Current Biology, Vol. 15, 1888–1898, November 8, 2005, ©2005 Elsevier Ltd All rights reserved. DOI 10.1016/j.cub.2005.09.046

Bicoid Determines Sharp and Precise Target Gene Expression in the *Drosophila* Embryo

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Summary

Background: The activity of the Bicoid (Bcd) transcription factor is a useful example of how quantitative information contained in a smooth morphogen gradient is transformed into discrete and precise patterns of target gene expression. There are two distinct and important aspects to this process: the "sharpening" of the posterior borders of the expression domains and the "precision" of where the target genes are expressed along the length of the embryo as the syncytial embryo begins to cellularize. Although the sharpening phenomenon was observed over a decade ago, it is still poorly understood.

Results: Here, we show that a Bcd reporter gene containing binding sites only for Bcd is expressed, like natural targets of Bcd, in a precise domain with a sharp boundary. Analysis of embryos expressing deleted forms of Bcd indicates that the sharpness of the Bcd target gene *hunchback*'s expression involves the glutamine-rich and C-terminal activation domains of Bcd. Furthermore, several artificial Gal4-derived transcription factors expressed as gradients in the embryo share Bcd's ability to drive precise target gene expression with sharp boundaries.

Conclusion: Thus, contrary to recent reports proposing that the Bcd gradient is not sufficient to establish precise positional information, we show that Bcd drives precise and sharp expression of its target genes through a process that depends exclusively on its ability to activate transcription.

Introduction

The involvement of gradients in pattern formation was first postulated by Morgan more than a century ago [1] and their importance revisited by Wolpert in the 1970s, when he proposed the French flag model for the functioning of a morphogen gradient [2]. According to this model, cells acquire distinct fates during development by responding to different thresholds of the morphogen in different ways [1, 2]. The first molecular evidence for the existence of such morphogen gradients was provided in 1988 by Driever and Nüsslein-Volhard, when they reported that a gradient of the transcription factor Bicoid (Bcd) was involved in development of the anteroposterior (A-P) axis of the *Drosophila* embryo [3, 4].

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The *bcd* mRNA is produced during oogenesis and anchored at the anterior pole of the embryo, where it is translated soon after the egg is laid. Unlike its mRNA, the Bcd protein can diffuse along the A-P axis, giving rise to a concentration gradient with its highest point at the anterior pole [3]. Bcd is a homeodomain-containing transcription factor that becomes active in the embryo when zygotic transcription begins about 1.5 hr postfertilization and egg laying [4–6]. High levels of Bcd promote the expression of genes such as *orthodenticle* (*otd*) in the most anterior part of the embryo, whereas lower levels allow expression of *hunchback* (*hb*) in the entire anterior half of the embryo (Figure 1). Molecular approaches have shown that the Bcd gradient behaves as predicted by Wolpert's French flag model. (1) Functional binding sites for Bcd, which differ in their number and affinity for the protein, are found in the regulatory regions of target genes [7, 8]. (2) Reporter genes placed under the control of low-affinity Bcd binding sites are expressed more anteriorly in the embryo than reporters placed under the control of high-affinity binding sites [6, 7]. (3) Increasing the amount of Bcd in the embryo by inserting additional copies of the *bcd* gene in transgenic females shifts the entire fate map of the embryo toward the posterior pole [4], as indicated by a shift in the expression of all the Bcd target genes tested [4, 6, 9, 10]. This Bcd-driven positional information is subsequently ensured along the A-P axis through the interplay of Bcd and some of its direct target gene products such as the Hb, Krüppel (Kr), and Giant proteins, which are themselves transcriptional activators and repressors [11, 12]. So, the establishment and interpretation of positional information by Bcd are closely related processes in the early embryo [13].

Recent studies, however, have questioned whether Bcd alone is sufficient to provide precise positional information along the A-P axis [14, 15]. Quantitative immunofluorescence microscopy indicated great variability in the position of the Bcd gradient along the A-P axis: a given level of Bcd was found at positions that varied by up to 30% of the egg length (EL) among a sample of embryos. By contrast, the same type of detection indicated that the position of the posterior border of Hb expression varied by only 4% of the EL. Because the precision of the *hb* target gene's expression was apparently far superior to the precision of morphogen expression, these observations suggested that the French flag model does not hold true for Bcd. Houchmandzadeh et al. proposed that another system must exist to filter out the "noise" associated with the variability of the Bcd gradient and establish precise expression of Bcd's targets. They found that the precise positioning of *hb* expression depends on another gene, *staufen* (*stau*), the product of which is involved in intracellular RNA localization and, in particular, in the anchoring of both *bcd* and *oskar* mRNAs at the poles of the embryo [16, 17]. Because most of Bcd's targets are also targets for the Hb protein [18], it was proposed that the precise positioning of *hb* expression by Stau

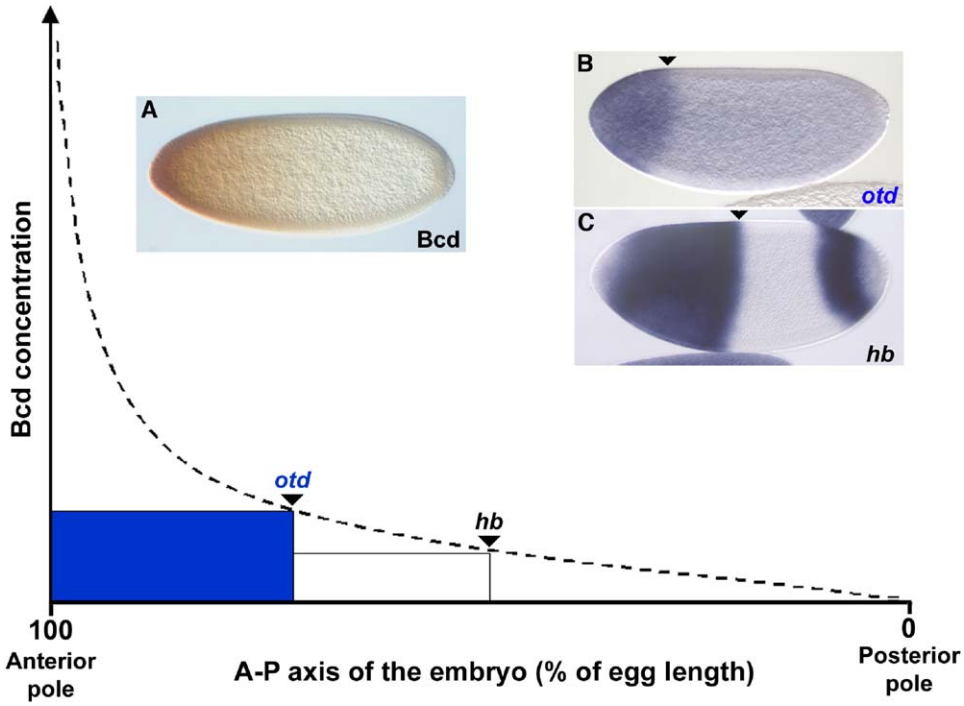

Figure 1. The Bcd Morphogen in the Blastoderm Embryo and the French Flag Model

The Bcd gradient with its highest point at the anterior pole revealed by antibody staining (A) and expression of the two Bcd target genes, *otd* (B) and *hb* (C), revealed by in situ hybridization performed with antisense probes. The position of the posterior border of expression is indicated with arrowheads. The concentration gradient of the Bcd protein (arbitrary units) is indicated on the graph by a dotted line as a function of the percentage of EL. According to the French flag model, the two target genes, *otd* (blue) and *hb* (white), are expressed at concentrations of Bcd above a specific threshold indicated by the arrowheads. Note that the posterior expression of *hb* is not dependent on Bcd. Anterior is at the left.

might contribute to establish precise expression of Bcd targets [14, 15].

