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Report

Precision of Hunchback Expression in the *Drosophila* Embryo

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Summary

Activation of the gap gene *hunchback* (*hb*) by the maternal Bicoid gradient is one of the most intensively studied gene regulatory interactions in animal development. Most efforts to understand this process have focused on the classical Bicoid target enhancer located immediately upstream of the P2 promoter [1–12]. However, *hb* is also regulated by a recently identified distal shadow enhancer as well as a neglected "stripe" enhancer, which mediates expression in both central and posterior regions of cellularizing embryos [13, 14]. Here, we employ BAC transgenesis and quantitative imaging methods to investigate the individual contributions of these different enhancers to the dynamic *hb* expression pattern. These studies reveal that the stripe enhancer is crucial for establishing the definitive border of the anterior Hb expression pattern, just beyond the initial border delineated by Bicoid. Removal of this enhancer impairs dynamic expansion of *hb* expression and results in variable cuticular defects in the mesothorax (T2) due to abnormal patterns of segmentation gene expression. The stripe enhancer is subject to extensive regulation by gap repressors, including Kruppel, Knirps, and Hb itself. We propose that this repression helps ensure precision of the anterior Hb border in response to variations in the Bicoid gradient.

Results and Discussion

hunchback (*hb*) is the premier gap gene of the segmentation regulatory network. It coordinates the expression of other gap genes, including *Kruppel* (*Kr*), *knirps* (*kni*), and *giant* (*gt*) in central and posterior regions of cellularizing embryos [15, 16]. The gap genes encode transcriptional repressors that delineate the borders of pair-rule stripes of gene expression. *hb* is activated in the anterior half of the precellular embryo, within 20–30 min after the establishment of the Bicoid gradient during nuclear cleavage cycles 9 and 10 (~90 min following fertilization) [3, 6, 17, 18]. This initial *hb* mRNA transcription pattern exhibits a reasonably sharp on/off border within the presumptive thorax [1–3, 5, 13]. This border depends on cooperative interactions of Bicoid monomers bound to linked sites in the proximal ("classical") enhancer (Figure 1A). However, past studies and recent computational modeling suggest that Bicoid cooperativity is not sufficient to account for this precision in *hb* expression [4–12].

The *hb* locus contains two promoters, P2 and P1, and three enhancers (Figure 1A) [1, 14]. The "classical" proximal enhancer [1, 3] and distal shadow enhancer [13] mediate activation in response to the Bicoid gradient. Expression is also regulated by a third enhancer, the "stripe" enhancer, which is located over 5 kb upstream of P2 [14]. Each of these enhancers was separately attached to a *lacZ* reporter gene and expressed in transgenic embryos. As shown previously, the Bicoid target enhancers mediate expression in anterior regions of nuclear cleavage cycle (cc) 12–13 embryos (Figures 1B and 1C) [1–3, 13], whereas the stripe enhancer mediates two stripes of gene expression at later stages, during cc14 (Figure 1D) [14]. The anterior stripe is located immediately posterior to the initial hb border established by the proximal and distal Bicoid target enhancers (see below).

BAC transgenesis was used to determine the contribution of the stripe enhancer to the complex *hb* expression pattern. For some of the experiments, we replaced the *hb* transcription unit with the *yellow* (*y*) reporter gene, which contains a large intron permitting quantitative detection of nascent transcripts (see [19]). The resulting BAC mimics the endogenous expression pattern (Figures 1E and 1F), including augmented expression at the Hb border. However, removal of the stripe enhancer from an otherwise intact *y*-BAC transgene leads to diminished expression at this border and in posterior regions (Figure 1G).

The functional impact of removing the stripe enhancer was investigated by genetic complementation assays. A BAC transgene containing 44 kb of genomic DNA encompassing the entire *hb* locus and flanking regulatory DNAs fully complements deficiency homozygotes carrying a newly created deletion that cleanly removes the *hb* transcription unit (see Figure S1 available online). The resulting adults are fully viable, fertile, and indistinguishable from normal strains. Embryos obtained from these adults exhibit a normal Hb protein gradient, including a sharp border located between *eve* stripes 2 and 3 (Figure 2B).

