Cell, Vol. 69, 237–249, April 17, 1992, Copyright © 1992 by Cell Press

Control of Drosophila Body Pattern by the hunchback Morphogen Gradient

Gary Struhl,* Paul Johnston,† and Peter A. Lawrence†
*Howard Hughes Medical Institute
Department of Genetics and Development
Columbia University College of Physicians and Surgeons
New York, New York 10032
†MRC Laboratory of Molecular Biology
Hills Road
Cambridge CB2 2QH
England

Summary

Most of the thoracic and abdominal segments of Drosophila are specified early in embryogenesis by the overlapping activities of the *hunchback* (*hb*), *Krüppel*, *knirps*, and *giant* gap genes. The orderly expression of these genes depends on two maternal determinants: *bicoid*, which activates *hb* transcription anteriorly, and *nanos*, which blocks translation of *hb* transcripts posteriorly. Here we provide evidence that the resulting gradient of hb protein dictates where the *Krüppel*, *knirps*, and *giant* genes are expressed by providing a series of concentration thresholds that regulate each gene independently. Thus, hb protein functions as a classical morphogen, triggering several distinct responses as a function of its graded distribution.

Introduction

One of the central problems in developmental biology is to explain how the body plan is first established. In Drosophila, most of the components required for specifying the basic pattern of head, thoracic, and abdominal segments have been identified, and in many cases their roles and modes of action have been determined (reviewed in Nüsslein-Volhard, 1991). Yet major uncertainties remain. One is the global control of thoracic and abdominal segmentation.

Proper development of the posterior half of the body normally depends on the activity of the maternal determinant nanos (nos) (Nüsslein-Volhard et al., 1987; Lehmann and Nüsslein-Volhard, 1991). *nos* mRNA is tightly localized at the posterior pole of the fertilized egg and is presumed to give rise to a gradient of nos protein soon after fertilization (Wang and Lehmann, 1991). Moreover, both genetical and embryological studies suggest that nos can function as a graded morphogen that specifies abdominal pattern (Lehmann and Nüsslein-Volhard, 1986; Wharton and Struhl, 1991). However, under certain conditions, the normal pattern of abdominal segments can be formed in the absence of nos activity (see below); hence, some other factor must be capable of generating posterior body pattern independently.

The best candidate for this factor is hunchback (hb) protein. The *hb* gene is transcribed during oogenesis, and the resulting transcripts are distributed uniformly in the egg (Tautz et al., 1987). Soon after fertilization, these transcripts are preferentially translated in the anterior half of the body because nos represses their translation posteriorly (Tautz, 1988). The *hb* gene is also transcribed under the control of the anterior determinant bicoid (bcd) (Tautz, 1988; Schröder et al., 1988; Driever and Nüsslein-Volhard, 1989; Struhl et al., 1989). Hence, bcd activates *hb* anteriorly, whereas nos represses it posteriorly; together, both activities ensure that the concentration of hb protein is maximal in the anterior half of the body and declines to undetectable levels in the posterior half.

The progressive decline in hb protein concentration from high to undetectable levels across the middle portion of the body appears to be critical for generating posterior body pattern. When hb protein is allowed to accumulate inappropriately in the posterior half of the body (e.g., by inactivating nos, deleting cis-acting nos response elements in *hb* mRNA, or generating high levels of *hb* transcripts under *hsp70* control), abdominal segmentation is blocked (Tautz, 1988; Hülskamp et al., 1989; Struhl, 1989a; Wharton and Struhl, 1991). Conversely, when hb protein expression is prevented in both halves of the body (e.g., by inactivating both the maternal and zygotic *hb* mRNAs by mutation or by causing ectopic nos activity at the anterior pole), many abdominal segments fail to form or have reversed polarity (Lehmann and Nüsslein-Volhard, 1987; Wharton and Struhl, 1989; Hülskamp et al., 1990). Finally, differential *hb* expression can suffice to generate posterior body pattern, even in the absence of the posterior determinant nos (Hülskamp et al., 1989; Irish et al., 1989; Struhl, 1989a). In this unusual circumstance, in which maternal *hb* transcripts are inactivated by mutation thereby obviating a requirement for nos, normal patterning depends on the formation of an hb gradient under bcd control (Hülskamp et al., 1990).

How does differential hb expression generate posterior pattern? The key to answering this question must lie in deployment of the gap genes *Krüppel* (*Kr*), *knirps* (*kni*), and *giant* (*gt*), which are activated in overlapping posterior domains and which control distinct portions of the abdominal segment pattern (Nüsslein-Volhard and Wieschaus, 1980; Carroll and Scott, 1986; Ingham et al., 1986; Frasch and Levine, 1987; Struhl, 1989b). Recent studies of these genes have led to the hypothesis that the anteroposterior differential of hb protein expression constitutes a morphogen gradient specifying where *Kr*, *kni*, and *gt* are expressed (Hülskamp et al., 1990; Eldon and Pirrotta, 1991; Kraut and Levine, 1991a, 1991b; see also Gaul and Jäckle, 1987, 1989). In these studies, the pattern of hb protein expression has been altered by mutations in *hb*, *nos*, or *bcd*; the resulting changes in the patterns of *Kr*, *kni*, and *gt* expression have suggested that high concentrations of hb protein block expression of all three of these genes, whereas lower concentrations allow *Kr* activity but still prevent *kni* and *gt* expression.

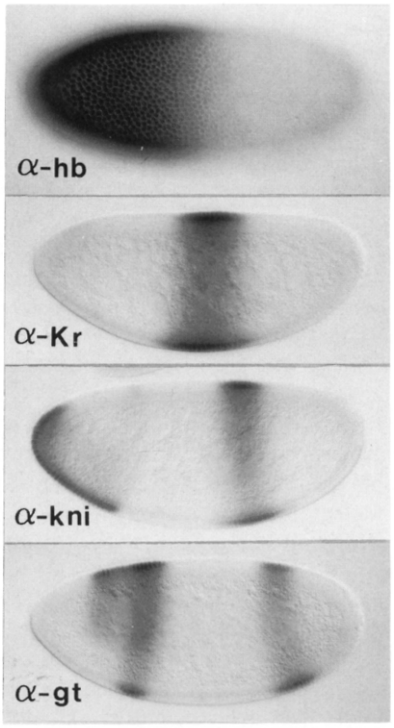

Figure 1. hb, Kr, kni, and gt Protein Expression in Wild-Type Embryos

The pattern of hb protein expression is shown in an embryo prior to nuclear cycle 13: the plane of focus is at the surface to illustrate the graded distribution of hb protein in the middle portion of the body. Note that the concentration of hb protein falls from maximal to undetectable levels over a distance of approximately 15 nuclei (the distance along the anteroposterior axis is around 80 nuclei in this embryo); detectable expression extends approximately two-thirds of the way down the body. The patterns of Kr, kni, and gt protein expression are shown in mid-cycle 14 embryos (stage 5(2); Lawrence and Johnston, 1989; the embryos are shown in optical cross section). Note that kni and gt are expressed in anterior as well as posterior domains. For both genes, anterior expression is completely dependent on transcriptional activation by bcd and falls within the domain of maximal hb expression (also under bcd control). In contrast, posterior kni and gt expression, as well as central Kr expression, depends critically on the progressive decline of hb protein beneath distinct threshold concentrations, which in turn depends on the absence of bcd activity. Here, and in the remaining figures, all embryos are oriented with the anterior end at the left and the dorsal side at the top.

In the experiments described here, we test this hypothesis by creating embryos in which the profile of the hb protein gradient is systematically altered, while all other known signaling systems are eliminated or held constant. In this way, it has been possible to assess how the boundaries of *Kr*, *kni*, and *gt* expression are influenced by the distribution of hb protein. We show that the hb gradient provides a series of distinct concentration thresholds that position the anterior boundaries of expression of all three genes, as well as the posterior boundary of *Kr* expression. Moreover, we show that these responses are independent and sufficient to generate the overlapping domains of gap gene expression that thereafter specify most aspects of posterior body pattern. Thus, hb controls thoracic and abdominal segmentation by acting as a classical gradient morphogen (Dalcq, 1938; Turing, 1952; von Ubisch, 1953; Sander, 1959, 1960, 1975).

