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Autonomous concentration-dependent activation and repression of *Krüppel* by *hunchback* in the *Drosophila* embryo

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SUMMARY

The subdivision of the anterior-posterior axis in *Drosophila* is achieved by a cascade of spatially regulated transcription factors which form short-range gradients at the syncytial blastoderm stage. These factors are assumed to have concentration-dependent regulatory effects on their target genes. However, there is so far little direct in vivo evidence that a single factor can autonomously activate and repress a given target gene. We have analysed here the regulatory capabilities of the gap gene *hunchback* by creating an artificial gradient of *hunchback* in the early embryo. This was achieved by providing the maternally expressed mRNA of *hunchback* with the anterior localization signal of the *bicoid* RNA. The effects of this artificial *hunchback* gradient were then studied in different types of mutant background. We show that under these conditions *hb* is autonomously capable of activating the target gene *Krüppel* at low concentrations and repressing it at high concentrations. In addition, we show that the artificially created *hunchback* gradient can organize a large part of the segment pattern, although it is expressed at a different position and in a different shape than the wild-type gradient of *hunchback*.

Key words: *Drosophila* segmentation, morphogen, gene regulation, gap genes, *hunchback*, transcription factor

INTRODUCTION

The gap gene *hunchback* (*hb*) is one of the key regulatory genes in the early *Drosophila* embryo. It was shown that it can formally act as a morphogen in the sense that it has a concentration-dependent effect on pattern formation (Hülskamp et al., 1990; Struhl et al., 1992). However, there are a number of problems that complicate the full understanding of the *hb* function. First, *hb* shows both a maternal and a zygotic expression, which can at least partially replace each other (Lehmann and Nüsslein-Volhard, 1987). Second, some of the regulatory functions of *hb* are also provided by other gene products. The regulation of *Krüppel* (*Kr*), in particular, is controlled by a number of different genes. While *giant* (*gt*) and the maternal terminal system act as negative regulators of *Kr*, *bicoid* (*bcd*) and *hb* act formally both as positive regulators at low concentrations and as repressors at high concentration (reviewed in Hülskamp and Tautz, 1991).

The third complication in separating the different regulatory inputs arises from the fact that *bcd* is itself required for the zygotic activation of *hb* (Tautz, 1988). Therefore, in the absence of *bcd* one gets only the low concentration of maternal *hb* in the anterior region, but not the high concentration of *hb* that would be expected to act as a repressor of *Kr* expression. In other words, *bcd*$^-$ embryos are effectively double mutant embryos, lacking both the BCD protein and high concentrations of HB protein.

To overcome all these problems, we have sought to provide high concentrations of HB protein in the anterior region by a source that is independent of the *bcd* function and to study the effects of this in the absence of the interference from other genes. This was achieved by placing the 3′UTR of the *bcd* gene, which acts as an anterior localization signal for maternally expressed RNAs (Macdonald and Struhl, 1988; Macdonald et al., 1993), behind the *hb* gene. We show here that this construct provides relatively high concentrations of HB protein in the anterior region of the embryo and allows the study of its effects on the regulation of *Kr*. We find that *hb* alone is capable of regulating the *Kr* expression such that a functional *Kr* domain is generated. Thus, HB protein acts as an autonomous concentration-dependent morphogen which activates *Kr* at low concentrations and represses it at high concentrations. Furthermore, we show that *hb* alone can organize a large part of the segment pattern under these conditions, independent of the anterior organizer *bcd*.

MATERIALS AND METHODS

Construction of the P-element

The *hb-bcd*3′UTR-construct was constructed from a full-length cDNA of the maternal 3.2 kb *hb* transcript and includes approximately 1 kb of sequence upstream of the maternal P1 promotor as well as a part of the *bcd*3′UTR directly behind the stop codon of the *hb* coding region. To construct this, a 1.25 kb genomic *Bam*HI-*Eco*RI fragment was taken from pE8-B100A (Tautz et al., 1987) and cloned into the *Bam*HI-*Eco*RI site of Bluescript (Stratagene). This fragment extends from about 1 kb upstream of the P1 promotor into the leader of the 3.2 kb transcript. Next a 2.1 kb *Eco*RI-*Xho*I fragment from a *hb* cDNA

