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# Posterior stripe expression of *hunchback* is driven from two promoters by a common enhancer element

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## SUMMARY

**The gap gene *hunchback* (*hb*) is required for the formation and segmentation of two regions of the *Drosophila* embryo, a broad anterior domain and a narrow posterior domain. Accumulation of *hb* transcript in the posterior of the embryo occurs in two phases, an initial cap covering the terminal 15% of the embryo followed by a stripe at the anterior edge of this region. By in situ hybridization with transcript-specific probes, we show that the cap is composed only of mRNA from the distal transcription initiation site (P1), while the later posterior stripe is composed of mRNA from both the distal and proximal (P2) transcription initiation sites. Using a series of genomic rescue constructs and promoter-*lacZ* fusion genes, we define a 1.4 kb fragment of the *hb* upstream region that is both necessary and sufficient for posterior expression. Sequences within this fragment mediate regulation by the terminal gap genes *tailless* (*tll*) and *huckebein*, which direct the formation of the posterior *hb* stripe. We show that the tll protein binds in vitro to specific sites within the 1.4 kb posterior enhancer region, providing the first direct evidence for activation of gene expression by tll. We propose a model in which the anterior border of the posterior *hb* stripe is determined by tll concentration in a manner analogous to the activation of anterior *hb* expression by bicoid.**

Key words: *Drosophila*, *hunchback*, segmentation, gap gene, *tailless*, *huckebein*, autoregulation, transcription, repression

## INTRODUCTION

Segmentation in the *Drosophila* embryo is a hierarchical process that gradually divides the embryo into increasingly finer subsets of cells (Akam, 1987; Ingham, 1988). Three distinct maternal systems specify the anterior, the posterior and the termini of the embryo (Nüsslein-Volhard et al., 1987; St. Johnston and Nüsslein-Volhard, 1992). The first tier of zygotic segmentation genes, the gap genes, initiate the interpretation of these maternal patterning components in two overlapping steps: the primary gap genes respond directly to the maternally provided positional information and then, together with maternally encoded components, direct the expression of the secondary gap genes (reviewed by Hülskamp and Tautz, 1991). In the anterior of the embryo, the *hunchback* (*hb*) gene acts as a primary gap gene, as it responds directly to the gradient of maternally encoded bicoid (bcd) transcription factor; this *hb* expression at the anterior is required for the establishment of a region that later forms the third gnathal segment and the thoracic segments (Lehmann and Nüsslein-Volhard, 1987). In the posterior, where it is expressed in a stripe pattern, *hb* acts as a secondary gap gene, as it responds to the primary gap genes *tailless* (*tll*) and *huckebein* (*hkb*). This posterior expression of *hb* is required for the establishment of a region that later forms the junction of the seventh and eighth abdominal segments (Lehmann and Nüsslein-Volhard, 1987).

As might be inferred from the above summary, the pattern of *hb* transcript accumulation in the early embryo is complex and evolves rapidly (Bender et al., 1988; Tautz et al., 1987; Tautz and Pfeifle, 1989). Maternal *hb* mRNA is present at a low uniform concentration in the embryo until about nuclear cycle 10, when it begins to disappear from the posterior of the embryo, forming a shallow gradient. The first zygotic *hb* expression then commences with a burst of transcription throughout the anterior half of the embryo. By nuclear cycle 14, the resulting broad domain of *hb* mRNA decays and a central stripe appears at 50% egg length (EL; 0% EL is the most posterior position). In some embryos, a weaker *hb* stripe can also be detected at about 75% EL. Slightly later, early in nuclear cycle 14, a cap of *hb* mRNA appears at the posterior of the embryo. This posterior cap disappears rapidly and is replaced by late nuclear cycle 14 with a posterior stripe at about

15% EL. hb protein distribution generally matches the *hb* mRNA expression pattern, with the exception that the translation of maternal *hb* mRNA is restricted to the anterior half of the embryo, rather than reflecting the graded distribution of the transcript (Tautz et al., 1987; Tautz and Pfeifle, 1989). *hb* is expressed, then, in anterior and posterior domains corresponding roughly to the embryonic regions affected in a *hb* mutant (Lehmann and Nusslein-Volhard, 1987).

In addition to the spatial and temporal complexity of its expression, the *hb* gene has two transcription initiation sites, both of which are active in the blastoderm-stage embryo; we refer here to the distal and proximal sites as P1 and P2, respectively. Transcripts from each of these start sites have unique first exons and share a common second exon containing the *hb* coding region (Tautz et al., 1987; Bender et al., 1988; see Fig. 1).

We have investigated the regulation of *hb* expression in the posterior of the embryo. While the control of anterior *hb* expression by maternal bcd is well characterized (Driever and Nüsslein-Volhard, 1989; Driever et al., 1989; Tautz, 1988), much less is known about the zygotic control of *hb* posterior expression; that is, the regulation of *hb* as a secondary gap gene. We have analyzed the expression pattern of the two classes of *hb* transcript and defined the *cis*-regulatory elements that direct posterior *hb* expression. We show that at the posterior of the blastoderm-stage embryo, *hb* transcription is initiated from both the P1 and P2 sites, in contrast to previously published results (Schröder et al., 1988). The initial posterior cap contains only P2-initiated transcripts, while the posterior stripe contains transcripts deriving from both P1 and P2. Using both genomic rescue constructs and promoter-*lacZ* fusion genes, we have identified a 1.4 kilobase (kb) region in the *hb* upstream sequence that is both necessary and sufficient to direct expression in the posterior cap and stripe. We provide evidence that the tll protein directly activates expression of *hb* in the posterior by binding to specific sites in this 1.4 kb enhancer region.

## MATERIALS AND METHODS

### Plasmid construction

*hb* genomic DNA fragments (see Fig. 1) were cloned into the CaSpeR vector (Pirrotta, 1988) to make the KG and KM constructs. The 10E1 cosmid was isolated from the CosPeR library kindly provided by J. Tamkun. The Lac12, Lac8.0, Lac6.6 and Lac5.3 constructs are described in detail by Margolis et al. (1994).

To test the potential enhancer functions of upstream regulatory sequences, we used HZCaSpeR (Margolis et al., 1994), a P element transformation vector containing a basal promoter fused to the *lacZ* reporter. A 1.4 kb *Xba*I-*Sma*I fragment from the *hb* upstream region (see Fig. 1) was cloned into HZCaSpeR to make the HZ1.4 plasmid. Subfragments of the 1.4 kb fragment – a 340 bp *Sma*I-*Rsa*I fragment, a 526 bp *Rsa*I-*Eco*RI fragment and a 555 bp *Eco*RI-*Bgl*II fragment – were each cloned into HZCaSpeR to produce, respectively, the HZ340, HZ526 and HZ555 constructs. (The *Bgl*II terminus of the 555 bp fragment lies 46 bp upstream of the *Xba*I site at the distal end of the 1.4 kb fragment). In all HZCaSpeR constructs, the *hb* sequence was oriented with its downstream end toward the basal promoter.

The heat shock-inducible *hb* construct *Hs-hb* was made by cloning a 2.4 kb *Xba*I fragment containing the entire *hb* coding region (see Fig. 1) into the *Xba*I site downstream of the *Hsp70* promoter in the vector CaSpeR-*Hsp70* (Bang and Posakony, 1992).

### Germline transformation

P element-mediated germline transformation was carried out by standard methods (Rubin and Spradling, 1982), using $w^{1118}$ as the recipient stock. All transformant strains used in these experiments were maintained as homozygous stocks and at least three independent lines were examined in each experiment.

### Cuticle preparations

Larval cuticles were prepared by the method of Struhl (1989). To identify unambiguously the rescued embryos, we constructed the following transformant genotype: *y w; P[w$^+$, hb$^+$]/P[w$^+$, hb$^+$]; hb$^{14F}$ st e/TM3, y$^+$ Ser*. Cuticles of *hb* homozygous progeny embryos from these adults were identified in brightfield by their yellow color, scored in either phase contrast or darkfield for rescue of the *hb* phenotype and photographed in darkfield.