The Hb BAC transgene lacking the stripe enhancer fails to complement hb^-/hb^- mutant embryos due to the absence of the posterior *hb* expression pattern (Figure 2A), which results in the fusion of the seventh and eighth abdominal segments (Figure 2E) (see [14]). In addition, the anterior Hb domain lacks the sharp "stripe" at its posterior limit, resulting in an anterior expansion of Even-skipped (Eve) stripe 3 (Figure 2A; compare with Figure 2B) because the Hb repressor directly specifies this border [20–23]. There is also a corresponding shift in the position of Engrailed (En) stripe 5, which is regulated by Eve stripe 3 (Figure 2D; compare with Figure 2C) (e.g., [24]). The narrowing of En stripes 4 and 5, due to the anterior shift of stripe 5, correlates with patterning defects in the mesothorax (Figures 2E and 2F; compare with Figures 2G and 2H).

Quantitative measurements indicate significant alterations of the anterior Hb expression pattern upon removal of the stripe enhancer (Figure 3). There is an anterior shift at the midpoint of the mature pattern, spanning two to three cell

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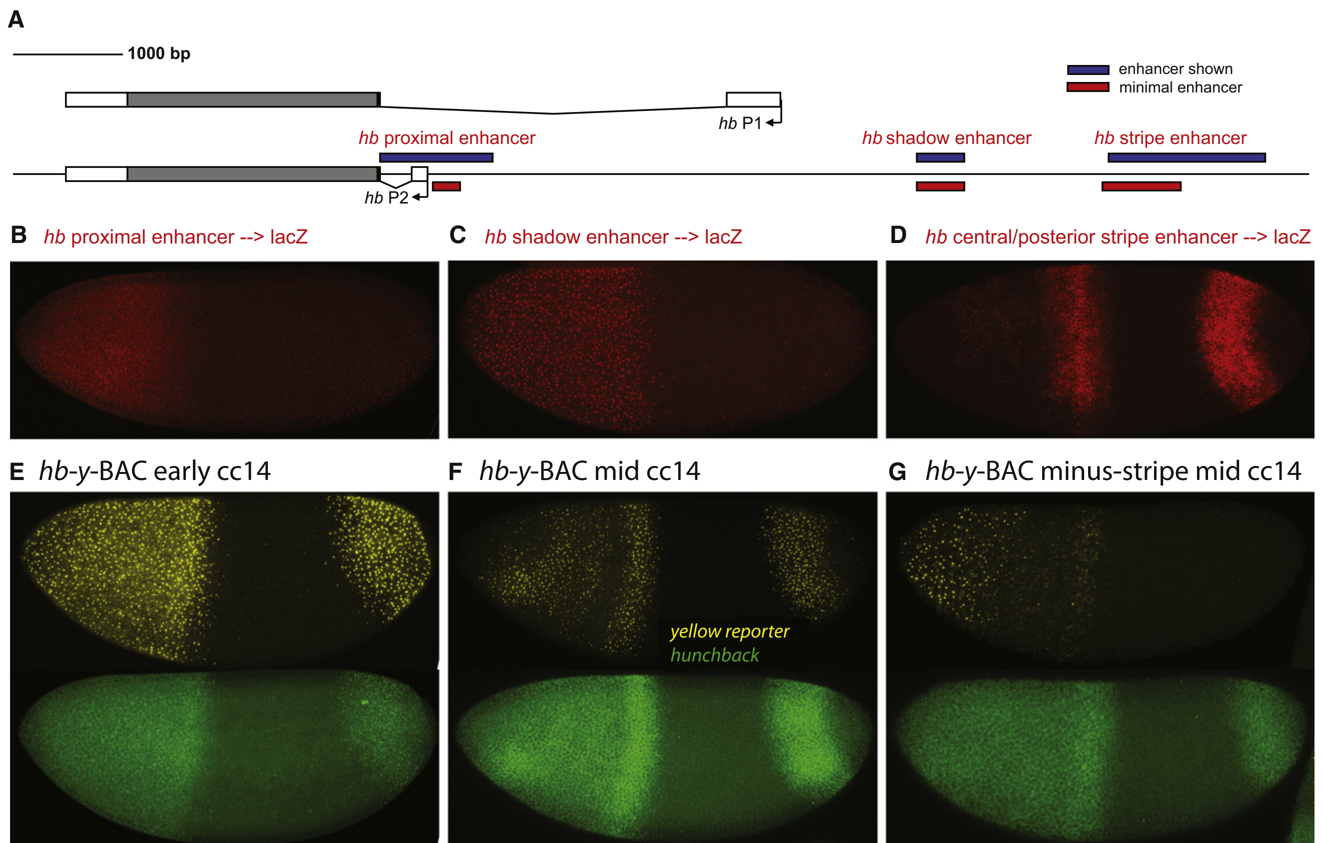