was cloned into this vector. The *Eco*RI site is identical to the one from the pE8-B100A clone, thus recreating the full leader sequence. The *Xho*I site is located within the coding region of *hb*, approx. 500 bp before the stop codon. For generating the region further downstream, we started from a 500 bp *Xho*I fragment of the *hb* gene that was in vitro mutagenized such that a *Bam*HI site was created directly behind the stop codon (Hülskamp, 1991). A 1.5 kb *Mlu*I-*Eco*RI fragment of the *bicoid* 3′ untranslated region (Berleth et al., 1988) was cloned into this *Bam*HI site and the whole *Xho*I fragment was cloned into the *Xho*I site of the above construct. The whole construct was then cloned into a modified Carnegie 20 vector (Rubin and Spradling, 1983) as a *Not*I-*Sal*I fragment.

P-element transformation and fly stocks

P-element transformations were basically done as described in Rubin and Spradling (1983), using the delta 2,3 P-element helper plasmid (Laski et al., 1986). The recipient flies for the construct were ry^{506}. Transformation efficiency was 3%, six independently transformed lines were established. The following alleles were used for the various backcrosses: hb^{14F}, ry^{506}; bcd^{E1}, tsl^{o35} and (*nos-bcd*3′UTR); tsl^{691}; ry^{506}. Cuticle preparations were made to score the phenotypes of the embryos. Whole-mount in situ hybridizations were done as described in Tautz and Pfeifle (1989).

RESULTS

Experimental design

It was previously shown that it is possible to localize a maternal transcript to the anterior end of the embryo by providing it with the 3′UTR of the *bcd* gene (Macdonald and Struhl, 1988; Gavis and Lehmann, 1992; Ephrussi and Lehmann, 1992; Macdonald et al., 1993). *hb* is maternally expressed and we have identified the enhancer element that is specifically required for this maternal expression (Lukowitz et al., 1994). It was therefore possible to produce a minimal *hb* construct that contains the maternal promotor and enhancer, the coding region and the *bcd* 3′UTR (called the *hb-bcd*3′UTR construct in the following). In addition, we have devised the construct such that its translation would not be under the control of the posterior morphogen *nanos* (*nos*), by excluding from the construct the ‘nanos response elements’ (NREs) that are also known to be located in the *bcd*3′UTR (Wharton and Struhl, 1991). The genetic effects and regulatory capabilities of this construct were then tested in wild-type flies and in different types of mutant background which will be discussed in turn.

Expression of the *hb-bcd*3′UTR-construct

To show that the RNA becomes localized in the expected way, we have done whole-mount in situ hybridization and antibody staining of embryos derived from mothers carrying the *hb-bcd*3′UTR construct. Fig. 1A shows that the RNA is indeed localized at the anterior end of the embryo. The HB protein that is produced from this RNA forms a concentration gradient from anterior to posterior (Fig. 1B). Interestingly, this gradient resembles much more the long-range BCD protein gradient (Driever and Nüsslein-Volhard, 1988) than the short-range zygotic HB protein gradient. Thus, the behaviour of the HB protein is similar to that of the BCD protein at these early stages. This observation suggests that either the diffusion coefficient, or the stability of the HB protein is higher during preblastoderm development.

The presence of the RNA and protein from the *hb-bcd*3′UTR construct does not interfere with normal development of the embryos, at least not in the anterior region. However, we have observed a variable degree of abdominal defects caused by the construct, which might be due to leakage of the hybrid RNA towards posterior. Ectopic expression of HB protein in the posterior region is known to lead to this type of defects (Hülskamp et al., 1989; Struhl, 1989). Unfortunately, this effect precludes the possibility of putting more than two copies of the hybrid construct into an embryo and thus to increase further the amount of HB protein in the anterior region, since the degree of abdominal defects becomes lethal under these conditions.