To determine how the information contained in the smooth gradient of Bcd is transformed into precisely sized domains of expression with sharp boundaries, we analyzed the sharpness of the posterior border and the precision of expression of three direct target genes of Bcd: *hb*, *otd* [8], and *Bcd3-lacZ*, a synthetic Bcd reporter gene [10]. We find that the presence of Bcd binding sites in a target promoter is sufficient for its sharp expression. We identify two activation domains of Bcd important for the sharpening of *hb* and show that artificial Gal4-derived transcription factors expressed, like Bcd, as maternal gradients in the early *Drosophila* embryo also drive sharp expression of Gal4 reporter genes. Interestingly, *otd*, the Bcd-dependent and the Gal4-dependent reporters are expressed as precisely as *hb* in wild-type embryos, and this precision is reduced in a $stau^{HL}$ mutant background. The effect of Stau is observed on all target genes tested and thus occurs upstream or at the level of the transactivation process. Bcd is thus sufficient to determine sharp and precise expression of its target genes through a process that depends only on its ability to activate transcription. We finally show that the *bcd* mRNA distribution pattern is much more variable in embryos from $stau^{HL}$ females than in wild-type embryos and propose that the effect of the $stau^{HL}$ mutant background on the precision of Bcd activity occurs through the anchoring of the *bcd* mRNA at the anterior pole of the embryo.

Results

Bcd Is Sufficient for the Sharpening of the Posterior Border of Its Target Genes

To gain insights into Bcd's morphogenic activity, we analyzed the expression of its direct natural targets *hb* and *otd*, and a synthetic Bcd reporter gene, *Bcd3-lacZ* [10]. This reporter places *lacZ* under the control of a simple promoter containing three strong artificial Bcd binding sites and no known binding site for other transcription factors. It allows the intrinsic ability of Bcd to activate transcription to be detected in vivo [19, 20]. In situ hybridization indicated that the expression patterns of these three target genes vary substantially during the first half of cellularization (Figure 2). Using Nomarski microscopy, we classified embryos into two stages based on the position of their basal membrane: early embryos were blastoderm embryos that had not started cellularization yet, and midstage embryos were embryos that had started but not reached 50% of the process. In early embryos, the three target genes, *hb*, *otd*, and *Bcd3-lacZ*, were expressed in anterior domains of different sizes that had a diffuse posterior border, their expression diminishing over several nuclei (Figures 2A–2C, brackets). During the first half of cellu-

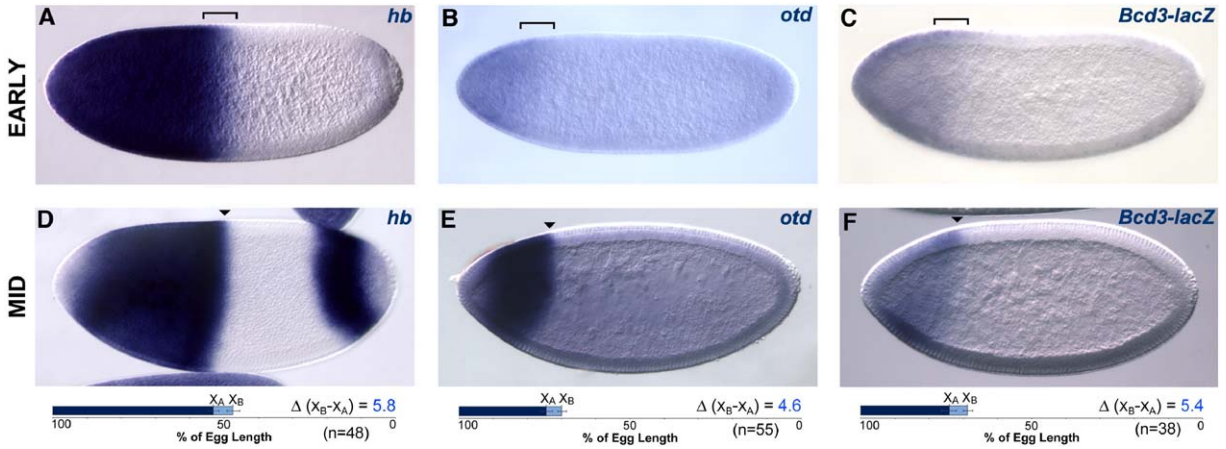

Figure 2. The Shape of the Posterior Border of Bcd Targets Is Modified during Blastoderm Development

In situ hybridizations were performed on wild-type early-stage (A–C) and midstage (D–F) embryos with *hb* (A and D), *otd* (B and E), and *lacZ* (C and F) antisense probes. Embryos carried the *Bcd3-lacZ* transgene (C and F). Diffuse borders of expression in early embryos are indicated by brackets. Sharp borders of expression in mid embryos are shown with arrowheads. (D–F) Sharpening was measured (see Figure S1 and Supplemental Experimental Procedures) on 30 to 50 (n) midstage embryos, and average measurements are indicated at the bottom. Anterior is at the left.

larization, however, the posterior boundary of *hb* and *otd* extended to not more than one or two nuclei and appeared much sharper than in early embryos (Figures 2D and 2E, arrowhead). We quantified the sharpness of the expression domains (i.e., the distance between the most posterior position of highest expression and the most anterior position of lowest expression) as a proportion of EL along the A-P axis (see Figure S1 and the Supplemental Experimental Procedures available with this article online). We found that this distance was 5.8% EL for *hb* and 4.6% EL for *otd*. We observed a similar sharpness of expression (5.4% EL) for *Bcd3-lacZ* (Figure 2F, arrowhead and Figure S1), indicating that sharpening clearly occurs when the promoter lacks binding sites for specific transcription factors other than Bcd. We conclude that Bcd binding sites are sufficient in a promoter to induce the sharpness of its expression domain posterior border.

The Sharpness of the *hb* Posterior Border Is Reduced in *bcd* Mutants

The existence of transgenic flies expressing deleted versions of the *bcd* gene [21] offered the opportunity to identify domains of Bcd involved in the sharpening of *hb* expression. We performed in situ hybridization on midstage embryos expressing these truncated versions of Bcd and lacking wild-type Bcd. In most cases, no change in the shape of *hb* posterior border was observed (not shown). Interestingly, however, when either the C (*Bcd-ΔC*) or both Q and C (*Bcd-ΔQC*) activation domains of Bcd (Figure 3) were deleted [20], the *hb* border was much more diffuse than normal (see brackets in Figures 3B and 3C). Quantitative measurements indicated that the sharpness of the *hb* posterior border was 13.6% EL in *Bcd-ΔC* embryos and 19.6% EL in *Bcd-ΔQC* embryos (compare Figures 3B and 3C; Figure S2).

