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Differential regulation of the two transcripts from the *Drosophila* gap segmentation gene *hunchback*

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The *Drosophila* gap gene *hunchback* (*hb*) is required for the establishment of the anterior segment pattern of the embryo, and also for a small region of the posterior segment pattern. The *hb* gene encodes two transcripts from two promoters which show a differential regulation, although they code for the same protein product. The 3.2-kb transcript is expressed during oogenesis and forms an anterior–posterior gradient during the early stages of development. The first zygotic expression of *hb* during cleavage stages 11–12 is due to the 2.9-kb transcript. Its expression is under the control of the anterior pattern organizer gene *bicoid* (*bcd*) and it appears to be necessary and sufficient for the anterior segmentation. The 3.2-kb transcript is expressed again at syncytial blastoderm stage in the anterior yolk nuclei, as well as in an anterior stripe which is posteriorly adjacent to the domain of the 2.9-kb transcript, and as a posterior stripe. Using *hb*-promoter/*lacZ* fusion gene constructs in combination with germ line transformation, we have delimited a regulatory region for the 2.9-kb transcript to ~300 bp upstream of the site of transcription initiation and show that this region is sufficient to confer the full regulation by *bcd*.

Key words: hunchback/transcription regulation/segmentation gene/maternal control

Introduction

The anterior segment pattern, the posterior segment pattern and the terminal regions of the *Drosophila* embryo are organized by three groups of maternal effect genes (Nüsslein-Volhard *et al.*, 1987), which regulate the expression of the gap class of segmentation genes at defined positions along the longitudinal axis of the embryo (Knipple *et al.*, 1985; Gaul and Jäckle, 1987; Tautz *et al.*, 1987; Tautz, 1988). Gap gene activities are thought to co-ordinate the spatial patterns of the pair rule genes at the next level of the segmentation gene hierarchy, and to define the early domains of homeotic gene expression (reviewed in Akam, 1987).

Anterior segmentation of the embryo requires the maternal activity of the genes *bicoid* (*bcd*), *exuperantia* (*exu*) and *swallow* (*swa*) (reviewed in Nüsslein-Volhard *et al.*, 1987) and the zygotic activity of the gap gene *hunchback* (*hb*) (Lehmann and Nüsslein-Volhard, 1986; Bender *et al.*, 1986). The *bcd* protein forms a morphogenetic gradient in the early embryo (Driever and Nüsslein-Volhard, 1988a), while both *exu* and *swa* appear to be required for the correct localization of the *bcd* activity (Frohnhöfer and Nüsslein-Volhard, 1987; Driever and Nüsslein-Volhard, 1988b). All three genes were tested for their effects on the *hb* gene expression and it was shown that only *bcd* is necessary for the zygotic activation of *hb* (Tautz, 1988). This and previous genetic evidence suggested a direct regulatory effect of *bcd* on *hb* (Frohnhöfer and Nüsslein-Volhard, 1986; Tautz, 1988).

The *hb* gene encodes two transcripts of 3.2 kb and 2.9 kb, which are under the control of separate promoters (Tautz *et al.*, 1987). We have examined the early spatial and temporal patterns of expression of the two *hb* transcripts by *in situ* hybridization with transcript-specific probes. The results suggest that the expression of the 2.9-kb transcript is responsible for the formation of most of the anterior pattern. In this paper we have therefore focused on the regulation of this transcript. We have fused various parts of its promoter region to the *Escherichia coli lacZ* gene and have produced germline transformants with these constructs, an approach which was first exploited for the segmentation gene *fushi tarazu* (Hiromi *et al.*, 1985). We have delimited the *cis*-regulatory control region required for the normal expression of the 2.9-kb transcript to a fragment containing ~300 bp upstream and ~400 bp downstream of its transcription start site. This region is sufficient for the apparently normal zygotic activation of this transcript under the control of *bcd*.

Results

Differential expression of the two hb transcripts

We have examined the spatial and temporal patterns of expression of the two *hb* transcripts by *in situ* hybridization with transcript-specific probes (see Figure 1) to tissue sections of early embryos. The 3.2-kb transcript-specific probe detects the maternal *hb* transcripts, which are at first homogeneously distributed throughout the egg (Figure 2a). Shortly after they form an anterior–posterior gradient (Figure 2b) and eventually disappear before the blastoderm stage (Figure 2c). The 2.9-kb transcript can be first detected at stage 11–12 (staging refers to nuclear division cycles, Foe and Alberts, 1983) in the anterior half of the embryo and in the yolk nuclei in this region (Figure 2d). The 3.2-kb transcript can be detected again at stage 13–14, where it forms an anterior stripe at ~53% egg length and a posterior stripe. Interestingly, it is also expressed in the anterior yolk nuclei (Figure 2e and f). The anterior stripe of the 3.2-kb transcript is roughly located at the position of the second thoracic segment in the blastoderm fate map (Figure 2e,f) and is presumably the one which overlaps the expression domain of the gap gene *Krüppel* (*Kr*) at this stage (Tautz *et al.*, 1987). Its exact position and width has, however, yet to be determined in relation to other markers. The posterior stripe is in the region of abdominal segments 7 and 8, which are the segments that are fused in *hb* mutant larvae (Lehmann