### Heat-shock regimens

Embryos between 1.5 and 2.5 hours of development (25°C) were heat shocked for 30 minutes in a water bath at 37°C [*Hs-tll* experiments (Steingrímsson et al., 1991)] or 35°C (*Hs-hb* experiments), and then aged for a further 60 minutes (*Hs-tll*) or 30 minutes (*Hs-hb*) at 25°C before fixation.

### In situ hybridization

In situ hybridization to embryos using the Genius kit (Boehringer Mannheim) was performed as described by Tautz and Pfeifle (1989) with the modifications of Jiang et al. (1991). After development of the phosphatase reaction, embryos were dehydrated and mounted in Epon resin.

The *hb* antisense RNA probe ('full-length *hb* probe') was synthesized from a 3.35 kb, full-length plasmid cDNA clone of a *hb* P1 transcript (described by Margolis et al., 1994). The distal promoter-specific DNA probe ('P1-specific probe') was prepared by random primed labeling of a 500 bp fragment of the same clone containing only the *hb* P1-specific first exon. The proximal promoter-specific antisense RNA probe ('P2-specific probe') was synthesized from an 8 kb *Xba*I genomic DNA fragment containing the *hb* P2-specific first exon (see Fig. 1) cloned into pGEM4 (Promega Biotec) and linearized at an *Rsa*I site 526 bp from the downstream end. The *lacZ* antisense RNA probe was made from plasmid KSII+*lacZ* (kindly provided by S. Small).

### Mutant stocks and fly culture

The following mutant alleles were employed in this study.

*tor: tor$^{PM}$* (recessive loss-of-function) and *tor$^{4021}$* (dominant gain-of-function) (Schüpbach and Wieschaus, 1986; Klingler et al., 1988).

*tll: tll$^{PGX}$* [*Df(3R)tll$^{PGX}$*], which deletes chromosomal region 100A1,2-100B1,2 and thus defines the *tll* null phenotype; *tll$^{l49}$*, the strongest point mutant; and *tll$^{1}$*, a point mutant slightly weaker than *tll$^{l49}$* (Strecker et al., 1988; Pignoni et al., 1990).

*hkb: hkb$^{2}$* (intermediate strength) (Weigel et al., 1990).

*hb: hb$^{14F}$* and *hb$^{6N47}$* are both null alleles (Jürgens et al., 1984; Lehmann and Nüsslein-Volhard, 1987); *hb$^{D2}$* [*In(3R)hb$^{D2}$ (84B; 85A)*], exhibits both posterior loss-of-function and neomorphic phenotypes [our unpublished results; see Lindsley and Zimm (1992)].

Other chromosomes and mutations are described in Lindsley and Zimm (1992), with the exception of the heat shock-inducible *tll* construct (*Hs-tll*), which is described by Steingrímsson et al. (1991). Flies were grown on standard yeast-cornmeal-molasses-agar medium.

### DNA sequencing

Genomic DNA fragments carried in bacteriophage vectors were subcloned into the Bluescript KS(+) vector (Stratagene) for sequencing as described by Ellis et al. (1990). All reported sequence was determined on both strands. 12.6 kb of genomic DNA sequence for the *hb* region has been submitted to GenBank under accession number U17742.

### DNaseI footprinting

In order to express tll protein in *E. coli*, a DNA fragment encoding the first 113 amino acids of the protein (Pignoni et al., 1990) was generated

by the polymerase chain reaction and cloned into the pET3c expression vector (Rosenberg et al., 1987); the sequence of the *tll* insert was confirmed by DNA sequencing. After transformation of this construct into *E. coli* strain JM109 (DE3), expression of tll protein was induced by the addition of IPTG. Bacterial protein extracts for footprinting were prepared by the procedure of Kadonaga et al. (1987).

DNA fragments to be footprinted were end-labeled and incubated with protein extracts from bacteria carrying either the tll expression plasmid or the parental pET3c vector with no insert. Protein-DNA complexes were treated with DNaseI according to Galas and Schmitz (1978) and then electrophoresed on a 5% polyacrylamide gel. To generate sequence standards, the A+G and C+T sequencing reactions of Maxam and Gilbert (1980) were performed on the same DNA fragments.

## RESULTS

### Promoter-specific patterns of *hb* expression

The location of the distal (P1) and proximal (P2) transcription initiation sites of the *hb* gene are shown in Fig. 1. The existence of two transcription start sites and of two domains (anterior and posterior) of *hb* transcript accumulation raises the question of whether differential initiation at the two sites plays a role in the temporal or spatial regulation of *hb* expression in the blastoderm-stage embryo. We analyzed the early embryonic expression patterns of the two classes of *hb* mRNA by in situ hybridization using transcript-specific probes (Fig. 2). We observed that the initial anterior domain of *hb* mRNA accumulation consists entirely of transcripts from P2 (Fig. 2B), while the later central stripe at 50% EL is composed of both P1 and P2 transcripts (Fig. 2E,F). Similarly, at the posterior, the initial cap of *hb* mRNA consists of transcripts from P2 only (Fig. 2B,C), while the later posterior stripe at 15% EL consists of both P1 and P2 transcripts (Fig. 2E,F). These results contrast with those of Schröder et al. (1988), who reported that the two stripes at 15 and 50% EL include only P1 transcripts. The discrepancy between our results and theirs is probably explained by the increased sensitivity of the enzymatic detection system used for our whole-mount in situ hybridizations (Tautz and Pfeifle, 1989) as compared to the autoradiographic detection used in their study.

### Rescue of the anterior and posterior segmentation defects of *hb* mutants

To investigate the contributions of different portions of *hb* upstream sequence to the normal function of the gene, we used P element-mediated germline transformation (Rubin and Spradling, 1982) to rescue the *hb* mutant phenotype. Three different genomic DNA fragments were tested (designated KM, KG and 10E1; see Fig. 1). All three contain both the P1 and P2 transcription start sites and all fully rescue the anterior segmentation defect of *hb* null embryos (Fig. 3D-F). Only the two larger fragments (KG and 10E1), however, rescue the posterior cuticular defects (Fig. 3E,F). These results indicate that the additional 1.4 kb of DNA present in the KG but not the KM construct (located between 3.0 and 4.4 kb upstream of P1) is necessary for *hb* function in the posterior.