Because the reduction *hb* posterior border sharpening in the *Bcd-ΔQC* and *Bcd-ΔC* lines might be due to the intrinsic anterior shift of the whole embryo's fate map in these backgrounds (compare Figure 2D with Figures 3B and 3C), we analyzed the shape of the *hb* posterior border in embryos from females homozygous for bcd^{E3}. This hypomorphic allele of *bcd* carries a point mutation in the homeodomain and induces also a significant shift of the whole fate map of the embryo toward the anterior (Figure 3D). It encodes a Bcd mutant protein that is affected in DNA binding rather than in transcriptional activation [6]. In embryos from bcd^{E3} females, the posterior border of *hb* sharpened over only 9.3% EL despite its even more anterior position than in the *Bcd-ΔQC* and *Bcd-ΔC* embryos (Figures 3D and S2). Together, these observations indicate that there is no correlation between the strength of the reduction in the sharpening of *hb* posterior border and the magnitude of the anterior shifts of fate map indicative of a weaker Bcd activity. These reductions of sharpening are therefore rather due to specific defects in the activities of the deleted forms of the Bcd proteins themselves.

Gradients of Transcription Factors Produce Sharp Borders of Target Gene Expression

To determine whether the reduction of *hb* sharpening observed in the *Bcd-ΔC* and *Bcd-ΔQC* backgrounds was due to the lack of specific sequences or to the lack of transcriptional activities carried by the deleted sequences, we took advantage of transgenic flies expressing Gal4-derived transcription factors in Bcd-like maternal gradients. These proteins activate transcription and some of them bear no other structural or functional similarities to Bcd [20]. Among them were Gal4-2Q, in which the Gal4 DNA binding domain is fused to two copies of the glutamine-rich (Q) domain of Bcd, and Gal4-3GCN4, in which the Gal4 DNA binding domain is fused to three copies of the yeast GCN4 activation domain. We asked whether these gradients of

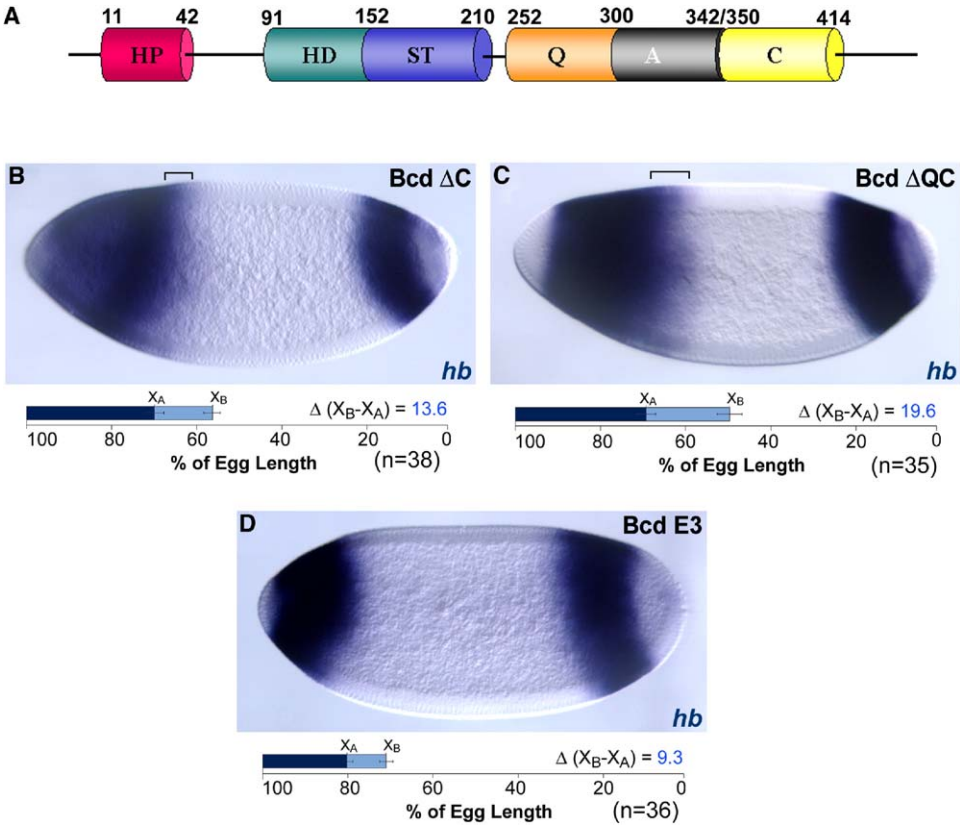

Figure 3. The Sharpness of the *hb* Posterior Border Is Reduced in the *bcd-⊿C* and *bcd-⊿QC* Mutants

(A) Domain structure of the 489 amino acid long Bcd protein: the histidine/proline-rich domain (HP; amino acids 11–42), the homeodomain (HD; amino acids 91–152), the serine/threonine-rich activation domain (ST; amino acids 153–210), the glutamine-rich activation domain (Q; amino acids 252–300), the alanine-rich repression domain (A; amino acids 301–342), and the C-terminal acidic activation domain (C; amino acids 350–414) are indicated.

(B–D) Midstage embryos were hybridized with an antisense *hb* probe. Mothers were homozygous for bcd^{E1} (null allele) and carried two copies of a transgene expressing the Bcd-ΔC protein (B) or the Bcd-ΔQC protein (C). Mothers were homozygous for bcd^{E3} (D). Bcd-ΔC is deleted from amino acids 344–489 and Bcd-ΔQC is deleted from amino acids 247–304 and 344–489 [21]. The bcd^{E3} allele encodes a mutant protein in which serine 126 is replaced by a leucine [6]. Diffuse borders of expression are indicated by brackets. Sharpening was measured (see Figure S1 and Supplemental Experimental Procedures) on (n) midstage embryos and average measurements are indicated at the bottom. Anterior is at the left.

Gal4-derived transcription factors could also drive sharp expression of their target genes. In early-stage embryos, expression of the Gal4-reporters under control of the Gal4-2Q or the Gal4-3GCN4 transcription factors appeared graded from the anterior pole of the embryo (Figures 4A and 4B, brackets). Interestingly, however, during the first half of cellularization, in midstage embryos, the posterior borders of reporter gene's expression sharpened to 6% EL for Gal4-2Q (Figure 4C) and to 6.7% EL for Gal4-3GCN4 (Figure 4D)—a similar extent to the sharpening of *hb*, *otd*, and *Bcd3-lacZ* expression (Figure S2). Similar qualitative results were obtained with fusion proteins bearing the other activation domains of Bcd ([20], data not shown). These observations indicate that Bcd shares its ability to induce the formation of a sharp border of expression with artificial transcription factors that diffuse as maternal gradient from the anterior pole of the embryo.

The Bcd-ΔC and Bcd-ΔQC proteins, which exhibit reduced *hb*'s sharpening ability, are able to drive expression of *hb* and must contain at least one additional activation domain that is not sufficient for sharpening. This is surprising since the known remaining activation domains in these proteins are Q and ST (Figure 3A), which are both able to drive sharp expression of the Gal4 reporters when fused to the Gal4 DNA binding domain (Figure 4C, data not shown, [20]). The ability of the protein to drive sharpening is obviously influenced either by the context of the Bcd protein in which the Q and ST activation domains remains or by the number of activation domains per se.