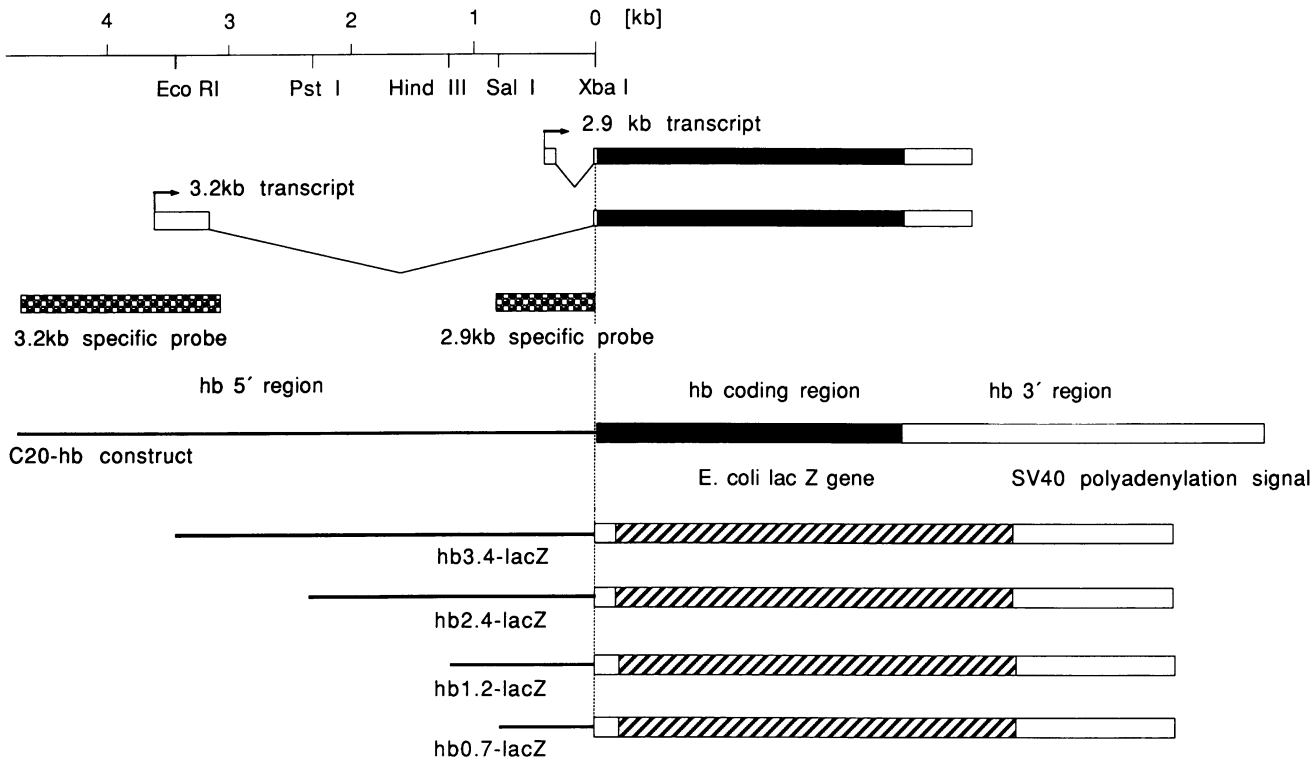

Fig. 1. *hb* transcripts for fusion gene transcripts. The structure of the two *hb* promoters and transcripts, which have been described earlier (Tautz *et al.*, 1987), is depicted at the top of the figure. The open boxes represent the non-translated regions, the coding region is drawn in black. The introns are indicated by thin lines. The two stippled boxes below indicate the DNA fragments which were used for the *in situ* hybridizations. The extent of the various constructs is shown underneath. The C20-*hb* construct is entirely derived from sequences of the *hb* region. The four fusion gene constructs consist of *hb* sequences (lines), *E.coli lacZ* gene sequence (cross-hatched) and SV40 sequence (open boxes).

and Nüsslein-Volhard, 1986; Bender *et al.*, 1987). While the 2.9-kb transcript disappears from the embryos with the beginning of gastrulation, the 3.2-kb transcript remains throughout germ band extension (Tautz *et al.*, 1987). Hybridization of the 3.2-kb transcript probe to *bcd* mutant embryos is shown in Figure 2g and h. The expression of the transcript is shifted towards anterior weak *bcd* alleles (Figure 2g) and replaced by an inverted posterior stripe in strong *bcd* alleles (Figure 2h). Antibodies directed against the *hb* protein (Tautz, 1988) showed that *hb* protein can be detected in the same regions where the *hb* transcripts are present, indicating that both transcripts are translated during blastoderm stage.

Differential functions of the two hb transcripts

The DNA segment encoding the *hb* gene was initially identified by P-element-mediated germline transformation and partial rescue of the *hb* mutation. This P-element construct (C20-*hb*) contains all transcribed regions, both promoters and ~1.5 kb of sequences upstream of the 3.2-kb transcription start site (Figure 1; Tautz *et al.*, 1987). Embryos of the hb^-;C20-hb^+ genotype show a fusion of abdominal segments 7 and 8 and the majority of them have defects in the second thoracic segment. Staining of embryos of the hb^-;C20-hb^+ genotype with the *hb* antibody shows that the zygotic *hb* protein expression occurs only in the anterior, and not in the posterior domain of these embryos. In addition, the broadening of the anterior domain at late blastoderm stage, which is presumably due to the expression of the 3.2-kb transcript (see above), is not observed (Figure 3c and d).

