textit{hb} and emphasizes the crucial early patterning role of \textit{hb} (Simpson-Brose et al., 1994).

**Ancestral patterning role of \textit{hb} for patterning of the thorax**

In the abdomen, flies still contain an ancestral patterning system that is redundant with \textit{bcd} since \textit{hb} and \textit{cad} can replace \textit{bcd} function (Hulskamp et al., 1990; Struhl et al., 1992; Schulz and Tautz, 1994; Rivera-Pomar et al., 1995). However, similar experiments have failed to characterize the relative contributions of \textit{hb} and \textit{bcd} in patterning the head and thorax. This results from the fact that activation of \textit{hb}\textsuperscript{PS4} is \textit{bcd}\textsuperscript{-}dependent. Thus, whenever \textit{bcd} activity is altered, \textit{hb}\textsuperscript{PS4} activity is also changed (Driever and Nusslein-Volhard, 1989; Struhl et al., 1989). To circumvent this problem, a system was developed that allows the study of the two morphogens (\textit{Bcd} and \textit{Hb}) independently of each other. \textit{bcd}\textsuperscript{-}dependent expression of \textit{hb}\textsuperscript{PS4} is mediated by the \textit{hb} P2 promoter; whereas maternal and late blastoderm expression of \textit{hb} is initiated by the \textit{hb} P1 promoter (Margolis et al., 1995). Therefore, a functional \textit{hb} transgene (\textit{hb}\textsuperscript{P1}) was constructed that does not mediate early \textit{bcd}\textsuperscript{-}dependent zygotic expression. However, \textit{hb}\textsuperscript{P1} is able to direct maternal as well as late zygotic expression, in particular the intense stripe of \textit{hb}\textsuperscript{PS4}. Therefore, this transgene uncouples the direct link between \textit{bcd} and \textit{hb}.

Experiments employing the \textit{hb}\textsuperscript{P1} promoter construct show that the standard “zygotic” phenotype of embryos born from heterozygous \textit{hb}\textsuperscript{+}\textit{+} parents \(\text{i.e.} \text{loss of T}_1, \text{T}_2, \text{and T}_3\) is in part caused by a decrease in the dose of \textit{hb}\textsuperscript{mat}. In fact, by restoring full maternal \textit{hb}\textsuperscript{mat} expression \(\text{i.e.} \text{two copies, by placing one \textit{hb}\textsuperscript{P1} transgene in the mother}\), the mutant phenotype of \textit{hb}\textsuperscript{PS4} is less severe, leading to the deletion of only the \textit{T}_2 and \textit{T}_3 segments while \textit{lb} and \textit{T}_1 segments are restored (Fig. 4). Therefore, in the presence of a normal \textit{hb}\textsuperscript{mat} dosage, \textit{hb}\textsuperscript{PS4} is necessary only for the development of \textit{T}_2 and \textit{T}_3, two segments that exactly overlap the domain of \textit{hb}\textsuperscript{PS4} expression.

Based on these results it can be hypothesized that: 1) the \textit{lb} and \textit{T}1 segments depend on a high maternal contribution of \textit{hb} and not on \textit{hb}\textsuperscript{PS4}, and 2) The real \textit{hb}\textsuperscript{PS4} defects are due to the late \textit{hb}\textsuperscript{PS4} stripes, such that the \textit{T}_2-\textit{T}_3 deletion seems to result from the failure to activate the \textit{hb}\textsuperscript{PS4} stripe and the fusion of \textit{T}1-\textit{T}8 depends on the posterior stripe. This strongly suggests that the early \textit{bcd}\textsuperscript{-}dependent \textit{hb}\textsuperscript{PS4} domain does not play a fundamental role. As the \textit{hb}\textsuperscript{PS4} stripe is autoregulated (in \textit{hb}\textsuperscript{PS4} mutants, this stripe is absent), the only role of \textit{bcd}\textsuperscript{-}dependent \textit{hb}\textsuperscript{PS4} might be to drive the high levels of \textit{hb} expression necessary to ‘kick in’ this autoregulation. Thus, if strong \textit{hb}\textsuperscript{mat} contribution could be driven, the \textit{bcd}\textsuperscript{-}dependent \textit{hb}\textsuperscript{PS4} could be made obsolete.