Figure 1. Summary of *hb cis*-Regulatory DNAs

(A) The *hb* locus contains two promoters, P2 and P1, and three enhancers. The proximal and distal shadow enhancers are targets of the Bicoid gradient, while the stripe enhancer is regulated by gap repressors (see text).

(B–D) *lacZ* antisense RNA in situ hybridization assays with transgenic embryos expressing proximal>*lacZ* (B), shadow>*lacZ* (C), or stripe>*lacZ* (D) transgenes. The proximal and shadow enhancers mediate broad expression in anterior regions, while the stripe enhancer produces central and posterior stripes of expression.

(E–G) Transgenic embryos expressing a y-BAC transgene containing a 44 kb genomic DNA encompassing the *hb* locus and associated regulatory DNAs. The *hb* transcription unit was replaced with the *yellow* (*y*) reporter. The wild-type y-BAC transgene exhibits broad anterior expression and a posterior stripe (E and F), whereas a mutagenized y-BAC transgene containing an internal replacement of stripe enhancer sequences with a non-regulatory spacer exhibits reduced expression of the central and posterior stripes (G). The embryos were double stained for yellow nascent transcripts (y-BAC transgenes) (shown in yellow) and endogenous *hb* (shown in green).

See also Figures S1 and S2.

diameters. This boundary normally occurs at 47.2% egg length (EL; measured from the anterior pole). In contrast, removal of the stripe enhancer shifts the boundary to 45.6% EL. The border also exhibits a significant diminishment in slope. Normally, there is a decrease in Hb protein concentration of 20% over 1% EL. Removal of the stripe enhancer diminishes this drop in concentration, with a reduction of just 10% over 1% EL. The most obvious qualitative change in the distribution of Hb protein is seen in regions where there are rapidly diminishing levels of the Bicoid gradient. Normally, the transition from maximum to minimal Hb levels occurs over a region of 10% EL (43%–53% EL). Removal of the stripe enhancer causes a significant expansion of this transition, to 26% EL (27%–53% EL). We therefore conclude that the stripe enhancer is essential for shaping the definitive Hb border.

The preceding studies suggest that the proximal and distal Bicoid target enhancers are not sufficient to establish the definitive Hb border at the onset of segmentation during cc14. Instead, the initial border undergoes a dynamic posterior expansion encompassing several cell diameters due to the action of the stripe enhancer. This enhancer is similar to the *eve* stripe 3+7 enhancer [20–23]. Both enhancers mediate two stripes, one in central regions and the other in the posterior abdomen, and the two sets of stripes extensively overlap. Previous studies provide a comprehensive model for the specification of *eve* stripes 3 and 7, whereby the Hb repressor establishes the anterior border of stripe 3 and the posterior border of stripe 7 while the Kni repressor establishes the posterior border of stripe 3 and anterior border of stripe 7 [20–23]. Whole-genome chromatin immunoprecipitation (ChIP) binding assays [25] and binding site analysis identify numerous Hb and Kni binding sites in the *hb* stripe enhancer, along with several Kr sites (Figure S2).