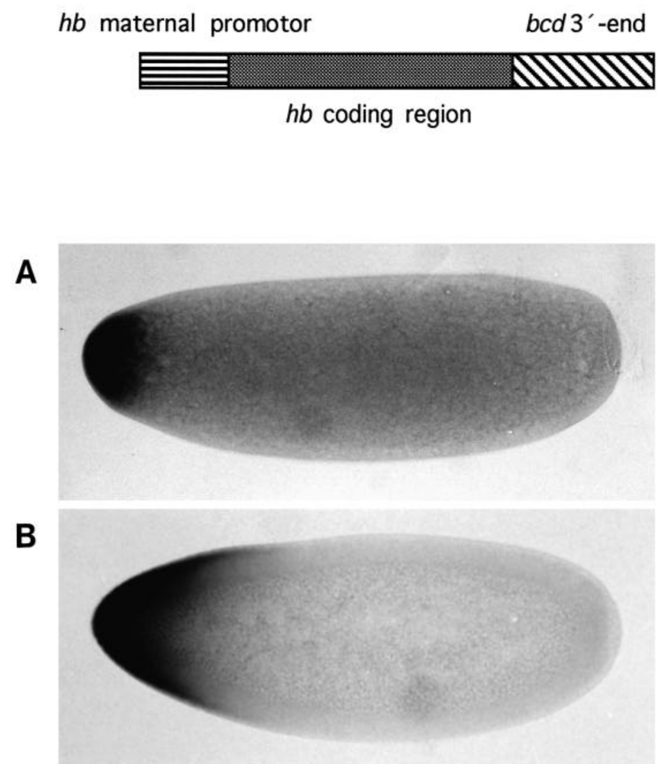

Fig. 1. Schematic drawing of the *hb-bcd*3′UTR construct and its expression characteristics. Top: The construct was assembled from three fragments, the maternal *hb* promotor, the *hb* coding region and a part of the *bcd* mRNA 3′-end. It contains no introns. Bottom: embryos carrying the *hb-bcd*3′UTR construct in hb_{zyg} mutant background. (A) Whole-mount in situ hybridization with the *hb* probe, (B) staining with the *hb* antibody. The level of detection in both cases is such that the normal maternal *hb* expression is not yet visible. Note that the RNA localization and the resulting protein gradient are very similar to those seen for *bcd* (Driever and Nüsslein, Volhard, 1988; St. Johnston et al., 1989).

Rescue activity for *hb*⁻

The *hb-bcd*3′UTR construct has a rescue activity for the zygotic *hb* mutant phenotype (hb_{zyg}). Amorphic hb_{zyg} mutant larvae lack in the anterior region the three thoracic segments and show an enlarged first abdominal segment. In addition, they lack the labium and show as an indirect consequence of this an incomplete involution of the more anterior head segments (Fig. 2A) (Lehmann and Nüsslein-Volhard, 1987). This mutant phenotype is partially rescued by the *hb*-