To test this model, a situation where \textit{Bcd} is no longer able to activate \textit{hb}\textsuperscript{PS4} \(\text{\textit{hb}\textsuperscript{P1} only in an \textit{hb}\textsuperscript{PS4} mutant background}\) was generated. In order to restore normal \textit{hb}\textsuperscript{PS4} expression, the dose of its activator, \textit{hb}\textsuperscript{mat} was increased via 4 \textit{hb}\textsuperscript{P1} transgenes; and the dose of its repressor, \textit{kni} (Pankratz et al., 1989), was reduced to one copy. In this context, most embryos develop thoracic structures posterior to \textit{T}_1; and a significant proportion exhibit complete rescue of the \textit{lb}, \textit{T}_1, \textit{T}_2, and \textit{T}_3 segments (Wimmer et al., 2000) (Fig. 4). Therefore, the role of \textit{bcd} in activating \textit{hb}\textsuperscript{PS4} can be replaced by adding higher levels of \textit{hb}\textsuperscript{mat} to activate \textit{hb}\textsuperscript{PS4} and form \textit{T}_1 and \textit{T}_3. Comitant with the rescue of \textit{T}_2 and \textit{T}_3, the \textit{hb}\textsuperscript{PS4} stripe reappears (as assayed by \textit{hb}\textsuperscript{P1-lacZ} expression).

These results demonstrate that the \textit{Bcd} and \textit{Hb} morphogenetic systems do not need to be directly linked in \textit{Drosophila}, which is consistent with the fact that \textit{bcd} is unlikely to exist in other insects. Further, they support the argument that the control of \textit{hb} by \textit{bcd} has been recently acquired phylogenetically and can be bypassed for proper thoracic segmentation.

Although thoracic development can be recovered when \textit{bcd} control over \textit{hb} is eliminated, \textit{bcd} may still be required for functional synergy with \textit{hb} to form the thoracic segments. This model can be tested in a situation where embryos lack \textit{bcd} but have high levels of \textit{hb}\textsuperscript{PS4} provided by an alternative mechanism. A maternally expressed gene was generated with a maternal promtor fused to the coding sequences of the Gal4 DNA binding domain and three copies of the yeast GCN4 activation domain (Janody et al., 2000). The mRNA is localized to the anterior pole of the embryo by the \textit{bcd} 3'UTR. Translation of this mRNA creates an A-P concentration gradient of the artificial Gal4-GCN4 transcription factor. By crossing \textit{bcd}\textsuperscript{-}mutant females containing this construct with males bearing a UAS-\textit{hb}

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**Fig. 4. Phenotypes of embryos in various \textit{hb} and \textit{bcd} backgrounds.**

(A) Wildtype. (B) Zygotic \textit{hb} mutant phenotype \(\text{(with 1/2 maternal contribution)}\). (C) Zygotic \textit{hb} mutant with normal maternal contribution restored by addition of one \textit{hb} copy driven by \textit{P}1 promoter. (D) Nearly complete rescue of \textit{hb} phenotype via \(4\) copies of \textit{hb}\textsuperscript{P1}, and reduction of \textit{hb}\textsuperscript{mat} contribution.

\text{Kni. (E)} Rescue of thoracic segments lost in \textit{bcd} mutant by driving \textit{hb} expression under control of artificial transcription factor. Abbreviations: \textit{Abd}, abdomen; \textit{an}, antennal; \textit{ic}, intercalary; \textit{lb}, labial; \textit{lr}, labrum; \textit{md}, mandibular; \textit{mx}, maxillary; \textit{oc}, ocular; \textit{T}1-3, thoracic segments 1-3 respectively; \textit{tel}, telson (posterior terminal structure).
transgene, a strong gradient of $\text{hb}^{\text{Gr}}$ expression is created in the absence of $\text{bcd}$ function. This genetic combination results in embryos that develop normal $T_2$ and $T_3$ segments (Wimmer et al., 2000). This $T_2$-$T_3$ rescue is likely due to the observed rescue of $\text{hb}^{\text{Gr}}$ expression in the absence of $\text{bcd}$ function. Therefore, $T_2$ and $T_3$ can form in the total absence of $\text{bcd}$, as long as $\text{hb}^{\text{Gr}}$ is activated. However, the $\text{lb}$ and $T_4$ segments, which were shown to depend on high levels of $\text{hb}^{\text{mat}}$, do not form in this situation (Fig. 4). Although there are high levels of $\text{hb}$ in this region of the embryo that gives rise to $\text{lb}$ and $T_1$, there is a delay in reaching high levels of $\text{Hb}$ protein expression from UAS-$\text{hb}$ and this expression is transient. It is likely that the formation of $\text{lb}$ and $T_4$, like the more anterior head segments, requires a synergy between $\text{bcd}$ and $\text{hb}$ as discussed below.

**Can $\text{hb}$ control head development?**

The above described experiments show that $\text{hb}$ is able to pattern thoracic and abdominal segments in the absence of $\text{bcd}$ and may thus control patterning of these regions in embryos with a more ancestral patterning system. However, since the pre-gnathal head segments are never rescued by manipulating $\text{hb}$ levels, at least one other factor must be invoked. The head gap gene $\text{orthodenticle (otd)}$ has been proposed to fulfill this role.