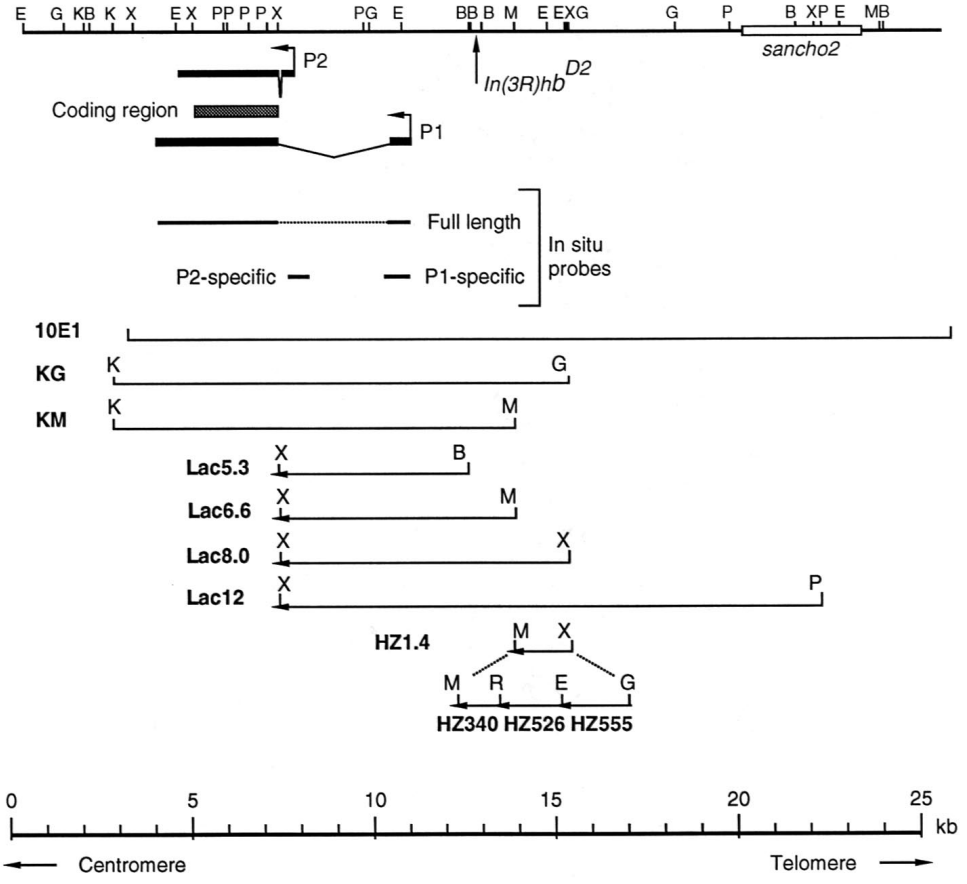


**Fig. 1.** Map of the *hb* genomic region. Bold lines below the restriction map indicate the structure of *hb* transcripts initiating from the proximal (P2) and distal (P1) promoters; shaded box represents the protein coding region, which is common to both classes of transcript (Tautz et al., 1987; Bender et al., 1988; our unpublished observations). Genomic DNA regions included in the various in situ hybridization probes are indicated. Thin lines below this represent the 10E1, KG and KM fragments used for the phenotypic rescue experiments, as well as the eight genomic DNA fragments used in *lacZ* reporter constructs. The position of the distal breakpoint of *In(3R)hb*$^{D2}$ is indicated. The *sancho2* transposable element insertion (Finnegan, 1992) shown upstream of *hb* was identified by Southern blotting and restriction mapping; it is present in DNA of the Canton S strain, but not of the Oregon R strain (our unpublished observations). Single-letter restriction enzyme code: B, *Bam*HI; E, *Eco*RI; G, *Bgl*II; H, *Hin*dIII; K, *Kpn*I; M, *Sma*I; P, *Pst*I; R, *Rsa*I; X, *Xba*I.

Additional evidence consistent with a role for the 1.4 kb fragment in posterior *hb* function is provided by phenotypic and molecular analysis of the $hb^{D2}$ mutation. This allele is associated with a chromosomal inversion that breaks 1.9 kb upstream of P1 (Fig. 1) and thus separates regulatory sequences upstream of this point (including the 1.4 kb fragment defined above) from the rest of the *hb* gene. Embryos bearing $hb^{D2}$ in *trans* to a null allele of *hb* exhibit only the posterior *hb* cuticular defects (Fig. 3C). This analysis demonstrates independently that sequences more than 1.9 kb upstream of P1 are necessary for the function, and presumably for the correct expression, of *hb* in the posterior of the blastoderm-stage embryo.

### A discrete upstream region controls *hb* posterior expression

To identify the transcriptional regulatory elements required for the posterior expression pattern of *hb*, we generated transformant lines carrying constructs in which fragments of *hb* DNA containing the P1 and P2 start sites, plus varying amounts of upstream sequence, were fused to a *lacZ* reporter gene (Fig. 1). The two largest constructs (Lac8.0 and Lac12) drive a pattern of *lacZ* transcript accumulation in the blastoderm-stage embryo that is essentially indistinguishable from that of endogenous *hb* expression (Fig. 4A,B and data not shown). The anterior *bcd*-dependent expression domain, the central stripe, and the posterior cap and stripe patterns all appear. In contrast, the smaller Lac6.6 and Lac5.3 constructs reproduce only the *bcd*-dependent anterior expression (Fig. 4C and data not shown); neither posterior expression nor the central stripe is observed. Thus, the additional 1.4 kb of DNA present in the Lac8.0 construct but not the Lac6.6 construct is essential for expression in the central stripe and for both the cap and stripe phases of posterior expression. This is the same 1.4 kb region identified in the phenotypic rescue assay (Figs 1,3).

To investigate whether this 1.4 kb region is not only necessary but sufficient to direct the posterior expression pattern, we prepared the HZ1.4 construct (Fig. 1). Transformants bearing this construct express *lacZ* in a pattern of central and posterior stripes (Fig. 4D,E) very similar to the stripes of endogenous *hb* expression (Fig. 5K). In the posterior, *lacZ* expression driven by the 1.4 kb fragment first appears in a terminal cap (Fig. 4D) and then develops into a stripe at about 15% EL (Fig. 4E), mirroring the pattern of endogenous *hb* transcript accumulation. Weak central expression driven by the 1.4 kb fragment first appears in early nuclear cycle 14 (Fig. 4D) and then matures into a robust stripe at 50% EL (Fig. 4E). These results indicate that the 1.4 kb fragment contains an enhancer capable of conferring on a heterologous promoter a pattern of expression that closely mimics, both spatially and temporally, the normal *hb* posterior pattern.

To localize more precisely the regulatory elements responsible for directing the posterior *hb* transcription pattern, we divided the 1.4 kb enhancer region into three non-overlapping restriction fragments of 340, 526 and 555 bp, which were used to construct additional *lacZ* reporter genes (Fig. 1). We found that the 340 bp fragment drives a strong central stripe of *lacZ* expression at 50% EL, while in the posterior it generates a distinct stripe of variable intensity at 15% EL, without the prior appearance of a cap (Fig. 4F). The HZ526 construct, by contrast, reproduces the evolving posterior cap of expression observed for *hb* P2 transcripts, but gives no central expression. Initially, an intense cap of *lacZ* transcript appears that extends more anteriorly than does endogenous *hb* posterior expression;