Site-directed mutagenesis was used to examine the function of gap binding sites in the *hb* stripe enhancer (Figure 4). Since the full-length, 1.4 kb enhancer contains too many binding sites for systematic mutagenesis (Figures 1D and 4C), we identified a 718 bp DNA fragment that mediates weak but consistent expression of both stripes, particularly the posterior stripe (Figure 4B). Mutagenesis of all ten Hb

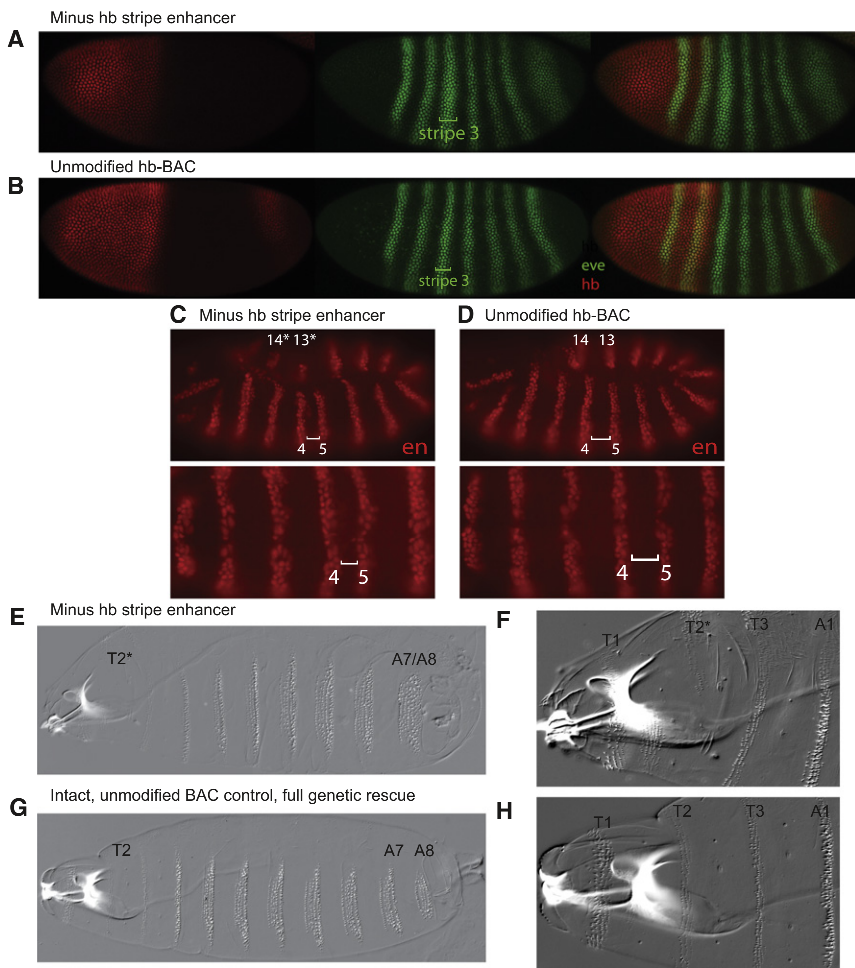

Figure 2. Developmental Consequences of Removing the *hb* Stripe Enhancer

hb− mutant embryos carrying either a mutant BAC transgene lacking the stripe enhancer (A, C, E, and F) or wild-type BAC transgene (B, D, G, and H). Both transgenes contain 44 kb of genomic DNA encompassing the *hb* transcription unit and flanking sequences. cc14 embryos were stained with antibodies against Hb (red) and Even-skipped (Eve; green). The wild-type BAC directs a normal Hb expression pattern and Eve pattern (B). In contrast, the mutant BAC transgene lacking the stripe enhancer exhibits reduced Hb expression in anterior regions, and loss of the posterior stripe (A; compare with B). The Eve pattern is also altered, with expanded patterns of stripes 3 and 7 (A). During germband elongation, the wild-type BAC transgene directs normal stripes of Engrailed (En) expression (D), whereas the mutant BAC lacking the stripe enhancer exhibits irregular spacing between En stripes 4 and 5 (C). The wild-type BAC transgene also produces completely normal cuticles (G and H), whereas the mutant BAC results in the variable loss of ventral mesothoracic (T2) pattern elements (E and F) and fusion of A7/A8 (E). Eve and En expression patterns were diagnostic of genotype, clearly discernible by eye, and this was confirmed by staining for the presence or absence of a labeled balancer.