Results

hb Protein Gradient

The early expression of hb protein depends on two independently controlled sources: maternal mRNAs that are initially distributed throughout the egg but are not translated posteriorly owing to nos; and zygotic mRNAs that are transcribed anteriorly in response to bcd. Together, these sources generate a pattern of differential hb expression in which the concentration of protein peaks in the anterior half of the body under bcd control and declines in a graded fashion to undetectable levels in the posterior half under nos control (Figure 1). Because nos and bcd act in distinct domains and have opposite effects on hb expression, the hb gradient can be subdivided into a lower (nos-dependent) and upper (bcd-dependent) half, each of which can be analyzed independently.

As shown in Figure 1, the hb gradient extends across a central interval of the body in which Kr, kni, and gt are expressed in a series of overlapping domains. In the experiments described here we are concerned principally with the role of the hb gradient in positioning the boundaries of central Kr and posterior kni and gt expression that falls within this interval. The *Kr*, *kni*, and *gt* genes are also activated anteriorly by bcd and repressed at the ends of the body by the "terminal" determinant system (see Nüsslein-Volhard et al., 1987; Nüsslein-Volhard, 1991). As described below, these additional levels of control have been eliminated by appropriate mutations in *bcd* and either *torso* (*tor*) or *torso-like* (*tsl*), two genes equally essential for terminal specification.

Control by the nos-Dependent Portion of the hb Gradient

In embryos lacking both *bcd* and *tsl* (or *tor*) function, hb protein derives solely from maternal *hb* transcripts that are uniformly distributed throughout the egg. Hence, the hb protein distribution can be manipulated simply by varying the maternal *hb* gene dosage or by eliminating nos activity, the only remaining regulator of hb expression. As shown in Figure 2 (left column), we performed three experiments to alter systematically the distribution of hb protein in these embryos. First, we eliminated hb protein expression by generating females with *hb* mutant germ cells (0×). Second, we varied the number of maternal copies of the *hb* gene from 1 to 2 to 4 (1×, 2×, 4×), thereby generating a series of gradients with different profiles (Figure 2 and Experimental Procedures). Third, we used a mutation in the *oskar* (*osk*) gene to eliminate nos activity (Lehmann and Nüsslein-Volhard, 1991), thereby causing hb protein to be expressed at uniform levels throughout the body. As described below, the distribution of Kr, kni, and gt protein in these embryos, as well as the resulting segmentation patterns, indicates that the nos-dependent portion of the hb gradient specifies abdominal segmentation by positioning the posterior Kr, anterior kni, and anterior gt boundaries.

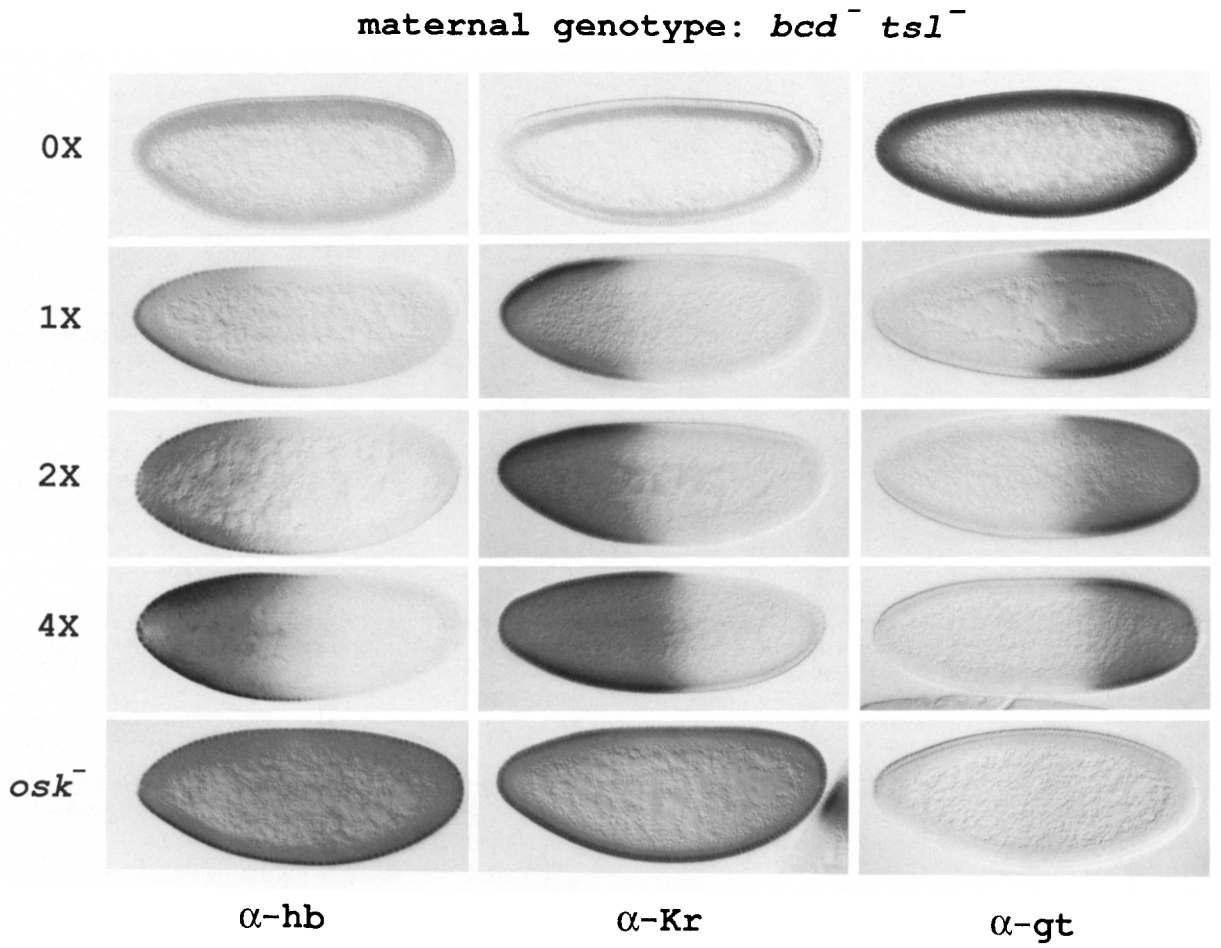

Figure 2. Control of the Posterior Kr and Anterior gt Boundaries by the hb Gradient

The patterns of hb, Kr, and gt protein expression are shown in embryos derived from *bcd tsl* females carrying 1, 2, or 4 copies of the *hb* gene (1×, 2×, and 4×) or containing 2 copies of the *hb* gene but lacking nos activity, owing to mutation in the gene *osk* (*osk*⁻). Also shown in the top row are embryos derived from *tor; bcd hb* oocytes obtained by pole cell transplantation (0×). As described in the text, these embryos are equivalent to embryos derived from *bcd tsl* females in that they lack both *bcd* and *tor* function; they differ, however, by the complete absence of hb protein. Note that the level of hb protein expression correlates with the maternal gene dosage in 1×, 2×, and 4× embryos (e.g., compare the intensity of hb staining in the anterior half of each embryo). The posterior limit of detectable hb protein expression also shifts posteriorly with each increase in gene dosage. (Because of the difficulties in recording low levels of protein expression photographically, we have measured the posterior limit of detectable hb protein expression directly; it falls at 39%, 35%, and 28% egg length [measured from the posterior pole] in 1×, 2×, and 4× embryos, respectively; see Experimental Procedures.) Note also that Kr and gt expression depend critically on the presence or absence, respectively, of hb protein and that the boundaries of each are positioned progressively more posteriorly as the maternal *hb* copy number rises from 1 to 2 to 4 (falling at 58%, 50%, and 45% egg length for Kr, and at 60%, 53%, and 47% egg length for gt; see Experimental Procedures). Finally, note that the posterior boundary of detectable hb protein expression determined by direct measurement extends at least 15% egg length further posteriorly than the Kr and gt boundaries measured in sibling embryos. All of the embryos showing Kr and gt protein expression are at the same stage of nuclear cycle 14 (stage 5(2)); however, all of the embryos showing hb expression are at an earlier stage (nuclear cycle 11 or 12).

Posterior Kr Boundary

As shown in the middle column of Figure 2, we find that the distribution of Kr protein depends on that of hb protein. In the absence of hb protein (0×), Kr is not expressed. In contrast, when a single *hb* gene is present maternally, Kr is expressed in a broad anterior domain, extending about halfway down the body (1×). Moreover, each increase in *hb* gene dosage (2×, 4×) causes a posterior shift in the boundary of Kr expression. Finally, ubiquitous hb expression (*osk*) gives rise to ubiquitous Kr expression. These results show that in the absence of the anterior and terminal determinants, a minimum concentration of hb protein is both necessary and sufficient for *Kr* gene activity. They also show that the graded distribution of hb protein controls where the boundary of *Kr* gene expression is positioned, presumably by determining where this concentration threshold occurs.