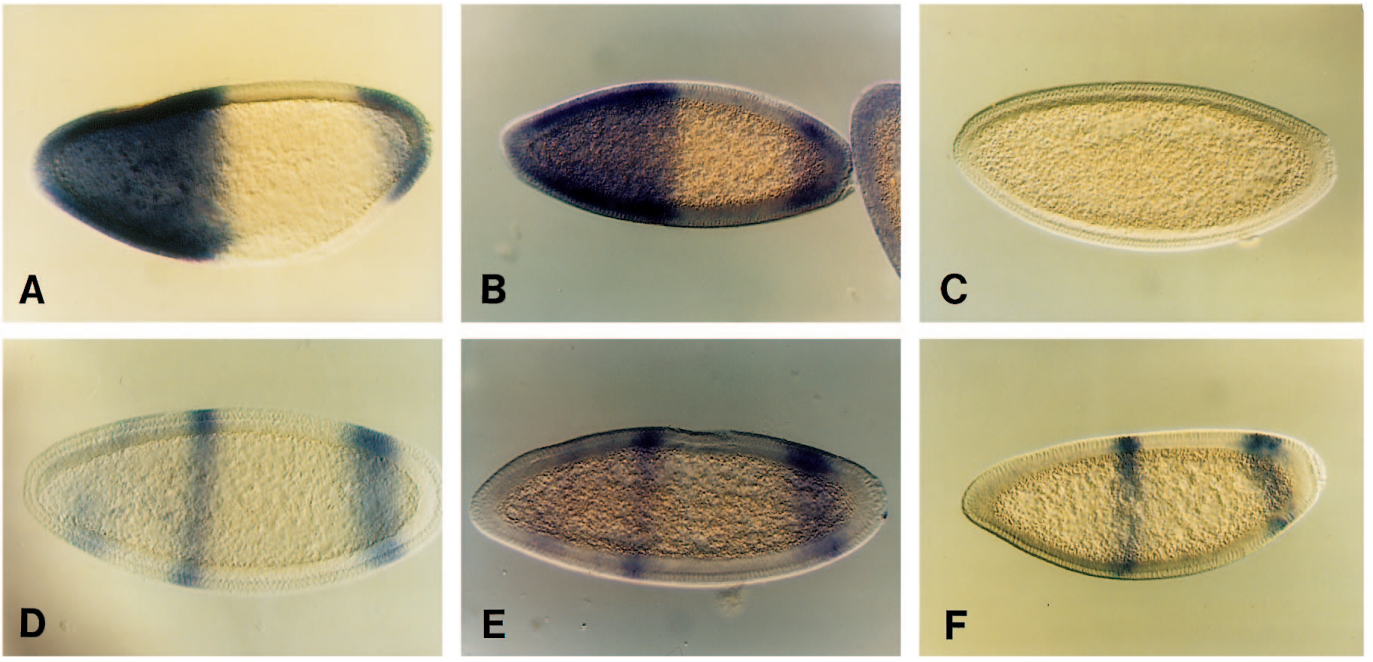


**Fig. 2.** Comparison of total and promoter-specific *hb* transcript distribution in wild-type embryos. Embryos in A-C are at early cycle 14, while those in D-F are at mid- to late cycle 14. (A,D) Embryos hybridized with the full-length *hb* probe. (B,E) Embryos hybridized with the P2-specific probe. (C,F) Embryos hybridized with the P1-specific probe. The posterior cap of *hb* mRNA (A) consists only of P2 transcripts (B,C); the posterior and central stripes (D) include both P1 and P2 transcripts (E,F). Anterior is to the left and dorsal is up. See Fig. 1 for diagram of probes.

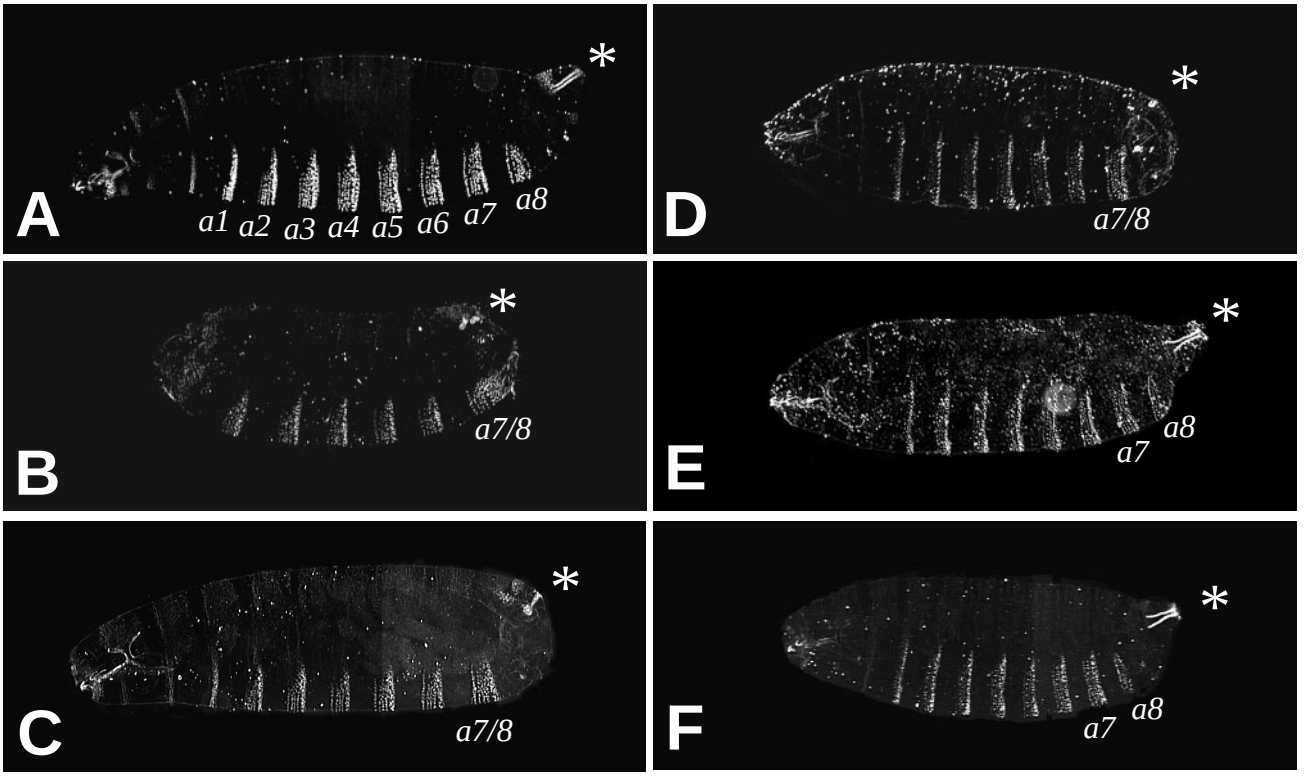


**Fig. 3.** Phenotypic rescue of *hb* mutants. Dark-field micrographs showing lateral or ventrolateral views of cuticle preparations of wild-type, mutant and rescued first-instar larvae. Anterior is to the left, dorsal is toward the top; the Filzkörper are indicated in each panel with an asterisk. Mutant and rescued cuticles (B-F) are $y^-$ and therefore of lower contrast than wild-type cuticle (A). (A) Wild type (Canton S). (B) $hb^{14F}$ homozygote showing the *hb* loss-of-function phenotype: a deletion of the third gnathal segment through the third thoracic segment; a deletion between the seventh and eighth abdominal denticle belts, resulting in their fusion (indicated by 'A7/8'); and reduced Filzkörper. (C) $In(3R)hb^{D2}/hb^{14F}$ embryo showing only the posterior deletion and Filzkörper defect. (D) *y w P[KM, w⁺]/+;* $hb^{14F}$ embryo showing rescue of the anterior (gnathal and thoracic) segmentation defect, but not the posterior (A7/A8) or Filzkörper defects. (E) *y w P[KG, w⁺]/+;* $hb^{14F}$ embryo showing full restoration (both anterior and posterior) of the normal segmental pattern of the cuticle. (F) *y w; P[10E1, w⁺];* $hb^{14F}$ embryo showing full rescue of *hb* segmentation defects. Despite the complete rescue of the *hb* cuticular defects by the KG and 10E1 constructs, *hb* null embryos carrying these transgenes are inviable.

i.e., to about 25% EL (Fig. 4G). This cap becomes refined into a mature posterior stripe at 15% EL (Fig. 4H). However, unlike the normal *hb* posterior stripe, the HZ526 stripe continues to progress anteriorly and to decrease in intensity; it can last be detected at the onset of gastrulation as a thin, weak stripe at approximately 30% EL (Fig. 4I). We were unable to detect any specific expression driven by the HZ555 construct (data not shown).

Since HZ526 reproduces the posterior cap and stripe, while HZ340 gives only the posterior stripe, it appears that the regulatory sequences controlling the early (cap) and late (stripe) phases of posterior expression are partially separable components of the complete posterior enhancer. At the same time, it should be noted that the 526 bp fragment includes all the elements necessary to generate the major features of the normal posterior *hb* expression pattern. Finally, our results indicate that the sequences required for the formation of the central stripe are entirely contained within the 340 bp fragment.