binding sites in this minimal enhancer resulted in a striking anterior expansion of the expression pattern (Figure 4D). This observation suggests that the Hb repressor establishes the anterior border of the central stripe, as seen for *eve* stripe 3 [22, 23]. There is no significant change in the posterior border of the central stripe or the anterior border of the posterior stripe, and repression persists in the presumptive abdomen (Figure 4D).

Mutagenesis of the Kni binding sites resulted in expanded expression in the presumptive abdomen (Figure 4E), similar to that seen for the *eve* 3+7 enhancer [22, 23]. More extensive depression was observed upon mutagenesis of both the Kni and Kr binding sites (Figure 4F). These results suggest that the Kr and Kni repressors establish the posterior border of the central Hb stripe and the anterior border of the posterior stripe. This depressed pattern is virtually identical to the late *hb* expression pattern observed in Kr^1;kni^{10} double mutants [22]. The reliance on Kr could explain why the Hb central stripe is shifted anterior of *eve* stripe 3, which is regulated solely by Kni.

The dynamic regulation of the zygotic Hb expression pattern can be explained by the combinatorial action of the proximal, shadow, and stripe enhancers (summarized in Figure 4G). The proximal and distal shadow enhancers mediate activation of *hb* transcription in response to the Bicoid gradient in anterior regions of cc10–13 embryos. The initial border of *hb* transcription is rather sharp, but the protein that is synthesized from this early pattern is distributed in a broad and shallow gradient, extending from 30% to 50% EL (see Figure 3C). During cc14 the stripe enhancer mediates transcription in a domain that extends just beyond the initial *hb* border. Gap repressors, including Hb itself, restrict this second wave of zygotic *hb* transcription to the region when there are rapidly diminishing levels of the Bicoid gradient, in a stripe that encompasses 44%–47% EL. The protein produced from the stripe enhancer is distributed in a sharp and steep gradient in the anterior thorax. It has been previously suggested that the steep Hb protein gradient is a direct readout of the broad Bicoid gradient [e.g., 4, 6, 7]. However, our studies indicate that this is not the case. It is the combination of the Bicoid target enhancers and the *hb* stripe enhancer that produces the definitive pattern.

It has been proposed that Hb positive autofeedback is an important feature of the dynamic expression pattern [2, 10, 14]. However, the mutagenesis of the *hb* stripe enhancer (e.g., Figure 4) is consistent with past studies suggesting that Hb primarily functions as a repressor [20–23]. The only clear-cut example of positive regulation is seen for the *eve* stripe 2 enhancer. Mutagenesis of the lone Hb-3 binding site results in diminished expression from a minimal enhancer [26]. It was suggested that Hb somehow facilitates neighboring Bicoid activator sites, and we sought to determine whether a similar mechanism might apply to the proximal Bicoid target enhancer. The two Hb binding sites contained in this enhancer were mutagenized, but the resulting fusion gene mediates an expression pattern that is indistinguishable from the normal enhancer (Figure S3). It is therefore likely that the reduction of the central *hb* stripe in hb^-/hb^- embryos is the indirect consequence of expanded expression of other gap repressors, particularly Kr and Kni (Figure S4).