Bcd's Ability to Activate Transcription Is Sufficient for Precise Target Gene Expression

To understand how the Bcd target genes are expressed in precisely sized domains, we quantified and compared the position of the posterior border of *hb*, *otd*, and *Bcd3-lacZ* expression in 30–50 midstage embryos (Figure 5). In agreement with Houchmandzadeh et al. [14], the position of the *hb* posterior border at midcellu-

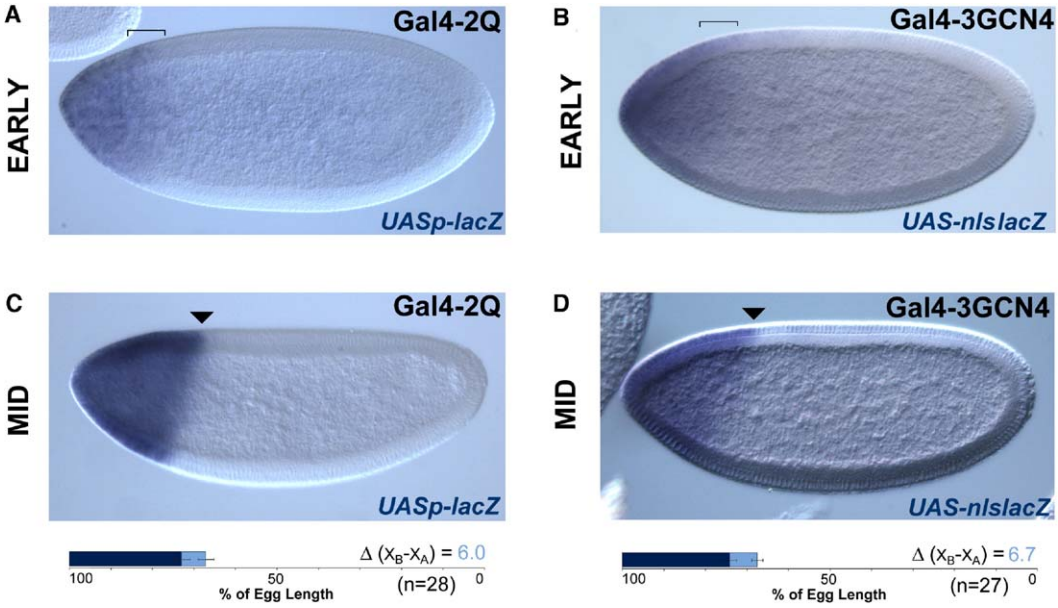

Figure 4. Gal4-Derived Transcription Factors Expressed in a Bcd-like Gradient Drive Sharp Expression of Target Genes

Females carrying four copies of a transgene expressing, in a Bcd-like maternal gradient, the artificial transcription factor Gal4-2Q (A and C) or Gal4-3GCN4 (B and D) were crossed with males carrying the transgene *UASp-lacZ* (A and C) or *UASnls-lacZ* (B and D). In situ hybridizations with an antisense probe for *lacZ* were performed on early-stage embryos (A and B) and midstage embryos (C and D) from these crosses. Diffuse borders of expression are indicated by brackets. Sharp borders of expression are indicated by arrow heads. Sharpening was measured (see Figure S1 and Supplemental Experimental Procedures) on (n) midstage embryos and average measurements are indicated at the bottom of (C) and (D).

larization varied from 48%–52% EL [50.2% ± 1.5% EL, Figure 5A]. In wild-type embryos, the locations of the *otd* and *Bcd3-lacZ* posterior borders were, respectively, at 72.1% ± 1.4% EL and 71.4% ± 1.6% EL (Figures 5B and 5C). These data indicate that, despite the observed variability of the Bcd gradient, the posterior borders of expression of these three Bcd direct targets are very precisely positioned in wild-type embryos. The promoter of *Bcd3-lacZ* does not contain binding sites for other transcription factors and, since addition of Hb binding sites drastically modifies its expression pattern [18], it is highly unlikely that it contains cryptic binding sites for Hb. The *Bcd3-lacZ* expression almost certainly reflects the intrinsic activity of the Bcd protein [10, 22], yet the Bcd gradient appears sufficient to induce its very precise positioning. This observation indicates that the mechanism allowing the precise positioning of expression of Bcd target genes, despite the reported variability of the Bcd gradient [17, 18], is a general feature of Bcd target genes. It can be driven solely by Bcd binding sites and does not necessarily involve Bcd's partner, the Hb protein.

To determine if any particular sequences of the Bcd protein were required for precision, we also quantified the precision of *UASp-lacZ* expression from the Gal4-3GCN4 and Gal4-2Q proteins: standard deviations were 1.7% of EL for Gal4-3GCN4 (Figure 5D) and 1.9% of EL for Gal4-2Q (Figure 5E). These values are very similar to the standard deviations obtained for the Bcd target genes (compare Figures 5A–5E) and indicate that the *UASp-lacZ* reporter gene was also expressed from the Gal4-2Q or the Gal4-3GCN4 artificial maternal gradients in very precisely sized domains. Together, these observations demonstrate that precise target gene expression can be driven by DNA binding transcription factors diffusing from the anterior pole of the embryo as a gradient. As the Bcd and Gal4-3GCN4 proteins have no sequence homologies and only share an ability to activate transcription, our experiments indicate that this property is sufficient for precision and that any other components in the system that contribute to this process must act upstream or at the level of transcriptional activation.

The Precision of Bcd- and Gal4-Derived Transcription Factors Is Reduced in Stau^{HL}

Although the *Bcd3-lacZ* reporter is expressed more anteriorly than *hb*, one could argue that its precision is due to the steeper Bcd gradient in this region where the requirements for accurate response might be less demanding. Because the Stau protein was identified as a potential component contributing to the precision of Hb expression, we asked whether the expression of more anterior Bcd target genes was affected in the *stau^{HL}* mutant background, a hypomorphic allele with the strongest effect on *hb* precision [14]. In agreement with Houchmandzadeh et al. [14], in embryos from *stau^{HL}* females, the position of the *hb* posterior border was much more variable than in wild-type embryos, as shown by the distribution of the posterior border positions among 56 midstage embryos (Figure 6A), which ranged from 45%–66% EL (mean position 52% EL, standard deviation 4%). Interestingly, higher variability than in wild-type embryos was also observed in em-