### The 1.4 kb enhancer region mediates regulation by the terminal system

The formation of the *hb* posterior stripe is regulated by the maternal terminal system (Brönner and Jäckle, 1991; Casanova, 1990; Nüsslein-Volhard et al., 1987). To determine whether the HZ1.4 posterior stripe is subject to the same genetic control as that of the endogenous *hb* gene, we examined HZ1.4 expression in embryos derived from mothers lacking *torso* (*tor*) function. These embryos exhibit virtually no posterior expression of either endogenous *hb* transcripts or *lacZ* transcripts from the HZ1.4 construct (Fig. 5A,B). Conversely, in embryos from mothers heterozygous for a dominant *tor* gain-of-function mutation (which causes expansion of the terminal domains), both *hb* and the HZ1.4 transgene show a dramatic expansion of expression, extending over most of the embryo (Fig. 5C,D). Thus, the 1.4 kb enhancer region mediates the same responses to changes in the activity of the terminal system as does the intact *hb* upstream region.

The genes of the maternal terminal system encode the components of a signal transduction pathway; activation of this pathway at the poles of the embryo leads to transcriptional activation of the gap genes *tll* and *hkb* (Brönner and Jackle, 1991; Pignoni et al., 1992; reviewed by St. Johnston and Nüsslein-Volhard, 1992). Both *tll* and *hkb* encode potential DNA-binding transcription factors and so could directly control the expression of downstream target genes (Pignoni et al., 1990; Brönner et al., 1994). Previous genetic studies of the *hb* posterior stripe

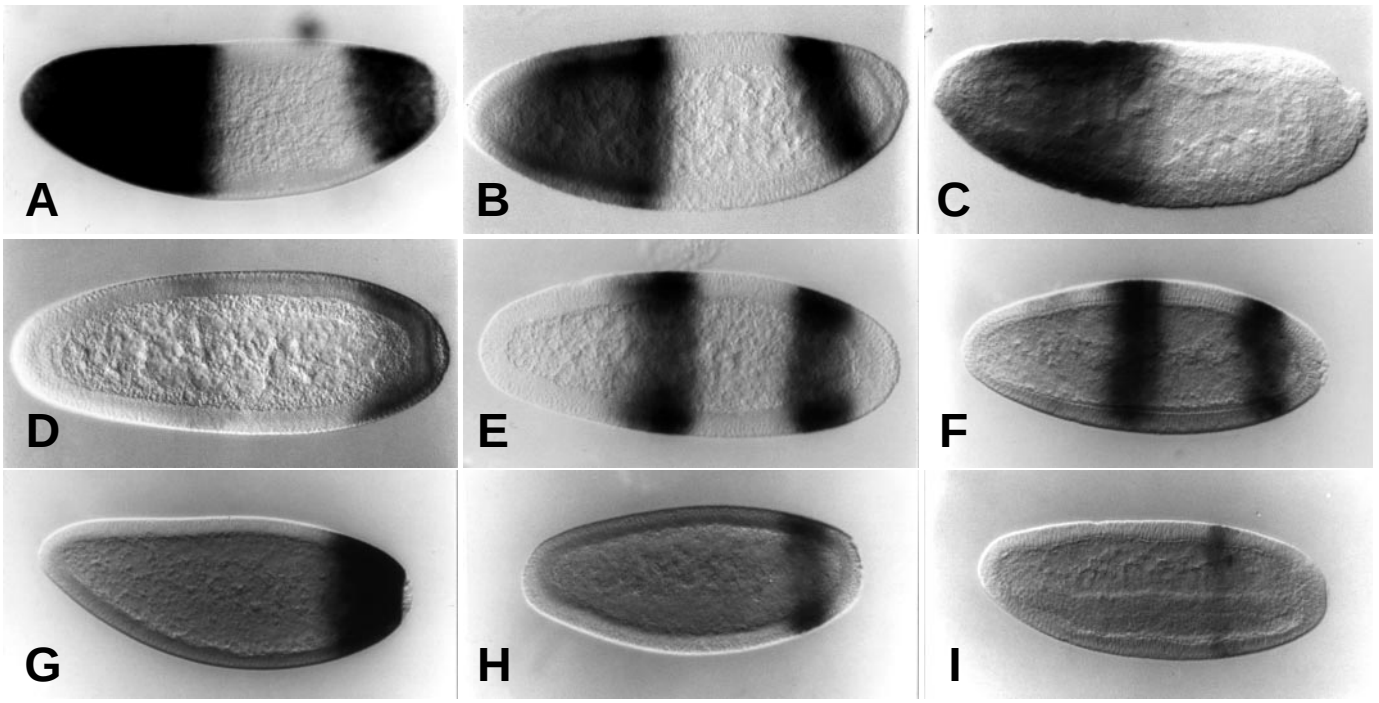


**Fig. 4.** Expression patterns of *lacZ* reporter transgenes, revealed by in situ hybridization with an antisense *lacZ* riboprobe. (A) *Lac8.0* embryo, late stage 4, showing posterior cap expression. (B) *Lac8.0* embryo, stage 6, showing the mature posterior stripe. (C) *Lac6.6* embryo, stage 5; note the absence of both the central and the posterior stripes. (D) *HZ1.4* embryo showing the initial posterior cap and the weak early central expression. (E) Mature posterior and central stripes of HZ1.4 expression. Unlike the endogenous *hb* central stripe, the HZ1.4 central stripe often lacks a sharp anterior border. (F) *HZ340* embryo, late stage 5. (G) *HZ526* embryo, showing the posterior cap. (H) *HZ526* embryo, showing the normal posterior stripe. (I) *HZ526* embryo, showing abnormally anterior final position of posterior stripe. Transformants carrying the HZCaSpeR vector alone show no specific expression of the *lacZ* reporter gene (data not shown).

have implicated *tll* as the activator and *hkb* as the repressor of *hb* expression in this part of the embryo (Tautz, 1988; Casanova, 1990; Brönner and Jäckle, 1991; Steingrímsson et al., 1991). To determine whether the *tll* and *hkb* genes exert their regulatory effects through the 1.4 kb posterior enhancer region, we tested the response of the HZ1.4 construct to mutations at these loci. Loss of *tll* function causes the loss of virtually all posterior expression of the endogenous *hb* gene, and the HZ1.4 reporter exhibits the same response (Casanova, 1990; Brönner and Jäckle, 1991; Fig. 5E,F; data not shown). Conversely, ectopic expression of *tll* under the control of an *Hsp70* promoter leads to a marked expansion, into the central domain of the embryo, of *hb* transcript accumulation (Steingrímsson et al., 1991; Fig. 5G). Again, the HZ1.4 reporter behaves similarly (Fig. 5H). These results indicate that activation of *hb* transcription by *tll* is mediated through the 1.4 kb posterior enhancer region. The HZ1.4 reporter construct also shows the same response as endogenous *hb* to loss of *hkb* function, exhibiting a broadened cap of expression that does not resolve into a stripe (Fig. 5I,J). In addition, it is worth noting that the central stripe of endogeneous *hb* expression and of *lacZ* expression from HZ1.4 is significantly broadened in *hkb* mutant embryos (Fig. 5I,J), indicating that the 1.4 kb enhancer region also mediates regulation of the central stripe by *hkb*.

### The 1.4 kb enhancer region appears to mediate direct regulation by the tll protein

The tll protein is a member of the nuclear receptor superfamily and contains a canonical nuclear receptor DNA-binding domain with two zinc fingers (Pignoni et al., 1990). We expressed the predicted DNA-binding portion of the tll protein (see Materials and Methods) in *E. coli* and used it in a DNase I footprinting assay (Galas and Schmitz, 1978) with the 555, 526 and 340 bp fragments of the 1.4 kb posterior enhancer region. Strong, medium and weak binding sites were identified in the 526 and 340 bp fragments, while only two weak sites were detected in the 555 bp fragment (Fig. 6). The strongest tll-binding sites in these fragments (approximately at positions −3600 and −3130) resemble the tll consensus binding site (AAAAGTCAA) identified in the *knirps* upstream region by Pankratz et al. (1992).