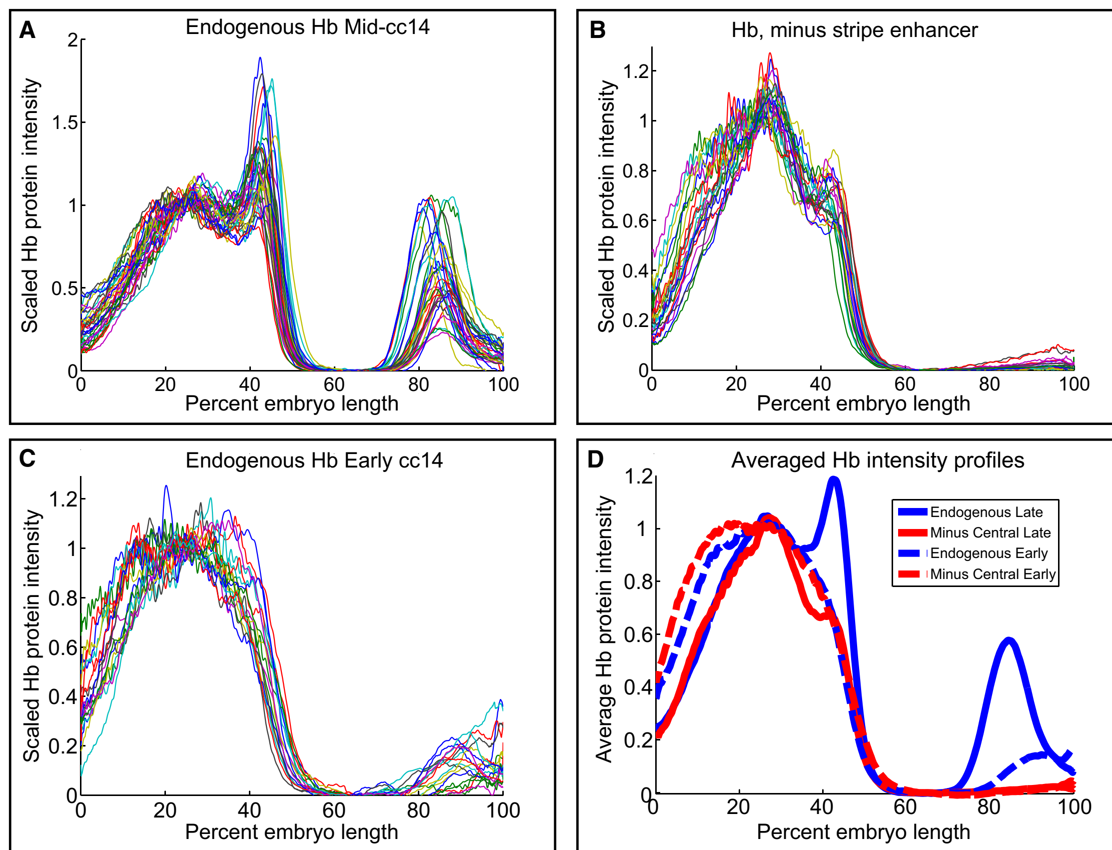

Figure 3. Quantitative Analysis of Hb Expression Profiles

(A and B) Profile of Hb protein late for 31 wild-type embryos (A) and for 23 transgenic rescue embryos lacking the stripe enhancer (B). The average position of the Hb boundary (as defined by the %EL at which the Hb intensity reaches 0.5) in endogenous embryos is 47.2% EL. In the minus stripe enhancer constructs the average position of the boundary is shifted anteriorly to 45.6% EL.

(C) In both cases, the Hb profile sharpens during cc 14.

(D) Removal of the stripe enhancer significantly affects the boundary region, as shown by comparing the average Hb profile for endogenous and the rescue constructs. A small amount of residual expression remains in central regions in some embryos. This expression may result from an incompletely knocked-out stripe enhancer, but more likely comes from a small amount of activation from the shadow enhancer, which drives some expression in this region in the enhancer>*lacZ* embryos (and which rapidly disappears).