Combined with the evidence from the *in situ* hybridizations shown above, this suggests that the C20-*hb* construct fails to express the 3.2-kb RNA correctly. The phenotypic effects seen in the hb^-;C20-hb^+ larvae therefore allows one to assign differential functions to the two *hb* transcripts. The 3.2-kb transcript has several functions: first the maternal expression, since it is the only transcript produced maternally (Tautz *et al.*, 1987); secondly, a function required for the correct formation of abdominal segments 7 and 8, since these are missing in the embryos of the hb^-;C20-hb^+ genotype; and third, an accessory function for the formation of the second thoracic segment, since this is affected in most of the embryos of the hb^-;C20-hb^+ genotype. The essential gap gene function required for the development of the anterior segment pattern seems to rest, however, on the 2.9-kb transcript, since its normal activation in the hb^-;C20-hb^+ embryos can rescue the anterior segment pattern.

Delimiting the regulatory control region of the 2.9-kb transcript

In order to delimit the regulatory sequence region necessary for the normal expression of the 2.9-kb transcript, we fused sequences upstream of the coding region of the *hb* gene to the *E.coli lacZ* gene in the P-element transformation vector Carnegie 20 (Figure 1). All constructs contain *hb* fragments ending at the *XbaI* site which lies 3′ to the splice acceptor site of the intron, but 5′ of the AUG initiation codon (Figure 1). The constructs therefore contain most of the 5′ untranslated leader sequence and the small intron of the 2.9-kb transcript. The *hb*3.4−*lacZ* fusion gene contains an