### The 1.4 kb enhancer region mediates *hb* autoregulation

Examining the response of the Lac12 and HZ1.4 constructs to changes in zygotic *hb* function revealed a role for *hb* in regulating its own expression. In embryos lacking zygotic *hb* activity, the central stripe of expression of both constructs is greatly reduced or abolished, while the posterior stripe is expanded and intensified (Fig. 7A,B). The opposite effect was observed when *hb* was overexpressed uniformly under the control of an *Hsp70* promoter: the posterior stripe was very greatly diminished and a strong central stripe appeared (Fig. 7C,D). These results indicate that *hb* autoactivates its expression in the central stripe and autorepresses its posterior stripe expression.

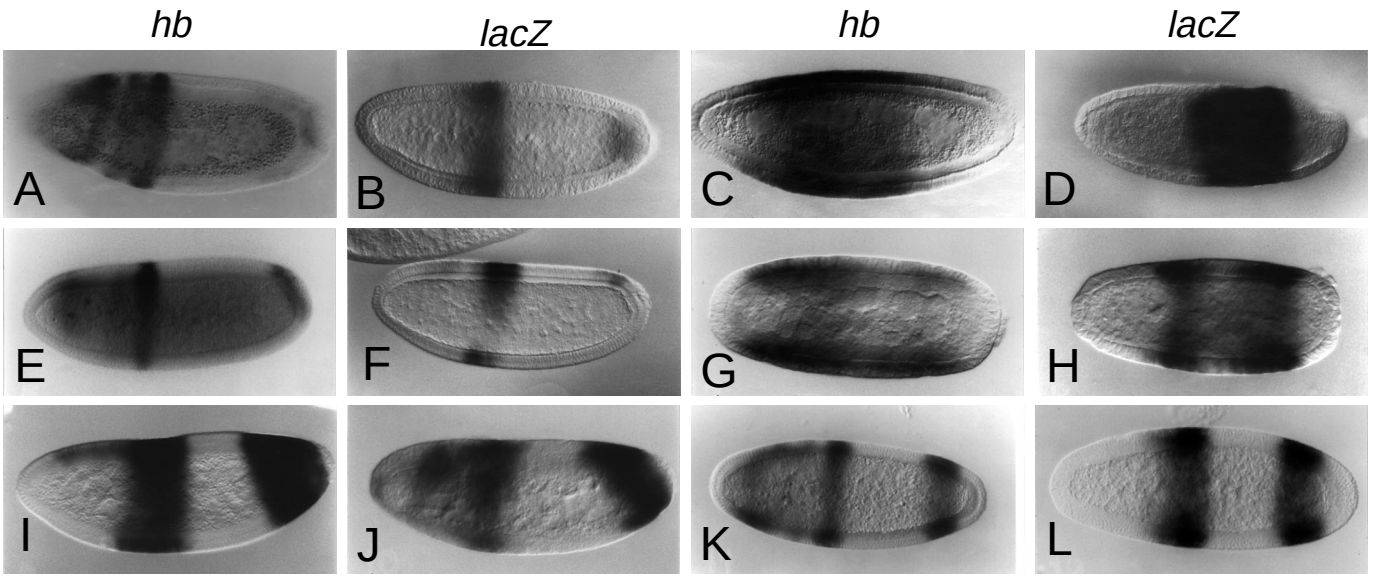


**Fig. 5.** Control of *hb* posterior stripe expression by the terminal system. Expression patterns of the endogenous *hb* gene (A,C,E,G,I,K) and of the HZ1.4 transgene (B,D,F,H,J,L) were detected by in situ hybridization. (A,B) Embryos derived from a cross of $tor^{PM}$ females to *HZ1.4* males. (C,D) Embryos derived from a cross between females heterozygous for $tor^{4021}$ and *HZ1.4* males. (E,F) Embryos of the genotype *HZ1.4; Df(3R)*$tll^{PGX}$. The residual posterior expression of both *hb* and the HZ1.4 transgene frequently observed in embryos of this genotype implies that *tll* is not the sole activator of posterior *hb* expression. (G,H) Embryos of the genotype *HZ1.4/+; Hs-tll/+*. (I,J) Embryos of the genotype *HZ1.4;* $hkb^2$. (K) Wild-type embryo. (L) *HZ1.4* embryo.

## DISCUSSION

We have identified and characterized a 1.4 kb region of *hb* upstream sequence that is both necessary and sufficient for the normal expression and function of the gene in the posterior of the blastoderm-stage embryo. The capacity of this region to confer posterior stripe expression on a heterologous promoter indicates that it contains a discrete transcriptional enhancer that directs the formation of the *hb* posterior stripe. The genes *tll*, *hkb* and, unexpectedly, *hb* itself act through this enhancer to control posterior *hb* expression. Since the tll protein binds in vitro to specific sites within this region, it is likely that tll is a direct transcriptional activator of *hb* in the posterior. The pattern and regulation of zygotic *hb* expression in the early embryo is summarized in Fig. 8.

### Control of *hb* posterior expression by the 1.4 kb enhancer region

Several lines of evidence support our conclusion that the 1.4 kb region is necessary for both expression and function of the *hb* gene. First, only genomic DNA fragments that include this region rescue both the anterior and posterior segmentation defects of *hb* null mutants; otherwise identical constructs lacking the 1.4 kb region rescue only the anterior defects. Second, the $hb^{D2}$ mutation, an inversion that separates *hb* upstream sequences (including the 1.4 kb region) from the rest of the gene, causes a similar loss of posterior function. Third, only *hb* promoter-reporter fusion constructs that include the 1.4 kb region recapitulate the *hb* posterior expression pattern. It is clear, then, that *cis*-regulatory sequences in the 1.4 kb region are essential for the expression of *hb* in the posterior of the blastoderm-stage embryo and that this expression is required for normal development of the 7th and 8th abdominal segments.

We also find that the 1.4 kb region is sufficient to direct reporter gene expression from a heterologous promoter in a pattern that mimics *hb* posterior expression. This is the basis for our further conclusion that this region contains the *hb* posterior stripe enhancer. Not only is the 1.4 kb enhancer region capable of reproducing the *hb* posterior expression pattern, it also responds like the endogenous *hb* gene to alterations in the activity of genes that control formation of the *hb* posterior stripe. First, as with *hb* itself, posterior expression of the HZ1.4 construct requires *tor* function and is expanded in *tor* gain-of-function mutants. Second, posterior expression of HZ1.4 requires *tll* activity, is expanded when *tll* is expressed ectopically and is repressed by *hkb*, again mimicking *hb*. Third, HZ1.4 expression is repressed in the posterior by *hb*, as is the Lac12 *hb* promoter-*lacZ* fusion gene. Fourth, in embryos derived from *bcd* mothers, the HZ1.4 construct, like *hb* itself (Tautz, 1988), exhibits a 'posterior stripe' pattern of expression at both embryonic termini (our unpublished results), suggesting that wild-type *bcd* activity overrides *tll* activation of *hb* at the anterior pole. The parallel responses of the HZ1.4 construct and the endogenous *hb* gene (or *hb* promoter-*lacZ* fusion genes) to alterations in activity of the maternal regulators *bcd* and *tor* and the zygotic gap genes *tll*, *hkb* and *hb* indicate that the 1.4 kb enhancer region contains all the response elements necessary to generate a posterior stripe.

A recent study by Lukowitz et al. (1994) obtained results consistent with ours; i.e., that a 1.1 kb genomic DNA fragment that includes the 526 and 340 bp fragments of the 1.4 kb region drives *lacZ* expression in the pattern of the posterior and central *hb* stripes. Within this 1.1 kb is a 650 bp island of sequence that is conserved betweeen *Drosophila melanogaster* and *D. virilis* (Lukowitz et al., 1994); most of this conserved sequence is present in the 526 and 340 bp fragments studied in detail here.