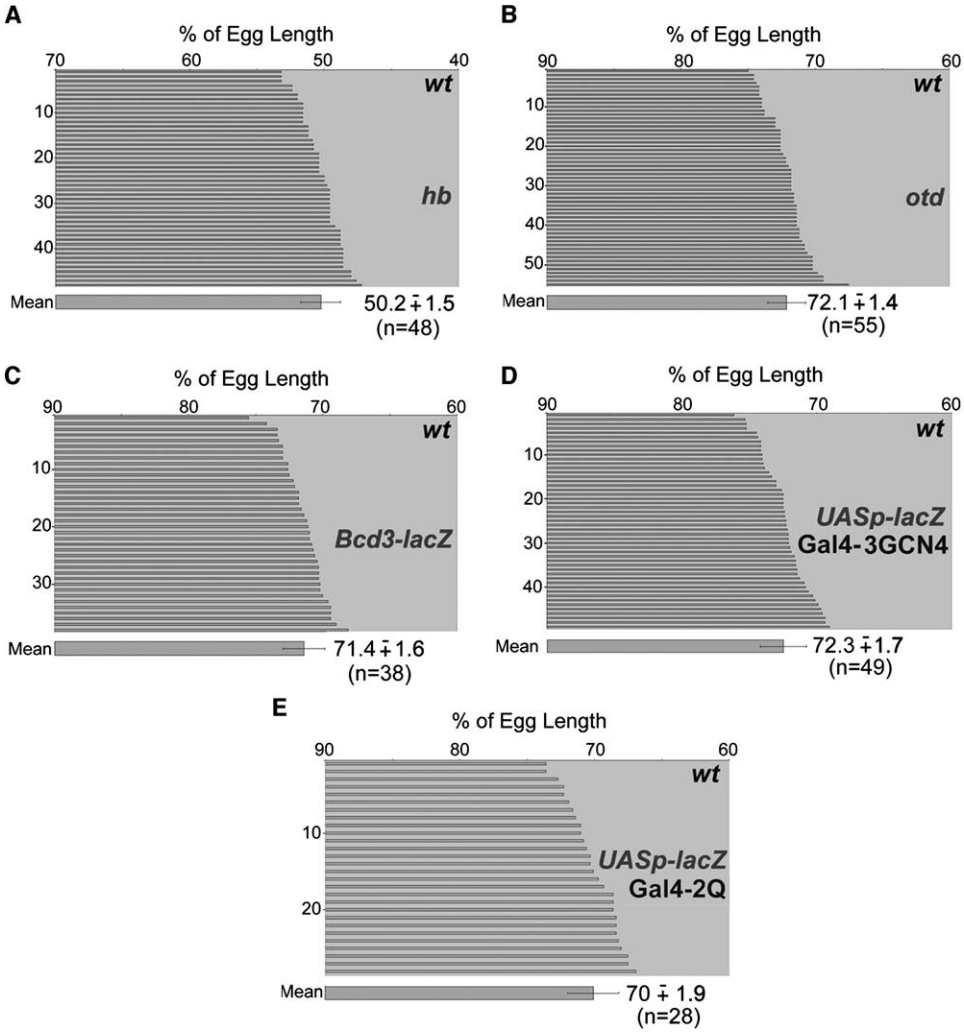

Figure 5. Precise Positioning of the Posterior Borders of Expression of Bcd's Target Genes during the First Half of Cellularization

In situ hybridizations with *hb* (A), *otd* (B) or *lacZ* (C–E) antisense probes were performed on embryos from wild-type females. Females carried two (D) or four (E) copies of a transgene expressing, in a Bcd-like maternal gradient, the artificial transcription factors Gal4-3GCN4 (D) or Gal4-2Q (E). Embryos carried the *Bcd3-lacZ* transgene (C) or the *UASp-lacZ* transgene (D and E). The distribution of the mean posterior border positions among (n) midstage embryos is given for each target gene and/or genetic background. Average values and standard deviations for the (n) embryos were calculated and are indicated at the bottom of each panel. Standard deviations observed were not significantly different ($p < 0.001$).

bryos from $stau^{HL}$ females for *otd* (Figure 6B), *Bcd3-lacZ* (Figure 6C), and *UASp-lacZ* expressed from the Gal4-3GCN4 artificial maternal gradient (Figure 6D). Standard deviations were 2.5% for *otd* (Figure 6B), 2.4% for *Bcd3-lacZ* (Figure 6C), and 3.1% for *UASp-lacZ* (Figure 6D). The standard deviations for these three genes were of similar magnitude (Figures 6B–6D) but were all clearly lower than the standard deviation obtained for *hb* (compare in Figure 6A with Figures 6B–6D). This reduction is likely linked to the more anterior position of the posterior borders of these three Bcd target genes along the A-P axis. Since the precision of *otd*, *Bcd3-lacZ* and *UASp-lacZ* expression was significantly reduced in the $stau^{HL}$ mutant context ($p < 0.01$; compare Figures 5B–5D with Figures 6B–6D), we conclude that the Stau protein contributed not only to the precision of *hb* expression but, more generally, to the precision of the Bcd morphogenic activity and to the precision of an artificial Gal4-3GCN4 transcription factor expressed as maternal gradient in the embryo.

The Involvement of Stau in the Precision of Bcd Morphogenic Activity

Bcd and Gal4-3GCN4 have no specific sequence homologies, but they share an ability to activate transcription and in our study, their mRNAs both contain the Bcd 3′ untranslated sequence required for anchoring at the anterior pole. Two processes may account for the conserved effect of Stau on Bcd and Gal4-3GCN4 morphogenic activity: either Stau affects the activities of both proteins and is directly involved in the general transcription process, or the reduced precision of target

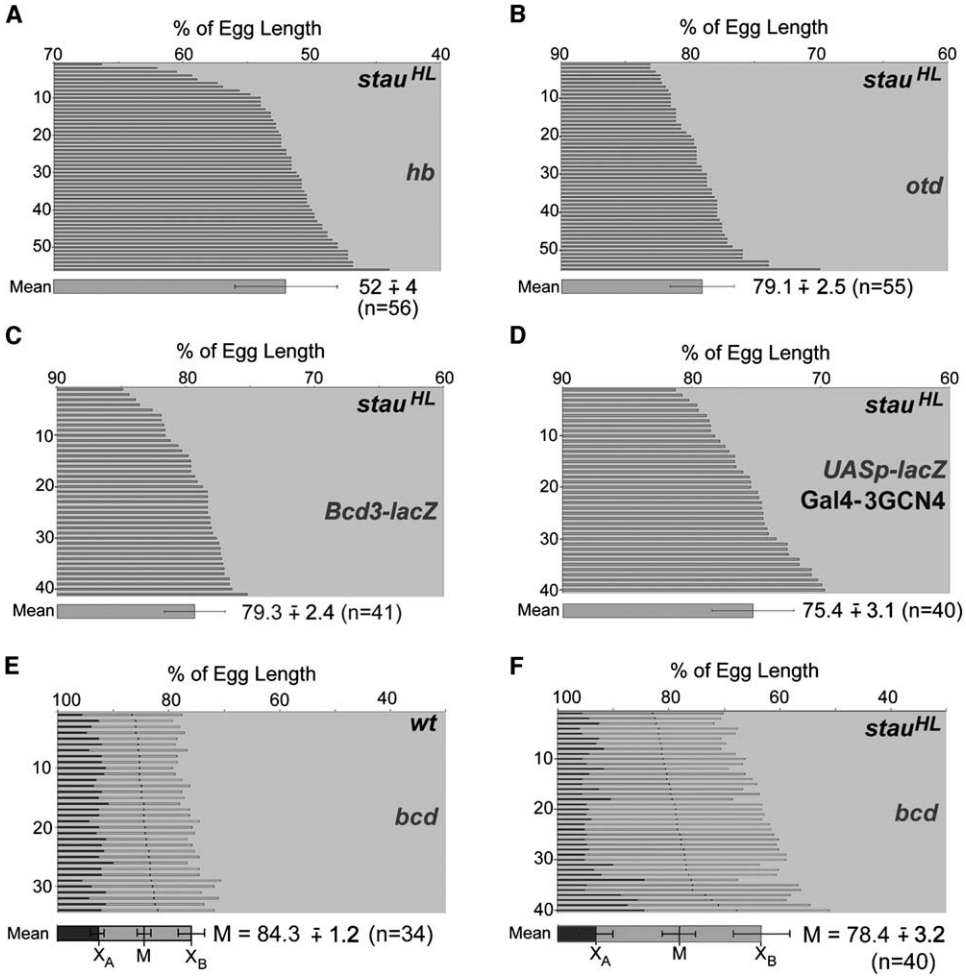

Figure 6. The Precision of Bcd's Target Gene Expression and *bcd* mRNA Distribution Is Reduced in the *stau*^{HL} Mutant Background

In situ hybridizations with *hb* (A), *otd* (B), *lacZ* (C and D), or *bcd* (E and F) antisense probes were performed on embryos from wild-type (E) or *stau*^{HL} (see [A]–[D] and [F]) females. Females carried two copies of a transgene expressing, in a Bcd-like maternal gradient, the artificial transcription factors Gal4-3GCN4 (D). Embryos carried the *Bcd3-lacZ* transgene (C) or the *UASp-lacZ* transgene (D). The distribution of the mean posterior border positions for (n) midstage embryos is given for each target gene and/or genetic background at the top of each panel. Average values and standard deviations for the (n) embryos of were calculated and are indicated at the bottom of each panel.