The *hb* stripe enhancer mediates expression in a central domain spanning 44%–47% EL, which coincides with the region exhibiting population variation in the distribution of the Bicoid gradient (e.g., [4]). Despite this variability, the definitive Hb border was shown to be relatively constant among different embryos. Previous studies suggest that the Kr and Kni repressors function in a partially redundant fashion to ensure the reliability of this border [9, 10, 22]. We have presented evidence for direct interactions of these repressors with the *hb* stripe enhancer, and suggest that a major function of the enhancer is to "dampen" the variable Bicoid gradient. Indeed, removal of this enhancer from an otherwise normal Hb BAC transgene results in variable patterning defects in the mesothorax, possibly reflecting increased noise in the Hb border.

Experimental Procedures

Drosophila Genetics

A new deletion of the *hb* coding region was generated using DrosDel collection lines as in [27–29] and Harvard Exelixis lines f07611 and f00586. Flies positive for hb-BACs (carrying w^+) were balanced over labeled balancers carrying either TM3*hb-lacZ* or TM3*actin-GFP*. The stable full genetic rescue line *hb-BAC/hb-BAC;hb*^{del10}/*hb*^{del10} was used as a control and compared to embryos from *hb-minus-stripe_enhancer-BAC/hb-minus-stripe_enhancer-BAC;hb*^{del10}/*labeled-TM3* balancers. One in four embryos lost the balancer, indicating appropriate genotype, as defined by the absence of the label or by posterior A7/A8 segmental defects (also seen in [14]).

Recombineering and Transgenesis

Recombineering was performed as described previously [13, 19, 30–35] with modifications described in the Supplemental Experimental Procedures. Primers used for recombineering and screening can be found in Table S1. All constructs were integrated into landing site VK33 on chromosome 3 [29], Bloomington Stock Center number 24871.

Immunohistochemistry and In Situ Hybridization

For antibody staining, embryos were collected in tightly timed ~2–3.5 hr collections for Hb and Eve, and 6–12 hr collections for en. We used mouse anti-hb monoclonal antibody 1G10 at a concentration of 1:10; rabbit anti-eve polyclonal #10900 at 1:2,000, and mouse anti-en monoclonal 4D9 at 1:40, antibodies kindly provided by Nipam Patel, and commercial Alexa Fluor secondaries (Molecular Probes). Embryos were fixed and stained using standard protocols. Fluorescent in situ hybridization was performed as described in [36]. Probes were generated from plasmids made using primers in Table S1 and in vitro transcription. Nuclei were counterstained with DAPI.

Cuticle Preparations

Cuticles were prepared by pipetting late-stage embryos or first-instar larvae onto a slide and removing excess fluid. These were mounted in a mixture of