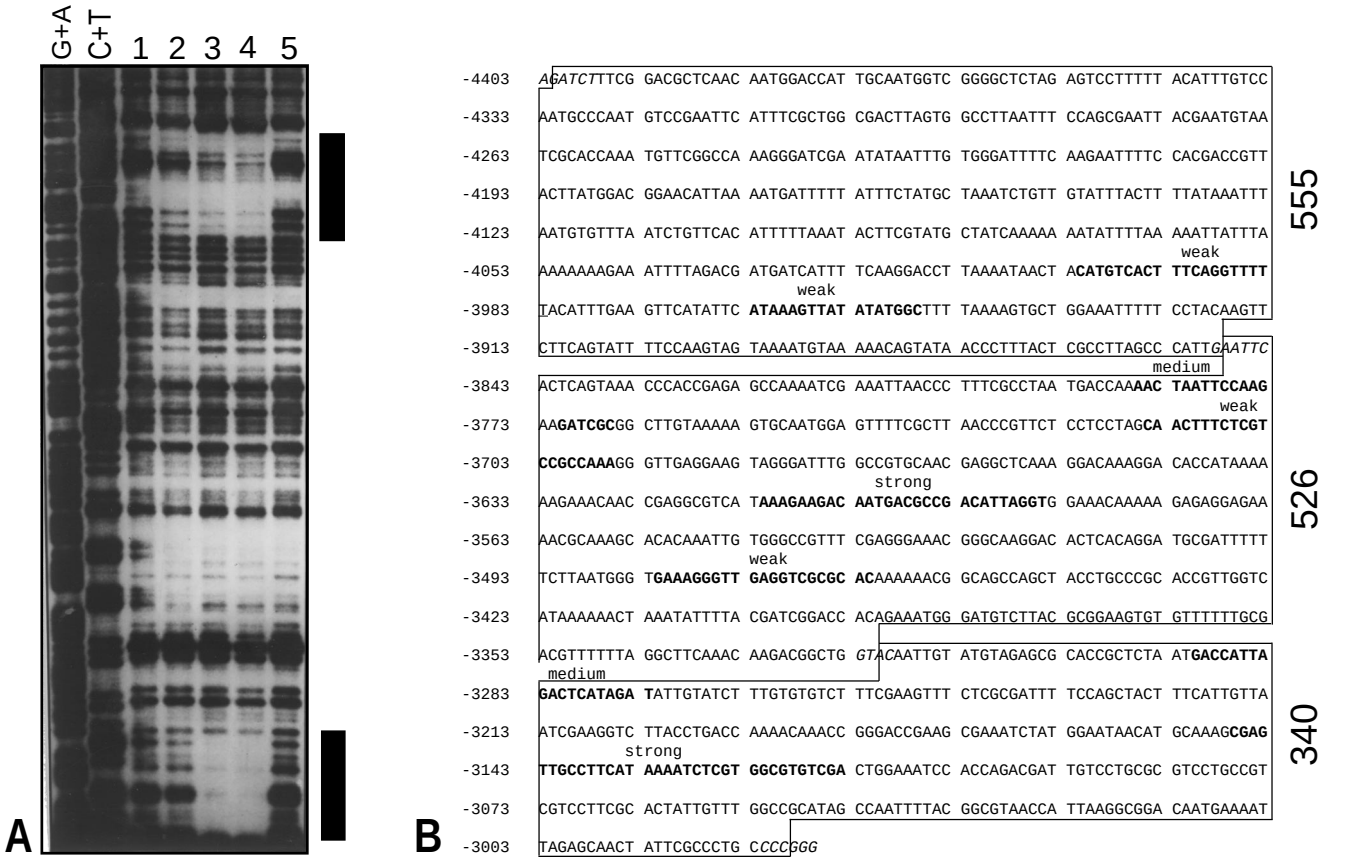


**Fig. 6.** The tll protein binds to specific sequences in the 1.4 kb posterior enhancer region. Binding sites for the DNA-binding domain of the tll protein were identified by DNase I footprinting. (A) Representative results, obtained with the 340 bp subfragment of the enhancer (see Fig. 1). Black bars represent regions protected from DNase I. Lane 1: no protein added; lanes 2-4: 4, 10, 20 μg of protein extract from bacteria expressing the tll DNA-binding domain; lane 5: 20 μg of protein extract from control bacteria not expressing tll protein. (B) Sequence of the 1.4 kb enhancer region, showing the restriction sites (italics) used to generate the 555, 526 and 340 bp subfragments. In vitro binding sites for the tll protein are shown in bold and designated as weak, medium, or strong. The strong site at approximately –3130 in the 340 bp subfragment corresponds to the lower footprint (black bar) in (A); the medium site (approximately –3280) corresponds to the upper footprint. The sequence is numbered with respect to the first transcribed nucleotide (+1) of the P1 (distal) transcription start.

## Molecular control of *hb* posterior expression

Our results show that *tll* activity controls posterior *hb* expression through the 1.4 kb enhancer region and that the tll protein binds to this region in vitro in a sequence-specific manner. The presence of strong tll binding sites in both the 526 and 340 bp fragments (each of which is sufficient to direct some aspect of posterior expression) and their absence in the 555 bp fragment (which by itself was inactive in our in vivo assays) suggests that tll activates posterior *hb* expression directly through these sites in vivo. Although *tll* gene activity is required for both positive and negative regulation of specific genes (Mahoney and Lengyel, 1987; E. S. and J. A. L., unpublished observations), in vitro DNA-binding assays have so far supported a direct role for tll protein only in repression (Hoch et al., 1992; Pankratz et al., 1992; Qian et al., 1993). Our results are the first to indicate that tll can function as a direct transcriptional activator.

Overall, the tll binding sites in the *hb* posterior enhancer are of lower affinity than those in the element driving the seventh stripe of *hairy* (*h*) expression in response to *tll* and those responsible for the repression of *kni* by *tll* (E. S. and J. A. L., unpublished observations). *h* and *kni* are regulated by *tll* at positions more anterior than the posterior *hb* stripe; presumably, higher-affinity tll binding sites are required to allow the lower concentrations of tll protein at these positions (Pignoni et al., 1992) to exert a regulatory effect. The correlation between binding site affinity and the position in a morphogen gradient where a threshold response occurs has been pointed out for the regulation of the *zen* and *twist* genes by the dorsal protein gradient (Jiang and Levine, 1993).

*hb* autoregulation and regulation of *hb* by *hkb* and *bcd* are all mediated by the 1.4 kb posterior enhancer region. It is possible that the hb, hkb and bcd proteins control *hb* expression directly by binding to target sequences within the 1.4 kb enhancer. As pointed out by Lukowitz et al. (1994), this region contains a number of matches to the consensus binding site for hb protein. It should be noted, however, that the one sequence indicated by Lukowitz et al. as a possible tll binding site was not identified in our footprinting assay.