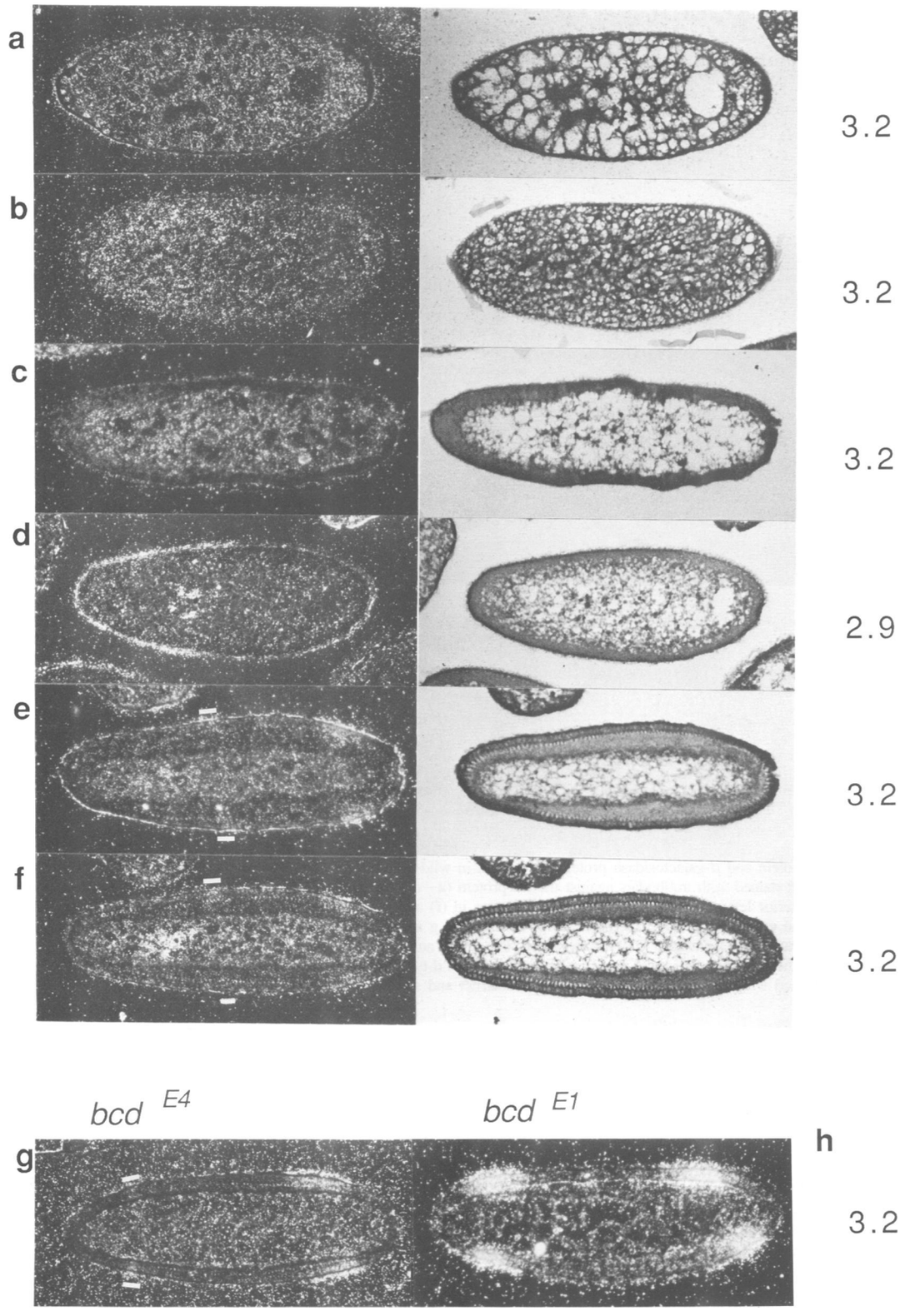

Fig. 2. *In situ* hybridizations with the transcript-specific probes. Pairwise dark-field images (left) and bright-field images (right) of the hybridized sections are shown. The sections in **a**, **b**, **c**, **e** and **f** were hybridized with the 3.2-kb transcript-specific probe, the section in **d** with the 2.9-kb transcript-specific probe. The orientation is anterior to the left and dorsal at the top. (**a**) Embryo prior to stage 4, showing a uniform distribution of the maternal *hb* RNA. (**b**) Embryo prior to stage 8 (pole cell formation), showing the formation of the gradient. (**c**) Embryo at stage 13, showing the virtual absence of the 3.2-kb transcript. (**d**) Embryo at stage 12, showing the zygotic expression of the 2.9-kb transcript. (**e**,**f**) Parallel sections of the same embryo at late stage 14, showing the first zygotic expression of the 3.2-kb transcript in an anterior stripe (indicated with white bars) and in the posterior stripe. The strong hybridization of the yolk nuclei in the anterior region is particularly evident in (f). (**g**) Embryo at stage 14 homozygous mutant for the weak *bcd* allele bcd^{E4} and hybridized with the 3.2-kb-specific probe. (**h**) Embryo at stage 14 homozygous mutant for the strong *bcd* allele bcd^{E1} and hybridized with the 3.2-kb-specific probe.