(E and F) The distribution of X_A (left edge of each light gray bar), X_B (right edge of each light gray bar) and the mean position, $M = [X_A + X_B]/2$ (middle position of each light gray bar), is indicated at the top. Ranking was performed on increasing order of mean positions.

genes expression in the *stau*^{HL} background is related to variation in anchoring of the Bcd and Gal4-3GCN4 encoding mRNAs at the anterior pole. We favored this second hypothesis, because the original function of Stau is precisely to regulate anchoring of the *bcd* mRNA at the anterior pole through its 3′ untranslated region [16, 17]. To test it, we compared the anterior localization of the *bcd* mRNA in early-stage embryos from wild-type and *stau*^{HL} females. In wild-type embryos the *bcd* mRNA is detected from the anterior pole to a mean position of 84.3% EL (Figure 6E and Figures S4A and S4B). In agreement with the previously identified role of Stau [16], the distribution domain of the *bcd* mRNA is larger and expands toward the posterior down to a mean position of 78.4% EL in embryos from *stau*^{HL} females (Figure 6F and Figures S4C and S4D). Quantitative measurements indicate that the limit of the *bcd* mRNA detection domain is fuzzy with a sharpening of 17% EL in wild-type (Figure 6E and Figures S4A and S4B) and 30% EL in *stau*^{HL} background (Figure 6F and Figures S4C and S4D). The variability of *bcd* mRNA localization is indicated by a standard deviation of 1.2% EL (Figure 6E) in wild-type embryos, a value significantly different ($p < 0.001$) from the 3.2% EL standard deviation observed in the *stau*^{HL} background (Figure 6F). These observations indicate that the *bcd* mRNA distribution is more variable among embryos in the *stau*^{HL} background than in wild-type. Although Houchmandzadeh et al. did not detect any variability in the average expression profiles of the Bcd protein in *stau*^{HL} background [14], the variability observed in *bcd* mRNA distribution (Figure 6F and Figures S3C and S3D) may likely contribute to the variability of the Bcd morphogenic activity in this genetic context.

Discussion

In the present study, we show that Bcd binding sites are sufficient in a promoter to allow its precise expression in an anterior domain with a sharp posterior border in the *Drosophila* embryo. Sharpening and precision of target gene's expression was also obtained when Gal4-derived transcription factors were allowed to diffuse in Bcd-like maternal gradients, indicating that the ability to activate transcription is sufficient for these processes. These observations also indicate that, despite the reportedly noisy nature of the Bcd-protein gradient [17, 18], the "filter" allowing the precision of its activity would have to be very general and not designed specifically to the Bcd-*hb* network. To gain more insights into the nature of this filter, we analyzed the expression of the Bcd-reporter and the Gal4-reporter in $stau^{HL}$ mutant background and observed that their precision was significantly reduced. This observation indicated that the effect of Stau in the precision of the Bcd-activity gradient occurs upstream or at the level of the activation process. Because the Stau protein was originally involved in the anchoring of the *bcd* mRNA at the anterior pole, we compared the *bcd* mRNA distributions in wild-type and $stau^{HL}$ mutant background. We observed that it was more diffuse in the $stau^{HL}$ background with a strong variability among embryos. We propose that such variability accounts for the reduction of precision observed for the Bcd and Gal4-dependent target gene's expression in this mutant background and that the involvement of Stau in this process is consistent with its well characterized activity in mRNA anchoring. At last, because our data put into question the existence of a filtering system allowing the precision of the Bcd-activity gradient despite the noisy nature of the Bcd-protein gradient, we discuss the possibility that the measurements of the Bicoid protein itself might be the source of the noise.

The Smooth Morphogen Gradient Induces Very Defined Domains of Expression

Expression of the direct target genes of Bcd appears graded in the early embryo and, at first, reflects the smooth gradient of the Bcd protein. Interestingly, it is during the first half of cellularization that the posterior borders of these expression domains resolve into sharp focus, dividing the embryo into an anterior domain where the target gene is on and a posterior domain where the target gene is off. Our data indicate that Bcd does not require other DNA binding transcription factors to induce this sharpening. Moreover, several transcription factors artificially expressed in a Bcd-like maternal gradient can also induce sharp expression of their target genes in the blastoderm embryo. Because activation domains of Bcd are required for the sharpening of *hb*, this process likely depends directly on the transcription process per se.

We can propose several hypotheses to explain how the sharpening of target gene expression during early cellularization might take place. Firstly, it might involve a repressor inhibiting Bcd's activity possibly through one of the repression domains identified in the Bcd protein [20, 23]. This repressor would be expressed as a gradient opposite to the Bcd gradient. Because sharpening occurs with both the Bcd and Gal4-3GCN4 proteins that have no sequence homologies, this repressor must act very generally on the transcription process. Another possibility could be that the response elements in Bcd target genes also contain binding sites for repressors. A recent study argues that most of the Bcd-dependent target elements contain Bcd binding sites in combinations with Hb and/or Kr sites [24]. However, since sharpening is observed with the Bcd-dependent and the Gal4-dependent reporters which do not contain binding sites for other transcription factors, the repressor involved in sharpening in these cases must not bind specifically to DNA. Secondly, cooperative interactions between activation domains and the transcriptional machinery might be involved [21]. Thirdly, chromatin modifications may be involved since during this period of development dramatic changes occur in the *Drosophila* embryo genome, which is at first intensively replicating in the absence of zygotic transcription and gradually becomes transcribed in the absence of replication [25]. Finally, the process of cellularization itself might play a role: it is possible that once Bcd is confined within individual cells its activity has a sharper threshold. However, since the cellularization process is occurring normally in the embryos with reduced *hb* sharpening, it is clearly not sufficient.

The Variable Bcd Gradient Induces Precise Expression

Our analysis of *Bcd3-lacZ* indicates that although it contains only Bcd binding sites, its domain of expression is as precisely reproducible from one embryo to another as those of *hb* and *otd*, the two other direct Bcd target genes tested. Unexpectedly, the domain of expression of *Bcd3-lacZ* does not mimic the wide variability in the Bcd-protein gradient described by Houchmandzadeh et al. [14] and Spirov et al. [15]. This variability of the Bcd-protein gradient was observed using immunofluorescence microscopy of whole mount embryos coupled to a quantitative detector of fluorescence. We also observed it frequently in immunochemistry experiments aiming to detect the Bcd-protein gradient (N.D., unpublished data). Immunofluorescence microscopy, like in situ hybridization, involves several crucial steps, such as the permeabilization and fixation of the embryo, that are subject to variation: we found that embryos processed together frequently exhibited a 2-fold variation in their staining level (data not shown). Despite these variations, two parameters can be analyzed after the staining procedure: the intensity of the staining and the frontiers that limit the domain of expression. Depending on the situation, these two parameters may be related or independent. In the case of *hb* and the Bcd target genes, which are expressed in a discrete domain with a clear-cut boundary, the domain of expression itself (i.e., the position of the posterior boundary) is not affected by variations in the intensity of staining. In the case of the Bcd gradient, by contrast, the domain of expression does not exhibit a clear cut boundary but is rather limited by a large graded domain