## Complexity of *hb* posterior expression

Previous analyses have interpreted the posterior expression of *hb* as a cap that is activated by tll and then repressed by hkb at the posterior pole to form a stripe (Brönner and Jäckle, 1991;

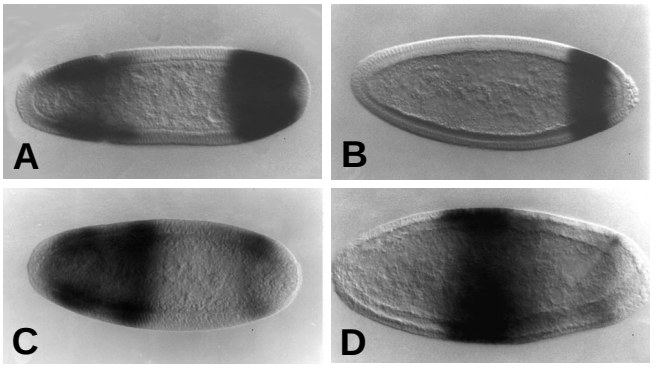


**Fig. 7.** Effect of loss and excess of *hb* function on expression of the Lac12 and HZ1.4 transgenes provides evidence for *hb* autoregulation. (A) Stage 6 embryo of the genotype *Lac12; hb$^{14F}$*. (B) Stage 5 embryo of the genotype *HZ1.4; hb$^{6N47}$*. (A,B) A central stripe of expression is lacking in these embryos and the posterior stripe is both expanded along the anterior-posterior axis and more intense than the posterior stripe in *hb*/+ and +/+ sib embryos (not shown). In addition, anterior expression of the Lac12 construct appears to persist longer than in wild-type embryos (A). (C,D) Double heterozygote embryos carrying one copy each of the Lac12 (C) or HZ1.4 (D) reporter gene and a *Hs-hb* gene, subjected to heat shock. These embryos exhibit expression patterns complementary to those shown in A and B: the central stripe is greatly enhanced (both expanded along the anterior-posterior axis and more intense) and the posterior stripe is severely reduced or eliminated. These expression patterns in *hb* mutant embryos may be compared to the corresponding patterns in wild-type embryos: For Lac12, expression in wild type is the same as that exhibited by Lac8.0 (see Fig. 4A,B); for HZ1.4, see Fig. 4D,E.

Casanova, 1990). Our data are consistent with this model, but show that the control of posterior *hb* expression is more complex.

First, tll and hkb are not the only regulators of posterior *hb* expression. As shown by the responses of the HZ1.4 construct to loss of *hb* function and overexpression of *hb*, *hb* negatively regulates its own expression in the vicinity of the posterior stripe. Also, in embryos homozygous for *tll* deficiencies, and thus totally lacking *tll* function, we still observe weak posterior expression of *hb* and *hb-lacZ* fusion genes. This suggests the existence of an additional activity in the blastoderm-stage embryo that can contribute to activation of *hb* expression in the posterior.

Second, the *hb* posterior cap and stripe are not just two temporal phases of the pattern; rather, each appears to be controlled and generated independently. Our promoter-specific in situ hybridization analysis revealed that the posterior cap expression is composed only of P2 transcripts, while the posterior stripe contains both P1 and P2 transcripts. When we assayed the regulatory capabilities of subfragments of the 1.4 kb posterior enhancer region, we found that the 526 bp fragment directed transcription in both the posterior cap and the stripe, while the 340 bp fragment directed transcription only in the posterior stripe. These results suggest that the 526 bp fragment directs transcription from the P2 promoter in both the posterior cap and stripe domains, while the 340 bp fragment directs transcription from the P1 promoter in the posterior stripe domain only.

## Establishing boundaries of gene expression

Studies of how spatially restricted patterns of gene activity are established in the early *Drosophila* embryo have revealed two general mechanisms for delimiting borders of gene expression. First, a border can be defined by a certain threshold concentration of a transcriptional activator, below which the target gene is not activated. Examples of this are the determination of the posterior border of *hb* anterior domain expression by a specific concentration of bcd protein, and the determination of the subterminal borders of *tll* and *hkb* expression by specific activation levels of the maternal terminal system (reviewed by Hülskamp and Tautz, 1991). In the second mechanism, a gene is activated in a rather broad domain; active repression on either side of this domain then establishes sharp borders. The best studied example of this is the second stripe of *even-skipped* (*eve*) expression, which results from activation by hb

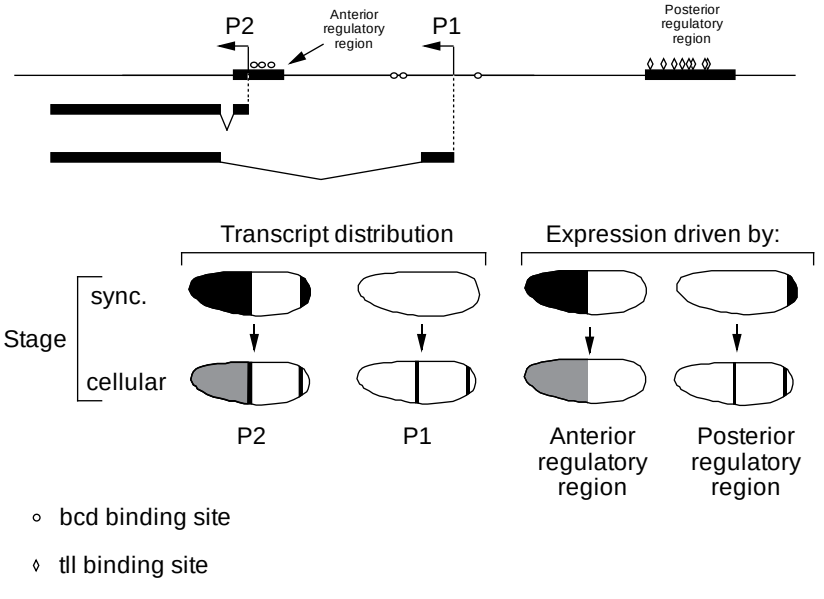


**Fig. 8.** Summary of the pattern and regulation of zygotic *hb* expression in the early embryo. The *hb* genomic region and transcript structure are shown schematically. Below (left) are shown the spatial distributions of P1 and P2 transcripts at the syncytial and cellular blastoderm stages. To the right, the components of this expression pattern are grouped on the basis of the regulatory regions that drive them. The posterior regulatory region (defined in this paper as the 1.4 kb posterior enhancer) directs expression in the posterior cap and stripe as well as the central stripe. The tll binding sites we have identified in this region are indicated by diamonds above the posterior regulatory region. The anterior regulatory region drives *hb* expression across the anterior half of the embryo in response to the maternal bcd gradient (Driever and Nüsslein-Volhard, 1989; Schröder et al., 1988). The bcd binding sites identified by Driever and Nüsslein-Volhard (1989) are marked by circles.

and bcd in a broad domain, followed by repression at the anterior and posterior borders by giant and Krüppel, respectively (Stanojevic et al., 1991).

Regulation of the posterior *hb* stripe appears to incorporate both of these mechanisms. There are no known genes that function to control the anterior border of the stripe; rather, this border appears to be established by a threshold concentration of the activating tll protein. The posterior border, in contrast, appears on the basis of genetic evidence to be established by repression by the hkb protein. These features of the regulation of *hb* posterior stripe expression in the anterior-posterior axis are similar to the generation of the lateral stripe of *rhomboid* (*rho*) expression along the dorsal-ventral axis. *rho* is activated and its lateral border established by a threshold concentration of dorsal protein; the ventral border of *rho* expression is established by repression by *snail* (*sna*) (Ip et al., 1992). Another similarity that relates posterior *hb*, lateral *rho* and *eve* stripe 2 regulation is that the *cis*-regulatory elements required to establish each of these stripes can be mapped to a discrete enhancer module of only several hundred base pairs (Fig. 1; Stanojevic et al., 1991; Ip et al., 1992). In the case of *rho* and *eve* stripe 2, these modules contain binding sites for both activators and repressors. We have demonstrated the presence of strong binding sites for the tll activator in the 526 and 340 bp fragments of the *hb* posterior enhancer; we predict that the hkb protein binds to sites in both of these elements.

### Evolution of *hb* blastoderm-stage expression

It is interesting to note that the two poles of the embryo show similar patterns of *hb* P1 and P2 promoter activity (Fig. 8): the posterior cap and the anterior *bcd*-dependent domain both contain only P2 transcripts, while the later posterior and central stripes (both formed at the non-polar edges of the initial broad domains of *hb* expression) are composed of both P1 and P2 transcripts. This correlation suggests that one of the two transcription initiation sites may have evolved later than the other, in concert with an addition to the expression pattern of either the anterior domain and the posterior cap, or of the central and posterior stripes. Since all four of these domains of expression are also observed in embryos of *Musca domestica* (Sommer and Tautz, 1991), analysis of *hb* gene expression and structure in more distantly related insects might shed light on this question.

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