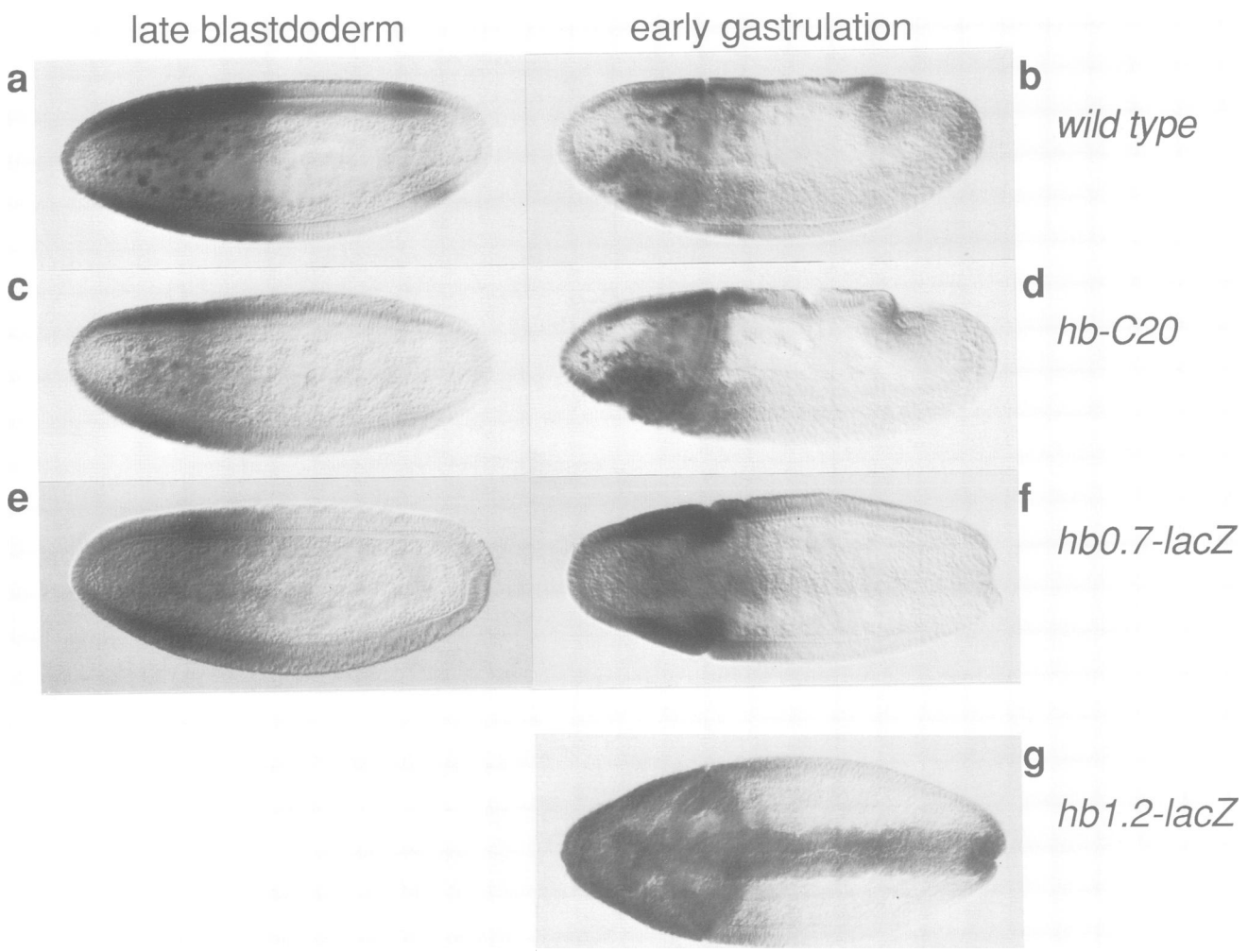

Fig. 3. Comparison of *hb* protein and β-galactosidase protein expression in wild-type and in *hb* mutant embryos containing the C20-*hb* gene construct. The embryos were stained with antibodies against the *hb* protein (**a**–**d**), or with antibodies against the β-galactosidase protein (**e**–**g**). The orientation of embryos is anterior left and dorsal up, except the embryos in (**f**) and (**g**), which are photographed ventrally to show the mesoderm anlagen. Embryos in **a**, **c** and **e** are at late stage 14, embryos in **b**, **d**, **f** and **g** are at an early gastrulation stage. (**a**),(**b**) Wild-type embryos. (**c**),(**d**) *hb* mutant embryos transformed with the C20-*hb* construct. The hb^{14F} null allele shows <10% of the wild-type level of *hb* staining, which explains the weak signal in the posterior region of these embryos. (**e**),(**f**) Wild-type embryos transformed with the *hb*0.7–*lacZ* fusion gene. The embryo in (**g**) was transformed with the *hb*1.2–*lacZ* gene fusion construct and shows the ectopic mesoderm expression.

additional 3.0 kb of upstream sequences, the *hb*2.4–*lacZ* fusion gene 2.0 kb, the *hb*1.2–*lacZ* fusion gene 0.8 kb and the *hb*0.7–*lacZ* fusion gene 0.3 kb respectively (Figure 1). The *lacZ* gene is fused such that its translation is initiated by its own AUG codon. A fragment of SV40, containing the polyadenylation signals for the major SV40 transcripts, is fused behind the *lacZ* gene.

Several independent transformant lines were established for each of the constructs in order to minimize the possibility of position effects due to the integration at sites which might confer repressing or enhancing effects. Such effects did indeed occur in some of the transformants, which showed ectopic expression in a segmentally repeated pattern. These lines were not used for further analysis.

All four constructs showed essentially the same results with respect to their expression in the anterior domain. β-galactosidase was expressed in the region which is normally covered by the 2.9-kb transcript but did not show the later expansion of the domain (Figure 3e and f), which was shown above to be due to the 3.2-kb transcript. This closely parallels the effect seen with the C20-*hb* construct. These results suggest that ~300 bp upstream of the transcription start site of the 2.9-kb transcript are sufficient for normal regulation of the expression of the 2.9-kb transcript in the anterior region.

The smallest construct, the *hb*0.7–*lacZ* fusion gene, showed no additional sites of expression at later developmental stages. This is in contrast to the three larger constructs, which showed additional sites of β-galactosidase expression in the nervous system, which is also stained with the *hb* antibody. A more detailed analysis of this expression pattern will be presented elsewhere. Interestingly, the three larger constructs also showed β-galactosidase expression in the mesoderm at the beginning of gastrulation (Figure 3g), a site which is marked neither with *hb* antibodies nor by *in situ* hybridization with the *hb* probe to tissue sections of wild-type embryos (see Discussion).