Anterior gt Boundary

The hb gradient also appears to position the anterior gt boundary. As shown in the right column of Figure 2, gt is expressed ubiquitously in the absence of hb protein (0×). However, when a single *hb* gene is present maternally, gt expression is blocked in a broad anterior domain (1×). Further, each increase in maternal *hb* gene dosage (2×, 4×) is accompanied by a posterior shift in the boundary of gt expression. Finally, ubiquitous hb expression (*osk*) prevents any detectable gt expression. Thus, in the absence of *bcd* and *tor* activity, gt is only expressed where

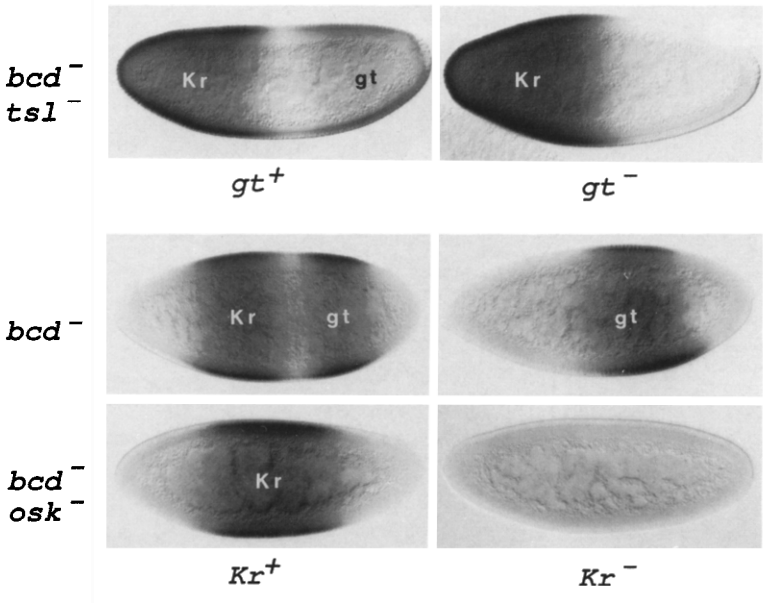

Figure 3. The Posterior Kr and Anterior gt Boundaries Are Positioned Independently by the hb Gradient

All six embryos shown were double stained for both Kr and gt protein expression using different immunohistochemical detection systems to generate distinguishable brown or blue-gray signals: the domains of Kr and gt protein expression are labeled on the micrographs. All of the embryos shown are at the same stage of nuclear cycle 14 (stage 5(2)). The upper panel shows that the posterior Kr boundary is correctly defined in *bcd tsl* embryos, irrespective of *gt* gene activity. The lower panel shows that the anterior gt boundary is initially set by the hb gradient, irrespective of *Kr* gene activity. In this case, gt expression has been compared in embryos derived from *bcd* or *bcd osk* females, which differ principally in the distribution of hb protein (hb protein expression is graded in *bcd* embryos [as it is in *bcd tsl* embryos, Figure 2] and uniform in *bcd osk* embryos [as it is in *bcd osk tsl* embryos in Figure 2]). Note that gt protein is expressed in a posterior stripe in *bcd* embryos but is not detected in *bcd osk* embryos, whether they are Kr^+ or Kr^-. This shows that the ability of the hb gradient to define initially the anterior gt boundary is not dependent on *Kr* gene activity. Note, however, that the anterior gt boundary has begun to shift anteriorly in the Kr^- embryo (as compared with its Kr^+ sibling). As described in the text, the boundary shifts progressively during the latter portion of nuclear cycle 14, indicating that *Kr* gene activity plays a significant role in maintaining the boundary initially defined under hb control.

the concentration of hb protein falls beneath a critical threshold. As in the case of Kr, this threshold dependence provides the means by which the hb gradient controls the pattern of gt expression.

Independent Control of the Posterior Kr and Anterior gt Boundaries

As shown in Figure 2, the *Kr* and *gt* genes are expressed in reciprocal anterior and posterior domains under hb control. Hence, hb might govern one of these genes directly, which in turn could control the other. Alternatively, it might deploy each gene independently, activating one (*Kr*), while repressing the other (*gt*). To distinguish between these possibilities, we have asked whether hb can control the expression of each gene in the absence of the other.

We tested the ability of hb to define the posterior Kr boundary in the absence of gt by generating gt^- embryos from *bcd tsl* mutant females. As shown in Figure 3 (upper panel), the absence of *gt* activity has little effect on the posterior boundary of Kr expression, indicating that the hb gradient positions this boundary independently.

Then we tested the ability of hb to define the anterior gt boundary in the absence of Kr by comparing gt expression in Kr^- embryos derived from *bcd* or *bcd osk* females (lower panel, right half). In both classes of embryos, early hb protein expression derives exclusively from maternal transcripts. However, in *bcd* embryos, expression of hb protein is down-regulated posteriorly under nos control (e.g., as in 2× embryos in Figure 2), whereas it persists throughout *bcd osk* embryos, owing to the absence of nos activity (e.g., as in *osk* embryos in Figure 2). As shown in Figure 3, the down-regulation of *hb* in *bcd* embryos is associated with posterior *gt* expression, whereas *gt* is completely repressed in *bcd osk* embryos where this down-regulation does not occur. More importantly, the same results are observed whether the embryos are Kr^+ or Kr^-. Hence, hb can repress gt expression and thereby define the anterior gt boundary irrespective of *Kr* gene activity.

This result does not eliminate the possibility that *Kr* gene activity may reinforce and stabilize the gt boundary in later embryos. Indeed, we observe a late change in gt expression in Kr^- embryos derived from *bcd* females that does not occur in their Kr^+ siblings: during the latter portion of nuclear cycle 14 (stages 5(2) and 5(3); Lawrence and Johnston, 1989), the anterior gt boundary shifts progressively anteriorly (e.g., compare gt expression in Kr^+ and Kr^- embryos derived from *bcd* females in Figure 3 in which a small shift is already apparent). Thus, although the anterior boundary of gt expression is initially positioned by the hb gradient, *Kr* gene activity is nevertheless required at a later time to maintain the position of the boundary.

Anterior kni Boundary

The posterior of the two kni domains normally falls between the central Kr and posterior gt domains, overlapping both but having distinct anterior and posterior boundaries (see Figure 1). We have tested the possibility that the hb gradient provides a distinct threshold that independently positions the anterior kni boundary by examining kni expression in embryos derived from *bcd tsl* females carrying 1, 2, or 4 copies of the *hb* gene. In the anterior third of these embryos, *Kr* is "on," whereas *gt* is "off," irrespective of whether they derive from females carrying 1, 2, or 4 copies of the *hb* gene (see Figure 2). In contrast, as shown in Figure 4, embryos derived from *bcd tsl* females with a single *hb* gene copy (1×) express readily detectable levels of kni protein anteriorly. However, barely detectable levels are observed in embryos from 2-copy females (2×), and no anterior expression is found in embryos from 4-copy females (4×). Thus, the hb gradient can provide a concen-

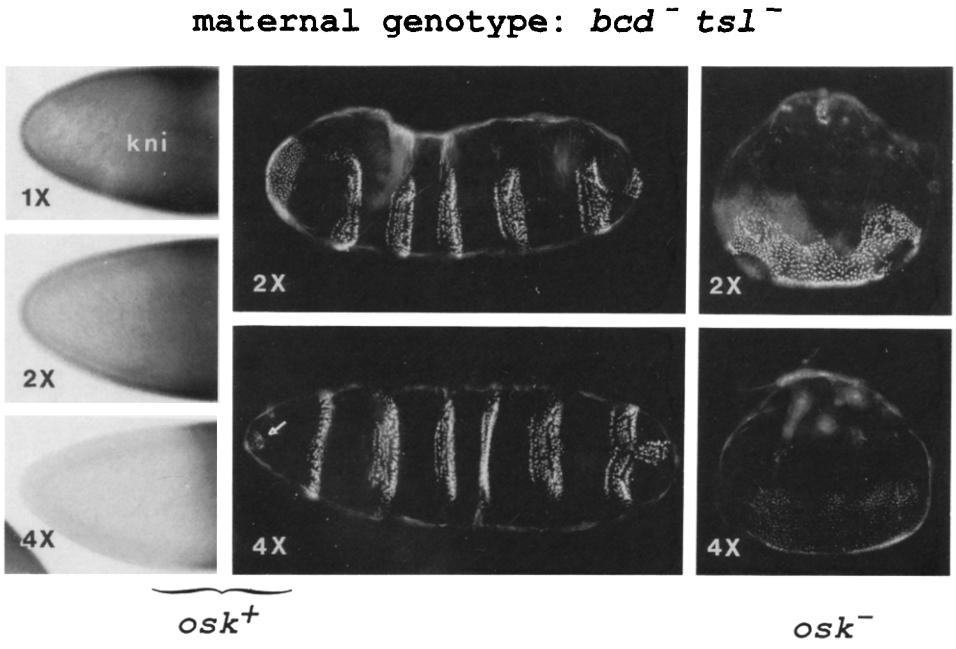

Figure 4. Control of kni Expression and Thoracic versus Abdominal Differentation by hb