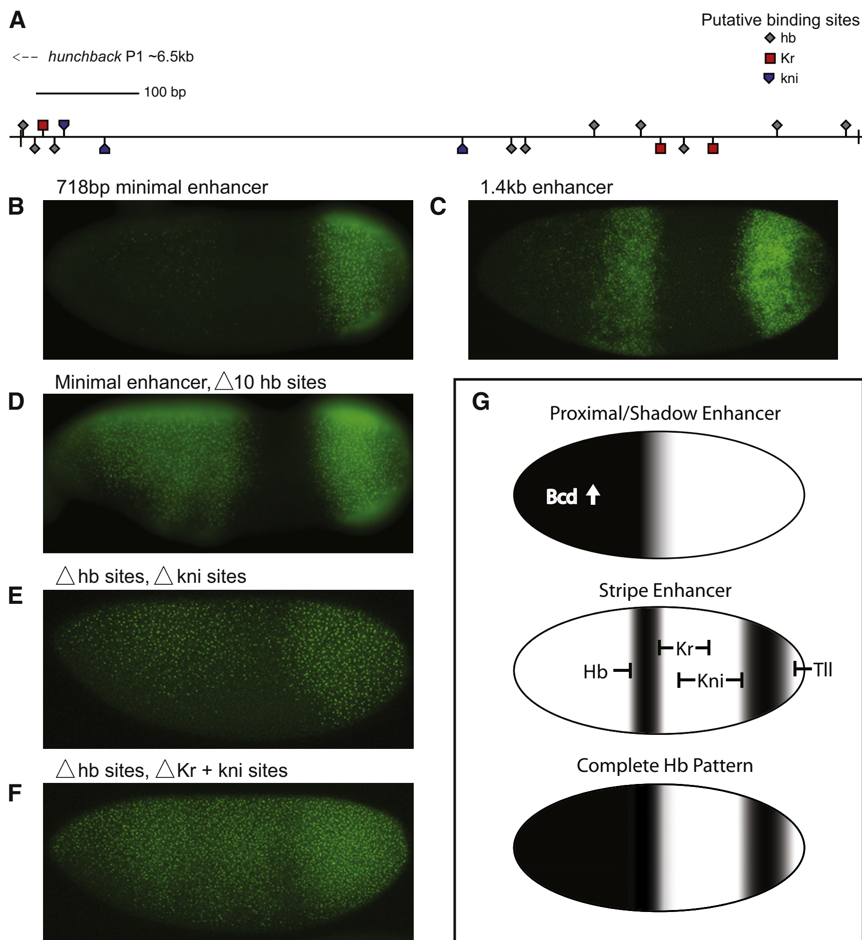

Figure 4. Analysis of the *hb* Stripe Enhancer

Transgenic embryos expressing different versions of the Hb stripe enhancer were attached to a *yellow* reporter gene. The 1.4 kb stripe enhancer contains >30 potential Hb sites, at least 8 potential Kr sites, and 6 potential kni sites and produces strong stripes of expression in central and posterior regions (C). A minimal, 718 bp stripe enhancer produces a weak central stripe and strong posterior stripe (B), putative binding site locations shown in (A). There is augmented expression and an anterior expansion of the anterior stripe>*yellow* staining pattern upon site-specific mutations in 10 Hb binding sites (D). There is a further expansion of the staining pattern upon mutagenesis of three putative kni sites (E) and even more dramatic expansion upon mutagenesis of the three putative Kr and three putative Kni sites (F); see Figure S2 for details on enhancer structure and specific binding sites. A proposed model is shown in (G). The complete Hb pattern is a composite of three enhancer inputs; the proximal and shadow enhancers rely on activation by anterior Bcd, while the stripe enhancer is instead ubiquitously activated and carved out by gap repressors. The composite boundary is both steeper and shifted posteriorly with the addition of the stripe enhancer's input.
See also Figures S2–S4.

glacial acetic acid mixed 1:1 with Hoyer's solution and dried for several days in an oven at 65°C for clearing. After 24 hr, coverslips were weighted to flatten the preps. These were imaged on an upright Zeiss Axiophot microscope with bright-field illumination, and grayscale images were later inverted and oversaturated for increased contrast using Adobe Photoshop.

Mutagenesis and Enhancer Testing

Mutagenesis was performed either by using a Stratagene QuikChange Site-Directed mutagenesis kit or by direct synthesis of the 718 bp enhancer fragment using Integrated DNA Technologies custom gene synthesis service, followed by subcloning into the enhancer testing vector. This vector is a modified version of the nE2G plasmid described previously [13], used with phiC31 targeted integration after injection into line BSC24871. Primers used can be found in Table S1; specific sites mutagenized are detailed in Figures S2 and S3.

Imaging and Hb Protein Quantification

High-resolution digital images (1,024 × 1,024, 12 bits per pixel) of fixed embryos were obtained on a Zeiss LSM 700 confocal microscope with a Plan Apochromat 20×/0.8 NA objective lens. Embryos were mounted in Prolong Gold (Invitrogen) and placed under a coverslip. The image focal plane of the embryos was chosen at the midsagittal plane for protein profile extraction. All images were taken with the same microscope settings. Hb protein profiles were extracted from confocal images of stained embryos by using software routines written in MATLAB (see Supplemental Experimental Procedures for details). Updated versions of the code used can be found at https://github.com/JacquesBothma. The source codes used to compute and plot the results from this publication are available at https://github.com/JacquesBothma/Hb_Stripe_Enhancer.

Supplemental Information

Supplemental Information includes four figures, Supplemental Experimental Procedures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2012.09.051.

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