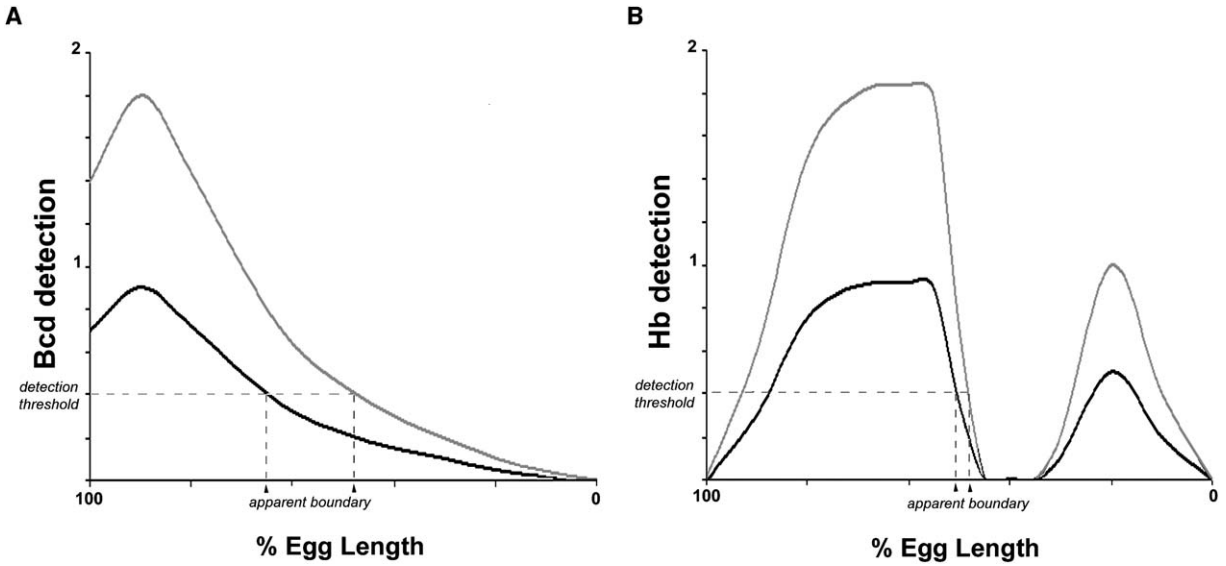

Figure 7. The Border of Bcd Expression Is Much More Sensitive to Experimental Variability Than Is the Border of Hb Expression

Expression of Bcd (A) and Hb (B) along the A-P axis are illustrated using arbitrary values for protein concentrations (y axis) as a function of the percentage of EL. Numerical values were obtained from [14]. The anterior pole is at 100%. In each case, the gray curve is deduced from the black curve by a simple 2X transformation. Arrows indicate the variation in the position of (A) the Bcd gradient and (B) the Hb posterior border for an arbitrary detection threshold of 0.4.

whose position is directly determined by the intensity of staining.

We wondered how variations in the intensity of staining might affect detection of the Bcd-protein gradient and detection of the expression domain of its main target protein Hb. To address this question, we performed a simulation that mimics a 2-fold increase in the signal detected either for Bcd (Figure 7A) or for Hb (Figure 7B). This simulation indicates that a 2-fold increase in the Bcd signal induces a wide (around 20% EL) movement of the gradient toward the posterior pole (Figure 7A). Interestingly, the same increase in the Hb signal shifts the plateau (i.e., the level of expression) along the y axis but induces only a 3% shift of the position of Hb toward the posterior pole (Figure 7B). This simulation indicates that even slight experimental variability in the immunodetection protocol could affect the observed position of the Bcd gradient much more than the position of the posterior border of its target gene expression. In light of the precise expression of a simple Bcd reporter gene, we propose that the Bcd-activity gradient is extremely precise and able, on its own, to drive very accurate positional information along the A-P axis of the embryo. The simulation illustrated in Figure 7 calls into question the degree of variability of the Bcd protein gradient itself, which needs to be revisited using experimental procedures that detect accurately a less than 2-fold variability in Bcd concentration.

Systematic Variation Introduced by Dosage and Temperature Have Little Effect on the Position of the *hb* Expression Border

Two additional sets of experiments were provided by Houchmandzadeh et al. to support the existence of a "filtering system" transforming the noisy Bcd-protein gradient in a precise Bcd-activity gradient [14]. They show that variation in temperature and in the gene dosage of Bcd have much less effects than expected on the position of Hb's expression border. In the light of our results, we would like to revisit these experiments and discuss their interpretation. As expected [5, 7], increases in the *bcd* gene dosage induced posterior shifts of the Hb posterior border [14]. Quantitative measurements indicated that these shifts decreased when the gene dosage of Bcd increased and were, therefore, smaller than expected if they corresponded to proportional increases in protein dosage and activity [14]. Although these observations could provide support for the existence of a filtering system for Bcd activity [14], they could also be explained by alternative hypotheses. Firstly, it is possible that increases in the *bcd* gene dosage do not allow corresponding quantitative increases of the Bcd protein itself in the embryo. For instance, the transgenes used might not express as much *bcd* mRNA as the endogenous gene (as frequently observed with transgenic insertions) and also, above a certain threshold of expression at the anterior pole, the translation of the *bcd* mRNA might be step limited. Secondly, the *hb* control region contains binding sites for transcription factors such as Hb itself. Importantly, the position of zygotic *hb* posterior border was shown to be precisely shifted toward the anterior in the absence of the Hb maternal protein (Hb^{mat}) [21]. Because Hb^{mat} is restricted to the anterior half of the embryo by Nanos [26], it is difficult to determine how its absence in the posterior half of the embryo might affect the extent of the posterior shift of *hb* expression border in situations where this border is precisely localized in the posterior

half of the embryo as it is the case with high *bcd* gene dosage (four to six copies). The second approach provided by Houchmandzadeh et al. to support the existence of a filtering system for Bcd activity shows that a reduction of temperature (from 29°C to 9°C) shifts the Bcd-protein gradient toward the posterior but has little effect on Hb expression [14]. An alternative hypothesis is that such a decrease in temperature may directly affect Bcd activity (for instance by reducing its DNA binding or transcriptional activity) and compensate its effect on the position of the Bcd-protein gradient. At last, as previously mentioned, the anterior restriction of Hb^{mat} might also be important to consider in this case, precisely because the expected shifts of Hb posterior border are localized in the region where the posterior border of Hb^{mat} is localized.

Experimental Procedures

Drosophila Stocks and Transgenics

Mutant stocks were bcd^{E1}, bcd^{E3} [26], and $stau^{HL}$ [16, 17] and were obtained from the Bloomington stock Center. P element-transformed lines were *Bcd3-lacZ* [10], *Bcd-⊿C* and *Bcd-⊿QC* [21], *UAS-placZ* [27], *Gal4-2Q*, and *Gal4-3GCN4* [20]. The Bcd-ΔC and Bcd-ΔQC proteins were expressed under the control of the *bcd* regulatory sequences [21]. The Gal4-2Q and Gal4-3GCN4 fusion proteins were expressed in Bcd-like gradients under the control of the maternal *nanos* promoter and the *bcd* 3′ untranslated region that allows the anchoring of their mRNAs at the anterior pole of the embryo. The *UAS-nlslacZ* was constructed by Thomas Lecuit by inserting five copies of an optimized Gal4 binding site upstream of the hsp27 minimal promoter driving the expression of a nuclear β-galactosidase in the pwnβ-E plasmid kindly provided by Ulrike Gaul.