The panel on the left shows kni protein expression at the anterior end of embryos derived from *bcd tsl* females carrying 1, 2, or 4 copies of the *hb* gene (1×, 2×, and 4×). Note that the level of kni protein declines progressively to undetectable levels as the maternal *hb* gene dosage is increased from 1 to 4 copies. High levels of kni protein can be seen accumulating in the middle portions of these embryos owing to the decline in maternally derived hb protein. As expected, the position of the interface between low and high kni expression, like that between anterior Kr and posterior gt expression (Figure 2), shifts posteriorly in response to increasing the maternal gene dosage. The middle panels show the segmentation patterns of pharate first instar larvae derived from *bcd tsl* females carrying 2 or 4 copies of *hb* (2×, 4×). Note that both larvae form a polarized series of several segments. In the 2× larva, all of these segments form bands of thick ventral hairs typical of normal abdominal segments; however, the anteriormost segment in the 4× larva (arrow) has formed a cluster of fine hairs characteristic of a thoracic segment. The panels on the right show larvae derived from embryos in which nos activity is also absent (owing to mutation in *osk*); note that these larvae have formed a lawn of unpolarized hairs lacking any overt sign of segmentation. Note also that the hairs are of the abdominal type when the mother carried 2 *hb* copies and of the thoracic type when the mother carried 4 copies.

tration threshold that independently positions the anterior kni boundary. This threshold is clearly distinct from the thresholds governing the neighboring Kr and gt boundaries.

Abdominal Segmentation

As shown above, the low end of the hb gradient is both necessary and sufficient to specify the orderly expression of Kr, kni, and gt in a series of overlapping domains. When hb protein is either not expressed or ubiquitously expressed, these genes respond homogeneously, either by being turned on or off throughout the body. Differential hb expression appears to be equally critical for abdominal patterning. Embryos derived from *bcd tsl* females carrying 2 copies of the *hb* gene make a polarized series of up to seven abdominal segments resembling the first seven abdominal segments of the wild-type larva (2×, Figure 4; see also Nüsslein-Volhard, 1991). However, when hb protein derived from two gene copies is expressed ubiquitously in embryos lacking *bcd* and *tor* function (e.g., owing to a block in nos activity caused by the *osk* mutation; 2×, Figure 4; see also Nüsslein-Volhard, 1991), or not at all (e.g., in embryos obtained from *tor; bcd hb nos* oocytes; data not shown; see Experimental Procedures), they give rise to an unpolarized lawn of abdominal hairs. Thus, it is the differential expression of hb protein that specifies abdominal pattern, presumably by its ability to generate spatially restricted patterns of *Kr*, *kni*, and *gt* gene expression.

Control by the bcd-Dependent Portion of the hb Gradient

As shown above, the nos-dependent portion of the hb gradient provides distinct thresholds that set the anterior gt and posterior Kr boundaries of expression. Moreover, the anterior boundary of kni appears to be dictated by a threshold concentration that is close to the maximal protein concentration normally derived from maternal *hb* transcripts. In the anterior half of the body, the concentration of hb protein increases far above this concentration, owing to zygotic activation of the *hb* gene under bcd control. In the experiments described below, we examined the role of the *bcd*-dependent portion of the hb gradient in specifying thoracic as opposed to abdominal differentiation and in positioning the anterior boundary of Kr expression.

Thoracic Differentiation

As described above, embryos derived from *bcd tsl* females develop a polarized series of up to seven abdominal segments resembling the first seven abdominal segments of the wild-type larvae. As shown in Figure 2, the concentration of hb protein expressed anteriorly in these embryos

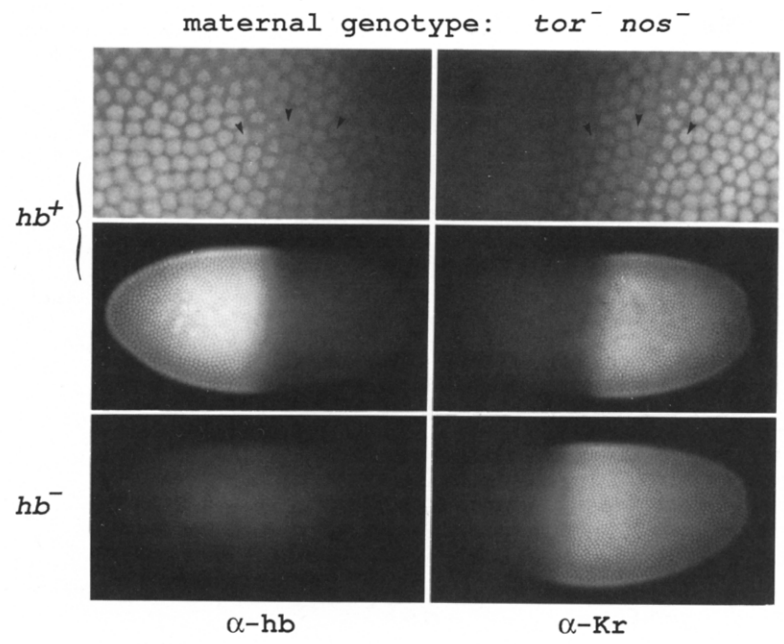

Figure 5. Zygotic hb Activity Plays a Significant Role in Defining the Anterior Kr Boundary

Sibling *hb*$^+$ and *hb*$^-$ embryos derived from *tor; hb nos/nos* mutant females are shown, double labeled, for hb and Kr protein expression. Both embryos are at the same stage of nuclear cycle 14 (stage 5(2)); at this stage, we can no longer detect hb protein expression derived from maternal *hb* transcripts by immunofluorescence. At higher magnification (top panel), the patterns of zygotically derived hb and Kr protein expression appear reciprocal (arrowheads mark the same nuclei in each micrograph). Note that the Kr boundary shifts anteriorly in the embryo lacking zygotic hb activity, but does not extend all the way to the anterior pole. The remaining restriction in Kr expression must be due to repression by other factors under bcd control, since it is not observed in the absence of *bcd* function (e.g., as in embryos derived from *bcd osk tsl* females, Figure 2).

can be increased by doubling the maternal gene dosage from 2 to 4. Under these conditions, we find that the anteriormost segment usually develops as a thoracic rather than an abdominal segment (e.g., as seen in the 4 × *osk*$^+$ embryo in Figure 4), suggesting that a 2-fold increase in the concentration of hb protein is sufficient to dictate a switch from abdominal to thoracic differentiation. We have tested this possibility by examining embryos derived from *bcd osk tsl* females carrying 2 or 4 copies of *hb*. As shown in the right column of Figure 4, embryos derived from 2-copy females (2 ×) differentiate a lawn of unpolarized abdominal hairs, while those derived from females carrying 4 copies (4 ×) differentiate a lawn of thoracic hairs. This 2-fold increase in the concentration of hb protein therefore appears to specify thoracic as opposed to abdominal differentiation. In wild-type embryos, this higher level of hb protein expression would require zygotic activation of the *hb* gene by bcd, indicating that the bcd-dependent portion of the hb gradient is responsible for specifying thoracic as opposed to abdominal differentiation. It is notable that the concentration threshold that dictates the choice between abdominal and thoracic differentiation coincides approximately with that required for completely repressing the *kni* gene (Figure 4).