Maternal regulation of the 2.9-kb transcript by bcd

The anterior activation of *hb* 2.9-kb transcript is essential

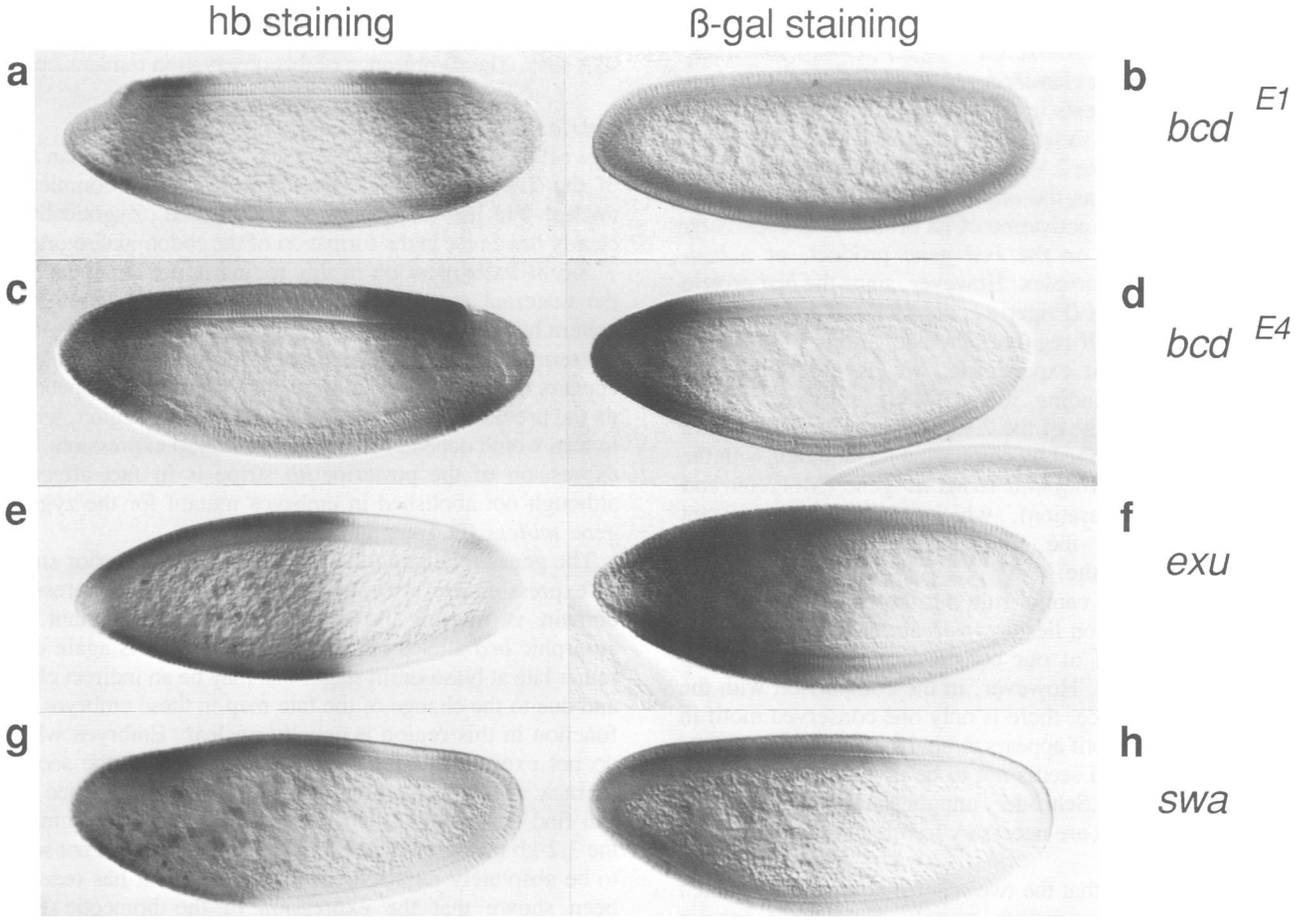

Fig. 4. Comparison of the *hb* protein and β-galactosidase protein expression in maternally mutant background. Embryos produced by females homozygous mutant for maternal genes were mated with wild-type males (**a**, **c**, **e**, **g**) or males carrying the *hb*1.2−*lacZ* fusion gene (**b**, **d**, **f**, **h**). The embryos are all at late stage 14 and are stained with an antibody against the *hb* protein (**a**, **c**, **e**, **g**), or with the β-galactosidase antibodies (**b**, **d**, **f**, **h**). Orientation of the embryos is as in Figure 3. (**a**),(**b**) Embryos coming from females homozygous mutant for the strong *bicoid* allele bcd^{E1}. (**c**),(**d**) Embryos coming from females homozygous mutant for the hypomorphic *bicoid* allele bcd^{E4}. (**e**),(**f**) Embryos coming from females homozygous mutant for *exuperantia*. (**g**),(**h**) Embryos from females homozygous for *swallow*.

for the gap gene function of *hb*. We have therefore tested the three known maternal effect genes which affect the anterior segment pattern, namely *bcd, exu* and *swa*, for their regulatory influences on the *hb*−*lacZ* gene fusion constructs. To analyse the β-galactosidase expression from the *hb* promoter in the mutant background, we mated males homozygous for the *hb*−*lacZ* fusion gene with females which were homozygous for either an amorphic or a hypomorphic *bcd* allele, or for *exu* or *swa* alleles. Embryos from these crosses develop the appropriate mutant phenotype and carry a copy of the *hb*−*lacZ* fusion gene. All constructs showed the same results in these experiments, proving that the smallest construct carries all the regulatory elements necessary for the regulatory effects described below.

Embryos from homozygous bcd^{E1} females develop the amorphic *bcd* phenotype (Frohnhöfer and Nüsslein-Volhard, 1986) and show an inverted duplication of the posterior *hb* domain (Tautz, 1988; Figure 4a), which is due to the expression of the 3.2-kb transcript (Figure 2h). These embryos showed no expression of the fusion gene constructs in the anterior *hb* domain (Figure 4b). This proves that *bcd* activity is essential for the normal expression of the 2.9-kb transcript at syncytial blastoderm stage. Embryos from homozygous bcd^{E4} females develop a weak *bcd* phenotype. Such embryos show a small domain of endogenous *hb* expression which is limited to the anterior tip of the embryos (Tautz, 1988; Figure 4c). The same pattern of expression was observed with the *hb*−*lacZ* fusion gene constructs (Figure 4d).