Whole-Mount In Situ Hybridization and Immunocytochemistry

Digoxigenin-labeled RNA probes and in situ hybridization were performed as in [19] from pKS derivatives containing the coding sequences of the different genes studied. The anti-digoxigenin antibody coupled to alkaline phosphatase (Roche Diagnostics) was preadsorbed and used at a 1/2000 dilution. Immunocytochemistry was performed as described in [28] with the anti-Bcd antibody [6] diluted to 1/500. Embryos were mounted in 80% glycerol and photographed by using Nomarski optics. The detailed procedures for measurements of posterior border sharpening and position are described in Figure S1 and in the Supplemental Experimental Procedures.

Supplemental Data

Supplemental Data include supplemental results, experimental procedures, and four figures and are available at http://www.current-biology.com/cgi/content/full/15/21/1888/DC1/.

Acknowledgments

We are deeply grateful to Geneviève Almouzni, Claude Desplan, Pierre-Antoine Defossez, Edith Heard, Fabiana Heredia, Thomas Lecuit, and Ken Sekimoto for helpful discussions and critical readings of the manuscript. O.C. was supported by the French Ministry of Youth, Education and Research (Elève Normalien) and N.D. by INSERM. This work was supported by grants from the Curie Institute, the "*Fondation pour la Recherche Médicale*," the "*Association pour la Recherche sur le Cancer*," and the French Ministry of Youth, Education and Research (*ACI Biologie du Développement et Physiologie Intégrative*) to N.D.

Received: June 28, 2005
Revised: September 20, 2005
Accepted: September 21, 2005
Published: November 7, 2005

References

1. Wolpert, L. (1996). One hundred years of positional information. Trends Genet. *12*, 359–364.
2. Wolpert, L. (1969). Positional information and the spatial pattern of cellular differentiation. J. Theor. Biol. *25*, 1–47.
3. Driever, W., and Nüsslein-Volhard, C. (1988). A gradient of Bicoid protein in *Drosophila* Embryos. Cell *54*, 83–93.
4. Driever, W., and Nüsslein-Volhard, C. (1988). The Bicoid protein determines position in the drosophila embryo in a concentration-dependent manner. Cell *54*, 95–104.
5. Driever, W., Ma, J., Nüsslein-Volhard, C., and Ptashne, M. (1989). Rescue of *bicoid* mutant drosophila embryos by Bicoid fusion proteins containing heterologous activating sequences. Nature *342*, 149–154.
6. Struhl, G., Struhl, K., and Macdonald, P.M. (1989). The gradient morphogen Bicoid is a concentration-dependent transcriptional activator. Cell *57*, 1259–1273.
7. Driever, W., and Nüsslein-Volhard, C. (1989). The Bicoid protein is a positive regulator of *hunchback* transcription in the early Drosophila embryo. Nature *337*, 138–143.
8. Gao, Q., and Finkelstein, R. (1998). Targeting gene expression to the head: the Drosophila *orthodenticle* gene is a direct target of the Bicoid morphogen. Development *125*, 4185–4193.
9. Finkelstein, R., and Perrimon, N. (1990). The *orthodenticle* gene is regulated by Bicoid and Torso and specifies Drosophila head development. Nature *346*, 485–488.
10. Ronchi, E., Treisman, J., Dostatni, N., Struhl, G., and Desplan, C. (1993). Down-regulation of the *Drosophila* morphogen Bicoid by the Torso receptor-mediated signal transduction cascade. Cell *74*, 347–355.
11. Rivera-Pomar, R., and Jäckle, H. (1996). From gradients to stripes in Drosophila embryogenesis: filling in the gaps. Trends Genet. *12*, 478–483.
12. Arnosti, D.N., Barolo, S., Levine, M., and Small, S. (1996). The eve stripe 2 enhancer employs multiple modes of transcriptional synergy. Development *122*, 205–214.
13. Jaeger, J., Surkova, S., Blagov, M., Janssens, H., Kosman, D., Kozlov, K.N., Manu, M., Myasnikova, E., Vanario-Alonso, C.E., Samsonova, M., Sharp, D.H., and Reinitz, J. (2004). Dynamic control of positional information in the early Drosophila embryo. Nature *430*, 368–371.
14. Houchmandzadeh, B., Wieschaus, E., and Leibler, S. (2002). Establishment of developmental precision and proportions in the early Drosophila embryo. Nature *415*, 798–802.
15. Spirov, A.V., and Holloway, D.M. (2003). Making the body plan: precision in the genetic hierarchy of Drosophila embryo segmentation. In Silico Biol. *3*, 89–100.
16. StJohnston, D., Beuchle, D., and Nüsslein-Volhard, C. (1991). *staufen*, a gene required to localize maternal RNAs in the Drosophila egg. Cell *66*, 51–63.
17. Ferrandon, D., Elphick, L., Nüsslein-Volhard, C., and St Johnston, D. (1994). Staufen protein associates with the 3′UTR of *bicoid* mRNA to form particles that move in a microtubule-dependent manner. Cell *79*, 1221–1232.
18. Simpson-Brose, M., Treisman, J., and Desplan, C. (1994). Synergy between the Hunchback and Bicoid morphogens is required for anterior patterning in Drosophila. Cell *78*, 855–865.
19. Bellaïche, Y., Bandyopadhyay, R., Desplan, C., and Dostatni, N. (1996). Neither the homeodomain nor the activation domain of Bicoid is specifically required for its down-regulation by the Torso receptor tyrosine kinase cascade. Development *122*, 3499–3508.
20. Janody, F., Sturny, R., Schaeffer, V., Azou, Y., and Dostatni, N. (2001). Two distinct domains of Bicoid mediate its transcriptional downregulation by the Torso pathway. Development *128*, 2281–2290.

21. Schaeffer, V., Janody, F., Loss, C., Desplan, C., and Wimmer, E.A. (1999). Bicoid functions without its TATA-binding protein-associated factor interaction domains. Proc. Natl. Acad. Sci. USA *96*, 4461–4466.
22. Janody, F., Sturny, R., Catala, F., Desplan, C., and Dostatni, N. (2000). Phosphorylation of bicoid on MAP-kinase sites: contribution to its interaction with the torso pathway. Development *127*, 279–289.
23. Zhao, C., York, A., Yang, F., Forsthoefel, D.J., Dave, V., Fu, D., Zhang, D., Corado, M.S., Small, S., Seeger, M.A., et al. (2002). The activity of the Drosophila morphogenetic protein Bicoid is inhibited by a domain located outside its homeodomain. Development *129*, 1669–1680.
24. Ochoa-Espinosa, A., Yucel, G., Kaplan, L., Pare, A., Pura, N., Oberstein, A., Papatsenko, D., and Small, S. (2005). The role of binding site cluster strength in Bicoid-dependent patterning in Drosophila. Proc. Natl. Acad. Sci. USA *102*, 4960–4965.
25. Sibon, O.C., Stevenson, V.A., and Theurkauf, W.E. (1997). DNA-replication checkpoint control at the Drosophila midblastula transition. Nature *388*, 93–97.
26. Frohnhofer, H.G., and Nüsslein-Volhard, C. (1986). Organization of anterior pattern in the drosophila embryo by the maternal gene *bicoid*. Nature *324*, 120–125.
27. Rorth, P. (1998). Gal4 in the Drosophila female germline. Mech. Dev. *78*, 113–118.
28. MacDonald, P.M., and Struhl, G. (1986). A molecular gradient in early drosophila embryos and its role in specifying the body pattern. Nature *324*, 537–545.