Anterior Kr Boundary

To study the role of the bcd-dependent portion of the hb gradient in positioning the anterior Kr boundary, we have analyzed Kr expression in embryos lacking nos and tor activity. In these embryos, hb protein is expressed at high levels anteriorly (under bcd control) and at moderate levels posteriorly (owing to the absence of nos). As shown in Figure 5, Kr protein accumulates in these embryos in a reciprocal pattern, off anteriorly and on posteriorly. Moreover, the overlapping and opposite distributions of hb and Kr protein expression observed at high magnification (Figure 5, top panel) are consistent with the notion that the distribution of hb protein defines the anterior Kr boundary. We therefore asked whether the position of this boundary depends on zygotic hb activity. As shown in Figure 5, the anterior Kr boundary shifts anteriorly in *hb* mutant embryos obtained from *tor; nos* females, establishing such a role.

One simple hypothesis to account for the control of the anterior Kr boundary is that high levels of hb activity could block Kr expression, just as lower levels suffice to block kni and gt expression. We have tested this as follows. Embryos were obtained from females that are triply mutant for three genes: *vasa* (*vas*), *tor*, and *exuperantia* (*exu*). The *vas* mutation blocks *nos* activity and, hence, is equivalent to a mutation in *nos* itself (Lehmann and Nüsslein-Volhard, 1991). The mutation in the *exu* gene interferes with the normal localization of *bcd* transcripts at the anterior pole (Berleth et al., 1988); the delocalized transcripts give rise to a shallow gradient of bcd protein that spans the anteroposterior axis (Fronhöfer and Nüsslein-Volhard, 1987; Driever and Nüsslein-Volhard, 1988; Struhl et al., 1989). In embryos derived from *vas tor exu* females, this gradient becomes the sole determinant of anteroposterior pattern, owing to the absence of both nos and tor activity. As shown in Figure 6, hb protein is expressed at uniform and high levels in these embryos, from which we infer that the concentration of bcd protein is sufficiently high throughout the body to activate the *hb* gene maximally. Under the hypothesis above, Kr expression should be completely repressed in these embryos. However, the majority of these embryos express Kr protein posteriorly (Figure 6). Hence, the simple explanation that peak levels of hb protein suffice to block Kr expression is inadequate.

We next asked whether the position of the anterior boundary of Kr expression observed in *vas tor exu* embryos depends on the concentration of hb protein, as is the case for the Kr boundary in *tor; nos* embryos (Figure 5). As shown in Figure 6, this is indeed the case: the anterior

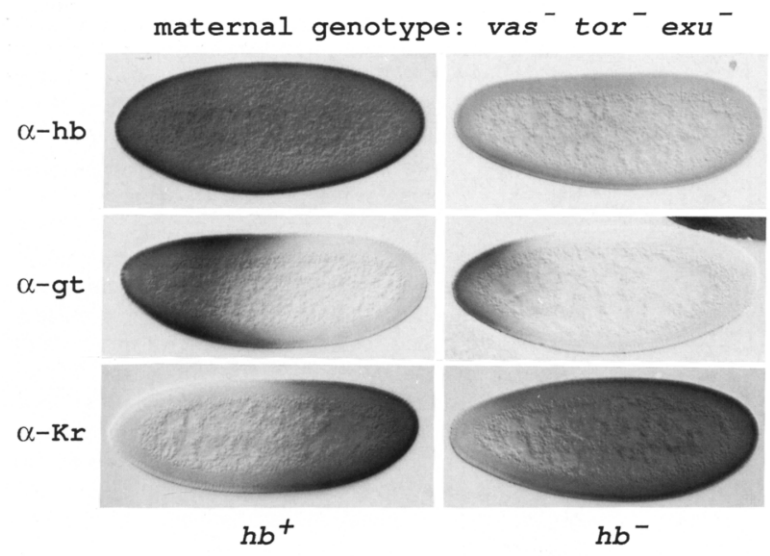

Figure 6. The Position of the Common Border between gt and Kr Expression in *vas tor exu* Embryos Depends on the Concentration of hb Protein

The patterns of hb, gt, and Kr protein expression are shown in hb^+ and hb^- embryos derived from *vas tor exu* mutant females. All of the embryos are at the same stage of nuclear cycle 14 (stage 5(2)). As shown in the top panel, the level of hb protein expression derived from maternal *hb* transcripts is close to the limit of detection at this stage, in contrast to the level of hb protein expression derived from zygotic transcripts, which is high. Note that in both cases, hb protein expression is uniform along the anteroposterior axis. Note also that the gt and Kr boundaries both shift anteriorly in hb^- embryos relative to hb^+ embryos. As described in Experimental Procedures, hb^- embryos were recognized as such by an independent marker. Some variation was observed in the patterns of both gt and Kr protein expression in these embryos. In particular, in hb^+ embryos, low levels of gt expression were sometimes detectable throughout the posterior half of the body, while, conversely, we sometimes failed to detect posterior Kr expression. We attribute this variation to small differences in the distribution of bcd protein, particularly its concentration posteriorly. The embryos shown exhibit patterns of protein expression representative of the majority of embryos of each genotype.

boundary of Kr expression shifts forward in these embryos in the absence of zygotic hb expression. Thus, it seems that the anterior Kr boundary is positioned in response to the distributions of at least two factors: the concentration of hb protein and at least one other factor that acts differentially in the anterior portion of the body.

A likely candidate for such a factor is gt protein, which is activated anteriorly under bcd control and which can repress *Kr* gene activity when ectopically expressed under the control of the *hsp70* promoter (Eldon and Pirrotta, 1991; Kraut and Levine, 1991a, 1991b). As shown in Figure 6, gt is activated in a broad anterior domain in embryos derived from *vas tor exu* females, presumably in response to the shallow gradient of bcd protein. Moreover, its pattern of expression appears to be reciprocal to that of Kr in sibling embryos. Finally, we observe that the position of the gt boundary depends on zygotic *hb* gene activity; when this activity is eliminated, gt expression shifts anteriorly in concert with the anterior shift in Kr expression (Figure 6). Thus, the anterior Kr boundary is not positioned simply by the decline in hb protein concentration from peak to intermediate levels. Instead, it may be set in response to the distribution of gt protein, which in turn depends on the distributions of both bcd and hb protein.

Discussion

The key attribute of a morphogen gradient is that the changing concentration of a single molecular species triggers a series of spatially distinct responses governing cell or body pattern (Dalcq, 1938; Turing, 1952; von Ubisch, 1953; Sander, 1959, 1960, 1975). The control of head and thoracic segmentation in Drosophila by the bcd protein provides a clear paradigm for such a gradient system. bcd protein has been shown to bind and activate the transcription of at least one target gene, *hb*, in a concentration-dependent fashion, thereby providing the means by which the bcd gradient controls where *hb* is expressed (Driever and Nüsslein-Volhard, 1989; Driever et al., 1989; Struhl et al., 1989). Moreover, the bcd gradient clearly has the instructional capacity to dictate other spatially distinct responses by the same mechanism (Driever et al., 1989; Struhl et al., 1989), and a number of potential target genes involved in head and thoracic differentation have been identified (Cohen and Jurgens, 1990; Dalton et al., 1989; Eldon and Pirrotta, 1991; Finkelstein and Perrimon, 1990; Kraut and Levine, 1991a, 1991b). Here we show that a second morphogen gradient controls many aspects of both thoracic and abdominal segmentation. The morphogen in this case is hb protein that is expressed as a gradient under the joint control of bcd and the posterior determinant nos (Figure 7). Our experiments demonstrate that the hb protein gradient controls posterior body pattern by providing several distinct thresholds that govern the domains of expression of the gap genes *Kr*, *kni*, and *gt*.

Generation of the hb Gradient by bcd and nos

bcd and nos play distinct, albeit overlapping, roles in generating the hb gradient. bcd is required for the upper end of the hb gradient, which, we show here, plays a significant role in positioning the anterior boundaries of Kr and kni expression and in dictating thoracic as opposed to abdominal differentiation. Conversely, nos is essential for the lower end, which we show governs abdominal segmentation by defining the posterior Kr and anterior kni and gt boundaries. Because both systems influence abdominal patterning by their ability to control the concentration of hb protein, it is possible to create abnormal conditions in which either system can partially or completely substitute for the other (Hülskamp et al., 1989; Irish et al., 1989; Struhl, 1989a; Figure 4). Nevertheless, in the context of

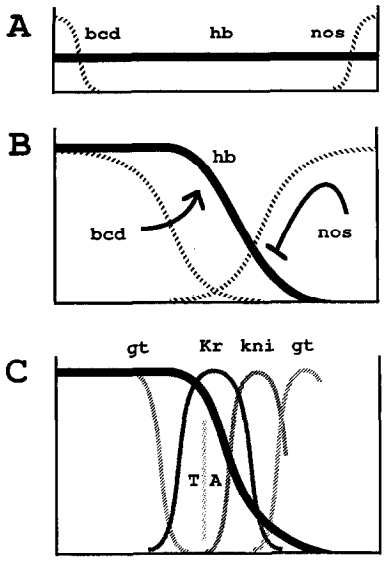

Figure 7. Generation and Function of the hb Morphogen Gradient

(A) *bcd* and *nos* maternal transcripts are tightly localized at the anterior and posterior poles of the unfertilized egg, in contrast to *hb* transcripts, which are ubiquitously distributed.