In *exu* and *swa* embryos, expression of β-galactosidase from the *hb*−*lacZ* fusion genes can be detected in the anterior region, but the size of the expression domain is altered with respect to wild-type. In *exu*, the expression domain is extended posteriorly and in *swa* it is extended posteriorly on the dorsal side and anteriorly on the ventral side. The same pattern is observed for the endogenous *hb* protein in these embryos (Figure 4e−h). Furthermore, the pattern of β-galactosidase and *hb* expression shows a similar degree of variation in different *swa* embryos from the same egg laying (data not shown). These observations support the argument that the qualitative activation of the 2.9-kb transcript is due to *bcd* activity, and that the influences of *exu* and *swa* on the anterior *hb* expression domain may be indirect and essentially mediated via their effects on the distribution of the *bcd* protein (Driever and Nüsslein-Volhard, 1988b).

Discussion

Role of the 2.9-kb transcript

The main reason for classifying *hb* as a gap gene is the loss of contiguous segments in the anterior region of *hb* mutant embryos. We have shown that organizing this pattern is a specialized task of the 2.9-kb transcript. This transcript may therefore be taken as the one which carries the gap gene function of *hb*. The activation of its promoter is apparently directly dependent on the *bcd* gene product, or a *bcd*-dependent protein complex. However, since the *bcd* protein contains a homeobox (Frigerio *et al.*, 1986), it appears likely that the protein itself regulates the activation of the 2.9-kb transcript. With our experiments, we have delimited the region for *bcd* binding to ~300 bp upstream of the transcription start site of the 2.9-kb transcript. This region can be further delimited by comparing its sequence with the sequence of the *Drosophila virilis hb* gene (M.Treier and D.Tautz, in preparation), which reveals a significant conservation over the first 100 bp upstream of the transcription start site.

Our experiments cannot rule out the possibility that the *bcd* regulatory region lies downstream of the transcription start site, since all of our constructs contain the 400-bp downstream region. However, in the comparison with the *D.virilis hb* sequence, there is only one conserved motif in this region. This motif appears to bind a protein at late stages of development and seems not to be involved in transcriptional activation (C.Schröder, unpublished). None the less, further experiments are necessary to rule out this possibility positively.

We have shown that the two other maternal genes which influence the anterior pattern, namely *exu* and *swa*, are not necessary for the activation of *hb* but only for its proper localization. It has been shown that the *bcd* protein is also delocalized in embryos maternally mutant for *exu* and *swa* (Driever and Nüsslein-Volhard, 1988b). In embryos maternally mutant for *exu*, one finds only little *bcd* protein in a rather shallow gradient (Driever and Nüsslein-Volhard, 1988b), which could explain the shift of the *hb* domain towards the posterior. In addition, this observation shows that only little *bcd* protein seems to be necessary to confer the full activation of *hb*. The situation seems to be more complex in embryos maternally mutant for *swa*, since a simple correlation between the shape of the *bcd* gradient in these embryos and the *hb* activation cannot be drawn. One should stress, however, that the *swa* phenotype is extremely variable and different embryos cannot be compared easily (Stephenson and Mahowald, 1987).

Our larger constructs which drive the transcription of the 2.9-kb RNA show two additional sites of expression of this transcript, namely in the developing CNS and in the early mesoderm. The early CNS expression may in some way be functional, since the respective cells also express *hb* protein in wild-type embryos. The regulatory signal for this expression must lie up to 0.8 kb upstream of the transcription start site, since the *hb*1.2−*lacZ* fusion gene still shows the effect. It is not known which genes are responsible for this activation. The observed mesoderm expression is clearly ectopic and does not parallel the normal wild-type expression of *hb*. This suggests that the constructs have lost the binding site for a selective repressor of *hb* expression in the mesoderm. Interestingly, the smallest of our constructs did not show this expression, although it should have lost the binding site as well. It may therefore be possible that our constructs can promote additional artificial transcriptional start sites. The significance of this observation remains open.

Role of the 3.2-kb transcript

The role of the 3.2-kb transcript is more complex than that of the 2.9-kb transcript. Its maternal role is completely unclear and has to be further investigated. Zygotically it clearly has a role in the formation of the abdominal segments 7 and 8. Its expression in this region is not dependent on the maternal genes determining the posterior segmented pattern but only on the *torso* group of genes, which organize the terminal regions of the embryo (Tautz, 1988). Since it appears only late at blastoderm stage, it may be dependent on the previous action of zygotic genes in this region, which in turn would depend on the maternal *torso* expression. The expression of the posterior *hb* stripe is in fact affected, although not abolished in embryos mutant for the zygotic gene *tailless* (D.Tautz,unpublished).

The genes regulating the formation of the anterior stripe of expression are also not yet identified. This expression domain is missing in embryos maternally mutant for amorphic *bcd* alleles but since its expression is again only rather late at blastoderm stage, this may be an indirect effect and due to the change of the fate map in these embryos. Its function in this region is equally unclear. Embryos which do not express this transcript show defects in the second thoracic segment at a high frequency. However, since one can find embryos with an intact second thoracic segment, the 3.2-kb transcript-dependent *hb* expression does not seem to be absolutely required for its formation. It has recently been shown that the expression of the homeotic gene *Antennapedia* (*Antp*) in parasegment 4, which is roughly the region of the 3.2-kb *hb* stripe, is directly dependent on *hb* (Harding and Levine, 1988). The regulation of *Antp* in this region is relatively complex and it was noted that additional spatial cues are necessary to mediate it (Harding and Levine, 1988). Such a cue could be provided by the secondary expression of the 3.2-kb transcript in this region.