$\text{otd}$ is a head gap gene that defines metamerization of head segments and specifies their identity. In the absence of $\text{otd}$ function, the ocular and antennal segments are missing (Cohen and Jurgens, 1990). The role of $\text{otd}$ as a determinant of head structures is highly conserved in evolution. There are four $\text{otx}$ genes in vertebrates that specify forebrain structures and the eye (Bally-Cuif and Boncinelli, 1997) and are expressed in anterior regions. Even in hydra, where there is no A-P axis, $\text{otf}$ is expressed around the mouth (along the oral-aboral axis) (Smith et al., 1999). In vertebrates, one of the functions of Otx is to antagonize the function of Cdx, the homologue of Cad (Isaacs et al., 1999), mostly at the transcriptional level. In

**Synergy between $\text{hb}$ and $\text{otd}$ for anterior patterning**

The conclusions reached by manipulations of the *Drosophila* embryo have been substantiated by experiments in other insects, where techniques for manipulating embryos have been recently developed. In *Tribolium* the functions of $\text{hb}$ and $\text{otd}$ have been tested by knocking down their respective messages with RNAi (Schroder, 2003) (Fig. 5). In this organism, double stranded RNA can be delivered to embryos through the mother, termed parental RNAi (pRNAi), resulting in a knock down of both maternal and zygotic components of expression (Bucher et al., 2002). This is important since, unlike in *Drosophila*, $\text{Tc-otd1}$ is expressed maternally as well as zygotically. When $\text{Tc-otd1}$ is knocked down in this manner, the embryos exhibit a range of phenotypes, the strongest being the loss of all head structures. This is much more severe than what is seen in

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**Fig. 5. Comparison of *Tribolium* pRNAi results and mutant phenotypes of *Drosophila* and *Nasonia* mutants.** See text for details.
Drosophila otd null mutants, which only lose the ocular and antennal segments. In fact, this phenotype is more reminiscent of a weak bcd mutant. There is a second Tc-otd paralog, otd2, which does not appear to play an important role in the early embryo, but rather act like the late Dm otd gene.

The Tc-otd/pRNAi phenotype is not as strong as what is seen in bcd mutant, indicating that another factor combines with otd to replace bcd function in the beetle embryo. The pRNAi phenotype of Tc-hb is consistent with this role: the more extreme cases are missing all thoracic segments as well as some of the gnathal head segments. The overlap of the two phenotypes indicates that hb and otd cooperate in setting up the axis of the Tribolium embryo.

Interestingly, there appears to be some lability in this proposed ancestral patterning mechanism. A zygotic loss of function mutation in the hbt ortholog of the wasp Nasonia vitripennis causes a loss not only of the thoracic and gnathal head segments, but also most of the pre-gnathal segments (Pultz et al., 1999). This phenotype is more severe than both the loss of maternal and zygotic function in Drosophilina and the Tribolium RNAi experiments (Fig. 5). This result indicates that the Nasonia embryo relies more heavily on input from hbt in patterning the anterior than either the fly or the beetle. The role of otd in this organism is currently being examined.

In conclusion, by manipulating the expression of genes in the context of the highly derived embryogenesis of the fly Drosophila, it is possible to gain insight into the mechanisms employed by insects of a more ancestral type. The application of emerging techniques such as RNAi (Hughes and Kaufman, 2000; Stauber et al., 1999), genetic transformation (Horn and Wimmer, 2000; Pelouquin et al., 2000; Grossman et al., 2001; Hediger et al., 2001; Kokoza et al., 2001; Heinrich et al., 2002) for manipulating the embryos of insects and arthropods other than Drosophila will allow hypotheses generated by these Drosophila experiments to be tested in a wide variety of organisms. Thus, the depth of knowledge obtained from the Tribolium research program can be supplemented with a dimension of breadth, allowing for a much clearer understanding of the evolution of developmental mechanisms in this incredibly diverse animal phylum.

Summary

The genetics of the establishment of the primary axes of the early embryo have been worked out in great detail Drosophila. However, evidence has accumulated that Drosophila employs a mode of patterning that is not shared with most insects. In particular, the use of the morphogenic gradient of the Bicoid homeoprotein appears to be a novel addition to the fly developmental toolkit. To better understand the ancestral mode of patterning that is probably more widely used by insects, several groups have used Evo-Devo approaches as well as sophisticated genetic manipulations of Drosophila to achieve some form of ‘de-evolution’ of this derived insect. Genetic manipulations of the beetle Tribolium and the wasp Nasonia have validated most of these results.

KEY WORDS: AP patterning, Evolution of Development

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References