(B) Shortly after fertilization, *bcd* transcripts are translated, and the resulting protein diffuses posteriorly, generating a gradient; similarly, *nos* transcripts are thought to generate an opposing gradient of nos activity, presumably its encoded protein. bcd activates zygotic *hb* transcription anteriorly, whereas nos translationally represses *hb* transcripts posteriorly. As a consequence, hb protein accumulates differentially, declining in a graded fashion from high, uniform levels at the anterior end to undetectable levels at the posterior end.

(C) The hb protein gradient then provides a series of concentration thresholds that independently dictate where the anterior Kr, kni, and gt boundaries, as well as the posterior Kr boundary, are positioned in the posterior half of the body. The posterior kni boundary is governed in part by gt (and hence indirectly by hb) and by the terminal system, which also controls the posterior gt boundary. The domain of anterior gt expression (activated under bcd control) also depends on the concentration of hb protein. Finally, the hb gradient dictates the boundary between thoracic (T) and abdominal (A) differentiation, possibly by directly repressing bithorax complex gene activity.

wild-type development, they each play distinct and essential roles in generating the hb gradient.

hb as a Gradient Morphogen

Soon after the formation of the hb protein gradient, the *Kr*, *kni*, and *gt* gap genes are activated in a series of overlapping domains, each having distinct anterior and posterior boundaries located in the region of the body where hb protein expression declines from maximal to undetectable levels (e.g., Figures 1, 2, and 7). Prior studies suggested that the graded distribution of hb protein may influence the activity of these other gap genes (Hülskamp et al., 1990; Eldon and Pirrotta, 1991; Kraut and Levine, 1991a, 1991b). However, with the exception of the anterior kni boundary (Hülskamp et al., 1990), these studies failed to provide compelling evidence for a causal relationship between the changing concentration of hb protein and the spatial domains of expression of these target genes. Our experiments establish such a causal relationship for each of the three genes. Moreover, they show that each gene responds independently to the hb gradient.

In the case of the bcd gradient, as little as a 2-fold difference in the concentration of bcd protein appears sufficient to distinguish between on or off states of expression of its target genes (Struhl et al., 1989). This inference is based on the observation that successive 2-fold increases in *bcd* gene dosage shift the posterior boundaries of these target genes by an interval similar to that in which the expression of their products falls from peak to undetectable levels (discussed in detail in Struhl et al., 1989). A comparable relationship is also observed for the hb gradient: as the maternal gene dosage of *hb* is increased from 1 to 2 to 4, the anterior gt and posterior Kr boundaries shift by intervals of approximately 5%–10% egg length, similar to the interval in which the expression of each declines from maximal to undetectable levels for any given *hb* gene dosage. Hence, we conclude that relatively small differences in the concentration of hb protein are sufficient to distinguish between "all" or "none" states of subordinate gene expression.

A complicating issue in interpreting the role of the hb gradient is that the gap genes *Kr*, *kni*, and *gt*, once active, engage in extensive cross-regulation that generally tends to reinforce and stabilize the spatial relationships initially established under hb control (Jäckle et al., 1986; Gaul and Jäckle, 1989; Pankratz et al., 1989; Eldon and Pirrotta, 1991; Kraut and Levine, 1991b). For example, the hb gradient can initially define the anterior boundary of gt expression irrespective of *Kr* gene function (Figure 3). However, this boundary subsequently shifts anteriorly in the absence of Kr activity, reflecting a role for Kr in defining neighboring boundaries of gt expression (Figure 3; see also Eldon and Pirrotta, 1991; Kraut and Levine, 1991a, 1991b). This distinction between establishing and stabilizing the orderly expression of gap genes raises the question of when the hb gradient acts. As described previously (Tautz, 1988), the graded distribution of hb protein changes continuously from the first accumulation of protein prior to pole cell formation to the sharply defined bipartite pattern observed just prior to gastrulation. Localized *Kr*, *kni*, and *gt* transcription is first observed during nuclear cycles 11 and 12 (Knipple et al., 1985; Nauber et al., 1988; Eldon and Pirrotta, 1991; Kraut and Levine, 1991a), arguing that graded hb protein is active at this time, if not earlier. Conversely, hb protein derived from maternal transcripts is difficult to detect after the beginning of nuclear cycle 14, suggesting that from this stage on, the nos-dependent portion of the hb gradient is no longer a factor in sustaining the spatial relationships between zygotic hb, Kr, kni, and gt expression.

Cross-regulatory interactions may also allow the hb gradient to define additional boundaries of subordinate gene expression. For example, in embryos lacking both bcd and tor activity, the hb gradient specifies a tripartite pattern of kni expression (low anteriorly, high centrally, and low posteriorly; data not shown). The first boundary, between low anterior and high central expression, is governed by the progressive decline in hb protein concentration. However, the second boundary, between high central and low posterior expression, appears to depend on posterior gt expression, as it is eliminated in the absence of *gt* gene

activity (data not shown). Thus, the hb gradient appears to influence the posterior kni boundary indirectly by defining the gt boundary.

Finally, we note that there is some redundancy in function between the bcd and hb morphogen gradients. As shown previously by Hülskamp et al. (1990), bcd activity can suffice to activate low levels of *Kr* gene expression in the complete absence of hb protein, allowing *hb* mutant embryos to develop a few middle abdominal segments that they would otherwise not develop. However, the importance of this regulatory interaction is uncertain, because in the complete absence of bcd protein, the hb maternal gradient appears sufficient to activate Kr fully, to dictate the orderly expression of Kr, kni, and gt, and to generate normal abdominal pattern.

hb as Both an Activator and a Repressor of Transcription

A large body of evidence indicates that hb is a transcriptional regulator that directly binds defined DNA targets and activates or represses gene expression as a consequence (Tautz et al., 1987; Stanojevic et al., 1989; Treisman and Desplan, 1989; Small et al., 1991; Hoch et al., 1991; Qian et al., 1991). It therefore seems reasonable to propose that hb, like bcd, controls posterior body pattern by acting as a concentration-dependent transcriptional regulator. However, all of the known and presumed targets of bcd action appear to be activated in response to bcd protein, whereas in at least two clear cases, *gt* and *kni*, the response to graded hb protein appears to be transcriptional repression. We therefore suggest that these genes are initially turned "on" by one or more ubiquitous transcriptional activators (see also Kraut and Levine, 1991b) and that the hb protein antagonizes these activators by interfering either with their binding to the DNA or with their interactions with other components of the transcriptional machinery.

In contrast, we also show that hb behaves as an activator of *Kr* transcription. Although this possibility was initially suggested by the experiments of Hülskamp et al (1990), the directness of the interaction was subsequently called into question by the observation that hb can repress *gt*, which in turn can repress *Kr* (Eldon and Pirrotta, 1991; Kraut and Levine, 1991a, 1991b). Although our experiments do not demonstrate that hb directly activates *Kr* gene expression, they do show that the activation is not mediated indirectly by blocking repression by gt. In the absence of other candidate repressors that might serve such an intermediate role, we suggest that hb functions directly as a transcriptional activator as well as a repressor.

The mode of action of hb in defining the anterior Kr boundary is more complex. Peak levels of hb protein expression are not sufficient to repress Kr expression, although they appear to influence regulatory relationships between bcd, gt, and Kr. As described previously, bcd activates gt, while Kr and gt appear to engage in a relationship of mutual repression (Eldon and Pirrotta, 1991; Kraut and Levine, 1991a, 1991b). Although the mechanism is unknown, the regulatory balance between these factors appears to depend on the concentration of hb protein, providing the means by which the hb gradient positions the boundary between anterior gt and central Kr expression.