The transcript structure and the sequence of the *hb* protein strongly suggest that both transcripts code for the same protein, although we have shown above that different functions may originate from the two transcripts. This is particularly true for the anterior stripe of 3.2-kb expression, which overlaps functionally and physically the region of 2.9-kb expression. The 3.2-kb transcript codes additionally for a small peptide in its leader sequence. It was assumed that this may be involved in the translational regulation of this RNA (Tautz, 1988), although an independent function for this peptide cannot be excluded. Furthermore it is possible that there is a differential modification of the *hb* protein in different regions of the embryo, or that even the RNA itself takes part in the differential functions. Further experiments are needed to decide between these possibilities.

Materials and methods

Drosophila strains

The *bicoid* alleles bcd^{E1} and bcd^{E4}, the *exuperantia* allele exu^{PJ} and the *swallow* allele swa^{14} (kindly provided by Dr C.Nüsslein-Volhard) have been described in Frohnhöfer and Nüsslein-Volhard (1987). The strain used for transformation experiments was a ry^{506} line kindly provided by Dr G.Korge.

Fusion gene constructs

The *hb*3.4−*lacZ* fusion gene was constructed from *hb* upstream sequences, the bacterial *lacZ* gene fused to the SV40 trailer region (Hall *et al.*, 1983) and the Carnegie 20 vector containing the *rosy* marker gene (Rubin and Spradling, 1983). The *hb* upstream sequences were obtained from a subclone of *hb* DNA in pEMBL8 which contains the 6.2-kb *Eco*RI *hb* DNA fragment (Tautz *et al.*, 1987). The 3.4-kb *Eco*RI−*Xba*I fragment (Figure 1) was isolated after a *Bam*HI (from the polylinker region of pEMBL8) and *Xba*I digest. The *Xba*I site was end-repaired to provide a blunt end. The *lacZ* gene was obtained from pUClac 20 DNA (a gift from R.Renkawitz-Pohl) after a *Hin*dIII and *Pst*I restriction digest. After end-repair of the *Hin*dIII site, the *Hin*dIII−*Xba*I blunt-end-ligated *hb*−*lacZ* fragment was subcloned into a modified pUC19 plasmid which had changes in the polylinker region, namely the *Hin*dIII site changed into a *Not*I site and the *Sma*I site changed into a *Xho*I site. This allowed the *hb*−*lacZ* fusion to be excised by a *Not*I and *Xho*I restriction digest for subsequent recloning into the modified Carnegie 20 vector which contained a *Not*I site instead of the *Hpa*I site. The *hb*2.4−*lacZ* construct was constructed by isolation of a 6.8-kb *Pst*I fragment from the *hb*3.4−*lacZ* plasmid. This fragment was inserted into the modified pUC19 vector (see above). After a *Xho*I and *Not*I restriction digest a fragment containing the 6.8-kb *Pst*I fragment was inserted into the modified Carnegie 20 vector (see above). For construction of the *hb*1.2−*lacZ* fusion gene, the *Hin*dIII site of the pUC19−*hb*−*lacZ* construct (see above) was changed into a *Xho*I site. This allowed the excision of a *Not*I−*Xho*I fragment containing the *hb*1.2−*lacZ* fusion gene which was subcloned into the modified Carnegie 20 vector (see above). The *hb*0.7−*lacZ* plasmid was constructed by isolation of a *Sal*I/*Not*I fragment from the *hb*3.4−*lacZ* construct and insertion into the modified Carnegie 20 vector. All constructs were tested for their ability to produce β-galactosidase in a transient expression assay in *Drosophila* embryos (C.Schröder, unpublished). Transformed lines, however, were not probed for β-galactosidase activity, but stained with antibodies against β-galactosidase, which is more sensitive and gives more reproducible results. DNA preparations, ligation, restriction analysis and handling of DNA was done by standard methods (Maniatis *et al.*, 1982); the resulting fusion gene constructs are drawn schematically in Figure 1.

P-element-mediated transformation

Germline transformation was done essentially as described by Rubin and Spradling (1982). Embryos were injected with 100 μg/ml helper plasmid and 300−400 μg/ml of the Carnegie 20 construct. The recipient was the ry^{506} strain. Up to 100 flies of the F0 generation were backcrossed in groups of six with flies from the recepient strain. From each batch of flies, the ry^{+} transformant flies (F1 generation) were crossed in single pair matings to ry^{506} flies. F2 transformants from these crosses were mated *inter se* to establish eventually homozygous lines. Transformant flies from different batches of the F0 generation correspond to independent P-element integration events.

Antibody staining and in situ hybridization

Whole mount preparations of embryos, their staining with either *hb* antibodies (Tautz, 1988) or β-galactosidase antibodies (a gift of U.Gaul) and the analysis of the antibody staining pattern was identical to a published protocol (Gaul *et al.*, 1987), except that 4.1% formaldehyde was used for fixation steps instead of the 1.6% paraformaldehyde solution. *In situ* hybridization with the transcript-specific *hb* probes (for their location within the *hb* gene, see Figure 1) to tissue sections of blastoderm stage embryos was done as described earlier for other probes of the *hb* gene (Tautz *et al.*, 1987).

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