Control of Bithorax Complex Gene Expression by the hb Gradient

Regional differentiation of the thoracic and abdominal segments is controlled in large part by the selective activity of the bithorax complex (Lewis, 1978; Struhl, 1981), the distinction between thoracic and abdominal differentiation depending primarily on the *Ultrabithorax* (*Ubx*) gene. Hence, our finding that a 2-fold difference in the concentration of hb protein is sufficient to cause a discrete switch between abdominal and thoracic differentiation (Figure 4) raises the possibility that the anterior boundary of *Ubx* transcription is controlled directly by the ability of hb protein to bind and repress transcription of the gene. Indeed, Qian et al. (1991) identified a cis-acting *Ubx* enhancer that contains several hb DNA-binding sites and can direct an early *Ubx*-like pattern of expression, the anterior boundary of which depends on repression by zygotic hb activity. Perhaps the hb protein similarly defines the anterior boundaries of expression of the remaining bithorax complex genes, *abdominal-A* and *abdominal-B*, as suggested previously (White and Lehmann, 1986), and, hence, constitutes the graded repressor initially proposed by Lewis to control the differential activation of the bithorax complex (1978). The *hb* gene was initially identifed by Lewis as the *Regulator of postbithorax* gene because he obtained mutations that interfered with normal bithorax complex activity (Lewis, 1968). The existence of other unusual *hb* alleles, which cause dramatic defects in *Ubx* expression distinct from their effects on segmentation (White and Lehmann, 1986; Lehmann and Nüsslein-Volhard, 1987), provides additional evidence that hb protein interacts directly with the bithorax complex, instead of operating solely through its action on Kr, kni, and gt.

Cascading Gradients

In wild-type embryos, the bcd gradient is thought to control most aspects of head and thoracic pattern by directly regulating several subordinate genes. However, in the abnormal situation in which maternal *hb* transcripts are inactivated by mutation, the bcd gradient can direct the development of abdominal, as well as head and thoracic pattern, even though its realm of direct action is apparently limited to the anterior half of the body (Hülskamp et al., 1989, 1990; Irish et al., 1989; Struhl, 1989a). As shown here, it does so by activating high levels of a second gradient morphogen, hb, at the extreme posterior limit of its effective range. The resulting hb gradient in turn extends further posteriorly, triggering a series of additional responses, one of which, the differential repression of gt, generates a third gradient influencing yet more responses (e.g., the posterior boundary of kni; see above). Thus, bcd can organize the global pattern by generating additional morphogen gradients that operate in regions outside its immediate realm of action. The ability of bcd to organize the body plan by triggering a series of such gradients may exemplify how a single, spatially restricted morphogen can control global pattern.

Experimental Procedures

Control of the Posterior Kr and Anterior gt Boundaries by hb

Females of the following four genotypes were generated by conventional genetic crosses:

bcd^{E1} hb^{FB} tsl^{035}/bcd^{E1} tsl^{035}.
bcd^{E1} tsl^{035}.
HB547#4 HB547#6/CyO; bcd^{E1} tsl^{035}.
bcd^{E6}osk^{166} tsl^{035}.

Except where stated otherwise, only mutations directly relevant to the experiments are indicated (see Lindsley and Zimm, 1985, 1986, 1987, 1988 and references therein for descriptions of mutant alleles and balancers used). The *bcd^{E6}* mutation is an in-frame deletion of a portion of the homeodomain and acts as an amorph (Struhl et al., 1989). *HB547#4* and *HB547#6* are independent P element–mediated insertions of a 4.7 kb Bam fragment of *hb* genomic DNA into the second chromosome. This fragment contains genomic DNA including the *hb* coding sequence and its associated maternal and *bcd*-dependent regulatory sequences; it can rescue most aspects of the *hb* mutant phenotype, though it does not appear to include regulatory sequences relevant to activation of the *hb* gene under the control of the terminal system (Tautz et al., 1987; Schröder et al., 1988; Struhl et al., 1989; Hülskamp et al., 1989). In these experiments, the *HB547* insertions behave as extra copies of the maternal *hb* gene function.

Embryos aged 0–4 hr after egg laying were obtained from females of each genotype and fixed and stained in parallel for hb, gt, and Kr expression using standard immunohistochemical procedures (Macdonald and Struhl, 1986; see also below; rat α-hb, rabbit α-Kr, and rabbit α-gt antisera were provided by P. M. Macdonald, M. Levine, and V. Pirrotta, respectively). When stained for hb expression, embryos derived from the first three genotypes show a gradient of hb expression apparent as early as nuclear cycle 8 and persisting until the beginning of nuclear cycle 14 (stage 5(1); see Lawrence and Johnston [1989] for staging during nuclear cycle 14). The concentration of hb protein increases proportionally as the number of maternal copies of the gene rises from 1 to 2 to 4 (1×, 2×, and 4× in Figure 2; see below). Embryos derived from *bcd^{E6} osk^{166} tsl^{035}* females show ubiquitous hb expression during the same period (*osk* in Figure 2). These embryos were mixed, fixed, and stained together with wild-type embryos (from which they can be distinguished by the absence of pole cells) to control for the staining reaction. An example of such a control can be seen in Figure 2, which shows Kr staining in a *bcd osk tsl*–derived embryo (note the patterned expression of Kr in the neighboring wild-type embryo [lower right corner]).

We also find, unexpectedly, that hb is activated during the latter portion of nuclear cycle 14 (beginning during stage 5(2)) in embryos derived from females of the first three genotypes. This late expression occurs at the posterior pole and appears to depend on *nos* because it is absent in embryos derived from *bcd^{E6} osk^{166} tsl^{035}* females.

Although the concentration of hb protein can clearly be seen to be proportional to the maternal gene dosage in the anterior halves of 1×, 2×, and 4× embryos (e.g., Figure 2), it is not readily apparent from simple inspection that a similar proportional relationship exists in the posterior half of the body where hb protein expression is downregulated by *nos*. To assess this quantitatively, we have directly measured the position at which hb protein expression falls beneath the level of detection in embryos obtained from females of each genotype (the measurements were performed using a Zeiss axioplan microscope [bright field optics] equipped with a graticule; 15 embryos in nuclear cycles 11 and 12 were scored for each genotype). We observed that the boundary shifted from 39% to 35% to 28% egg length (measured from the posterior pole) in 1×, 2×, and 4× embryos, respectively, indicating a proportional increase in the concentration of hb protein at any given position along the body. Because of the difficulties in recording low levels of protein expression photographically, these direct measurements provide a more accurate reflection of the extent of the hb protein gradient than that apparent in the optical cross sections shown in Figure 2. Note, however, that the hb gradient can be seen to extend approximately two-thirds of the way down the body in the wild-type embryo shown in Figure 1 (in which the plane of focus is on the surface of the embryo), consistent with our measurements.

Because the boundaries of Kr and gt protein expression are graded rather than sharp, we measured the position at which the amount of protein begins to decline from peak levels as well as the position at which it falls beneath the level of detection and recorded the halfway point in between. In general, both proteins fell from peak to undetectable levels of expression within 10% egg length. For each boundary determination given in the legend of Figure 2, we examined 12 embryos at stage 5(2).

To generate embryos completely lacking early hb protein expression as well as *bcd* and *tor* function (0× in Figure 2), chimeric females carrying *tor^{RX}; bcd^{E1} hb^{FB}*germ cells were obtained by pole cell transplantation. Agametic female host embryos were generated by crossing wild-type females to *OvoD^{1}/Y* males. Donor embryos were obtained as the progeny of *tor^{RX}; bcd^{E1} hb^{FB}/TM2, tor^{+}* males and females. The *TM2, tor^{+}* balancer chromosome was obtained by P element–mediated insertion of an 11–12 kb EcoRI fragment carrying the intact *tor^{+}* gene (Casanova and Struhl, 1989). The *tor^{RX}*, *bcd^{E1}*, and *hb^{FB}* mutant alleles were chosen because they appear to be protein nulls; identical results were also obtained in preliminary experiments using the *tor^{PM}*, *bcd^{E1}* and *hb^{14F}* mutant alleles, which behave genetically as amorphs, although the *tor^{PM}* and *hb^{14F}* alleles encode antigenically detectable protein products (Casanova and Struhl, 1989; Tautz, 1988). Chimeric *OvoD^{1}/+* females carrying mutant germlines were initially identified because they laid eggs that did not hatch; the identification was then confirmed by mounting and inspecting pharate first instar larvae. Embryos derived from mutant germ cells differentiated a characteristic cuticular pattern of a large lawn of unpolarized abdominal denticles, followed posteriorly by a small lawn. This polarity in the cuticular pattern was unexpected because the only remaining determinant system operating in these embryos is *nos*, and its only known targets, *bcd^{E1}* and *hb^{14F}* maternal transcripts (Wharton and Struhl, 1989, 1991; Hülskamp et al., 1990), cannot encode protein. To test whether this polarity is generated by the action of *nos* on some other target molecule, we examined embryos derived from chimeric females carrying *tor^{RX}; bcd^{E1} hb^{FB} nos^{L7}* germ cells (in this case, the donor embryos were obtained as the progeny of *tor^{RX}; bcd^{E1} hb^{FB} nos^{L7}/TM2, tor^{+}* males and females). These embryos differentiated a single lawn of unpolarized abdominal hairs similar to that formed by embryos derived from *bcd^{E6} osk^{166} tsl^{035}* females (Figure 4), confirming that the polarity observed in embryos derived from *tor^{RX}; bcd^{E1} hb^{FB}* germ cells is *nos* dependent.

Females carrying *tor^{RX}; bcd^{E1} hb^{FB}* germlines were then pooled, and their embryos were fixed and stained by immunohistochemical techniques for hb, Kr, and gt. As expected, these embryos do not express any hb protein up until nuclear cycle 14 (0× in Figure 2); however, as in the case of embryos derived from *bcd^{E1} tsl^{035}* females carrying between 1 and 4 copies of the *hb* gene, we find that *hb* is activated in the vicinity of the posterior pole during the latter portion of nuclear cycle 14 (beginning during stage 5(2)). This late expression may account for the polarized cuticular pattern formed by these embryos. To control for vagaries in fixation and staining, similarly aged embryos derived from *bcd osk* mutant females were included along with the experimental embryos throughout the fixation and staining procedure. These control embryos could be easily distinguished from the experimentals because they lacked pole cells.

Independent Control of the Posterior Kr and Anterior gt Expression Boundaries

To test whether the hb gradient can define the posterior boundary of Kr expression independent of *gt* gene activity, *Df(1)62g18, gt^{-}/+; bcd^{E1} tsl^{035}* females were crossed to wild-type males, and their progeny were double stained for Kr and gt protein expression by immunohistochemistry as follows. After standard fixation, the embryos were incubated in MeOH containing 3% H_2O_2 for 15 min (Kellerman et al., 1990), washed briefly in MeOH, stained for Kr expression by standard immunohistochemical procedures using a horseradish peroxidase–conjugated goat α-rabbit antiserum and the horseradish peroxidase signal developed using the conventional diaminobenzidine color reaction, which generates an orange-brown signal. The embryos were then incubated in 0.2 M glycine–HCl (pH 2.5) containing 0.1% Triton X-100 to strip embryos of the initial antiserum (Kellerman et al., 1990) and subjected to a second round of antibody staining using the rabbit α-gt antiserum followed by the horseradish peroxidase–conjugated goat α-rabbit antiserum. The embryos were then stained again using the

diaminobenzidine color reaction, this time supplemented with nickel and cobalt ions (Lawrence and Johnston, 1989) to produce a blue-gray as opposed to an orange color.

To test whether the hb gradient can define the anterior boundary of gt expression independent of *Kr* gene activity, Kr^1/+; bcd^{E1} and Kr^1/+; bcd^{E6} osk^{166} females were generated by standard genetic means, crossed to Kr^1/+ males; their progeny were fixed, pooled together with wild-type embryos, and double stained for gt and Kr protein expression by immunohistochemistry as described above, except that gt staining was developed using the standard horseradish peroxidase substrate to yield an orange-brown reaction product and Kr staining was obtained using an alkaline phosphatase–conjugated goat α-rabbit antiserum and the Vector Labs "black" substrate kit, which generates a purple signal. The embryos shown in Figure 3 are representative, except that we occasionally observed thin stripes of gt staining on either side of the central Kr domain in Kr^+ embryos derived from bcd^{E1} osk^{166} females and on the anterior side of the central *Kr* domain in Kr^+ embryos derived from bcd^{E1}. However, these thin stripes of gt staining were never observed in sibling Kr^- embryos. We note that Kraut and Levine (1991a) reported similar results to those obtained by us: they were unable to detect gt expression in embryos derived from Kr^1/+; nos^{L7} females outcrossed to Kr^1/+ males, even though one-quarter of the progeny should lack *Kr* gene activity. However, in the absence of a positive control for gt staining, and, particularly, given that these authors also report observing posterior gt expression in a significant portion of Kr^+ embryos derived from nos^{L7} females in separate experiments, we do not regard their negative results as compelling.

Control of the Anterior kni Boundary by hb

Females of the following five genotypes were generated by conventional genetic crosses:

bcd^{E1} hb^{FB} tsl^{035}/bcd^{E1} tsl^{035}.
bcd^{E1} tsl^{035}.
HB547#4 HB547#6/CyO; bcd^{E1} tsl^{035}.
bcd^{E6} osk^{166} tsl^{035}.
HB547#4 HB547#6/CyO; bcd^{E6} osk^{166} tsl^{035}

Embryos derived from these females were fixed and stained in parallel for kni protein expression as described above using a rat α-kni antibody provided by J. Dubnau. Cuticles of pharate first instar larvae were mounted for compound microscopy in a mixture of 1:1 Hoyer's mountant:lactic acid (Struhl, 1984).

Control of the Anterior Boundary of Kr Expression by bcd-Dependent Zygotic Activity of hb

Females of the following three genotypes were prepared by standard genetic crosses:

tor^{RX}; hb^{FB} nos^{L7}/nos^{L7}.
vas^{PD} tor^{WK} exu^{PJ}.
vas^{PD} tor^{WK} exu^{PJ}; hb^{FB}/TM3, hb-β-gal.

The *TM3, hb-β-gal* balancer was obtained by using P element–mediated transformation to insert a *hb-β-gal* fusion gene onto a conventional *TM3, ri* p^p Sb Ser e balancer; the *hb-β-gal* fusion gene consists of three copies of the *bcd*-dependent regulatory region of the *hb* gene (−260 to −60 relative to the *hb* transcriptional start; see Struhl et al., 1989) placed in front of an *hsp70-β-gal* reporter gene, *HZ50PL*, which includes the *hsp70* TATA box, but lacks the heat shock–dependent regulatory elements (Hiromi and Gehring, 1987). β-gal expression derived from this gene is *bcd* dependent and can be detected by immunoreactivity as early as the beginning of nuclear cycle 14 (beginning of stage 5(1), approximately 60 min before the onset of gastrulation).

In the initial experiment (Figure 5), embryos aged 1–4 hr after egg laying were obtained from tor^{RX}; hb^{FB} nos^{L7}/nos^{L7} females crossed to hb^{FB}/+ males and then fixed and double stained for hb and Kr protein expression according to standard immunofluorescence procedures (e.g., Macdonald and Struhl, 1986); the rat α-hb antiserum and rabbit α-Kr antiserum were visualized using appropriate rhodamine and fluorescein secondary antibodies, respectively. In subsequent experiments (Figure 6), similarly aged embryos were derived from vas^{PD} tor^{WK} exu^{PJ} females crossed to wild-type males and from vas^{PD} tor^{WK} exu^{PJ}; hb^{FB}/TM3, hb-β-gal females crossed to hb^{FB}/TM3, hb-β-gal males. The embryos were then fixed and processed in parallel to detect both β-gal protein and either gt or Kr protein using the double-labeling immunohistochemical procedures described above (the second signal was generated using either a horseradish peroxidase–conjugated secondary antibody followed by the diaminobenzidine color reaction supplemented with nickel and cobalt ions, or an alkaline phosphatase secondary antibody followed by the Vector "black" substrate kit; similar results were obtained with both staining protocols. A rabbit α-β-gal antiserum from Cappel was used to detect β-gal protein expression.) In addition, sibling embryos were stained for hb expression as described above.

Acknowledgments

We thank J. Casanova, J. Dubnau, K. Howard, and R. Wharton for discussion, P. Fazio-Miceli and A. Nakanishi for technical assistance, J. Dubnau, M. Levine, P. M. Macdonald, and V. Pirotta for antibodies, W. Driever, M. Hülskamp, V. Pirrotta, T. Schupbach, and D. Tautz for stocks, and R. Axel, J. Dubnau, T. Jessell, and R. Wharton for comments on the manuscript. This work was supported by Howard Hughes Medical Institute, the McKnight Foundation for Neuroscience, and the Medical Research Council of Great Britain.

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Received October 15, 1991; revised February 6, 1992.

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