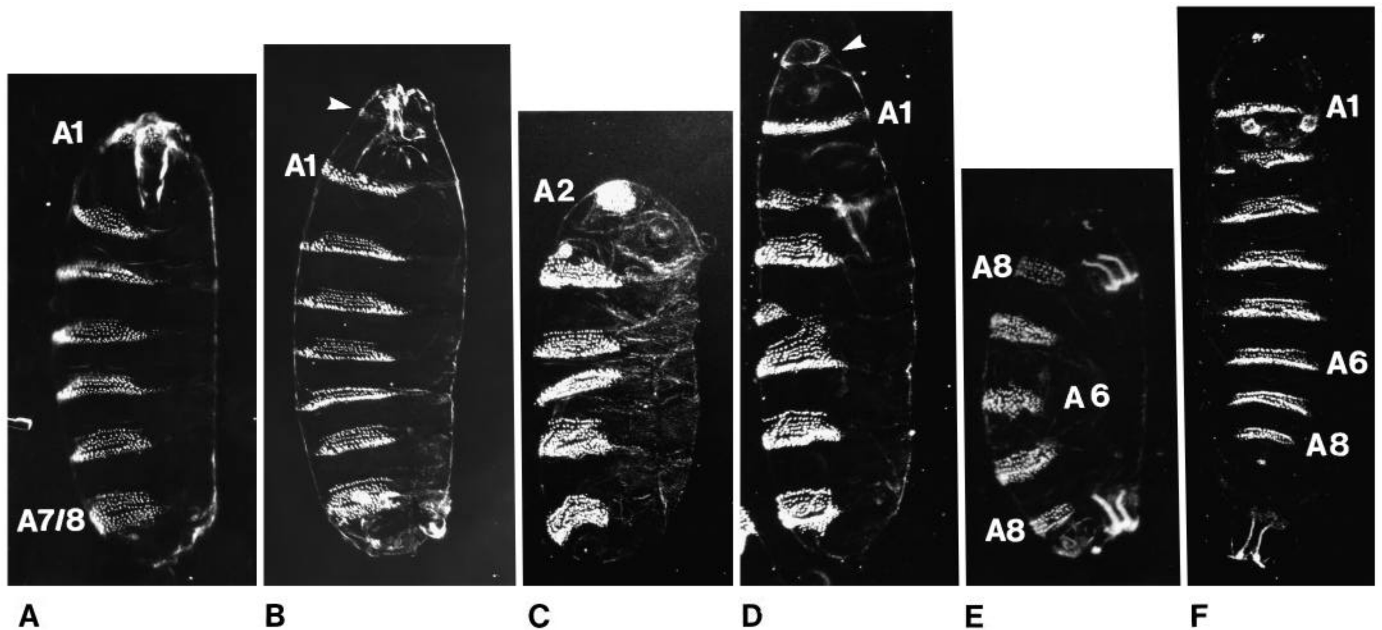

Fig. 2. Cuticle phenotypes of mutant and rescued embryos. (A) Amorphic *hb* phenotype. Note that embryos mutant for *hb* do not only show anterior defects, but also a fusion of abdominal segments A7 and A8 (Lehmann and Nüsslein-Volhard, 1987). This aspect of the phenotype, which does not depend on the anterior *hb* expression, serves as a marker for identifying the respective mutant larvae in A-D. (B) Partial rescue of the *hb* mutant phenotype by the *hb-bcd*3′UTR construct. Note that the head skeleton is restored in addition to one thoracic denticle belt (marked with arrowhead). (C) *bcd,tsl* double mutant phenotype and (D) rescue of this phenotype by the *hb-bcd*3′UTR construct. Note the appearance of a thoracic denticle belt (marked with arrowhead). The slight defect seen in abdominal segment A4 is an indirect consequence of the *hb-bcd*3′UTR construct (see text). (E) *nos-bcd*3′UTR construct mutant phenotype. The embryo shows a mirror-image symmetry around abdominal segment A6. (F) Rescue of the *nos-bcd*3′UTR phenotype by the *hb-bcd*3′UTR construct. Note that the whole anterior segment pattern (A1-A6) is fully restored.

*bcd*3′UTR construct. Embryos carrying this construct in the hb_{zyg} mutant background show a normal abdominal segment A1 and a normal formation of the labium which results in a correct involution of the head segments. If two copies of the construct are present, one can see in addition the rescue of one thoracic segment (Fig. 2B). These phenotypes are reminiscent of the hypomorphic *hb* phenotypes in the allelic series (Lehmann and Nüsslein-Volhard, 1987) proving that the construct can supply *hb* function.

Effects in *bcd*⁻

In view of the rescue effects of the *hb-bcd*3′UTR construct in *hb* mutant background, the effects seen in embryos coming from *bcd* mutant mothers (called *bcd* mutant embryos in the following) are unexpected. Instead of showing a partial rescue, the mutant phenotype is enhanced when the *hb-bcd*3′UTR construct is brought into *bcd* mutant embryos (Fig. 3C,D). We found that this effect is due to an ectopic activation of *gt* by the *hb-bcd*3′UTR construct. Although there is normally no anterior *gt* expression in *bcd* mutant embryos (Fig. 3A), an ectopic *gt* domain can be seen in the presence of the *hb-bcd*3′UTR construct (Fig. 3B). To show that this ectopic *gt* expression is responsible for the unexpected enhancement of the *bcd* phenotype, we genetically removed *gt* from this background. Under these conditions, a rescue effect of the *hb-bcd*3′UTR construct becomes apparent. In the embryo with only one dose of *gt*, the abdominal segments up to A1 are seen (Fig. 3E). Similarly, in the embryo lacking *gt* completely (Fig. 3F), only the typical *gt* phenotype in the posterior region is evident, while the anterior segments are rescued.

hb acts normally as a repressor of *gt* (Struhl et al., 1992) and an activation of an anterior *gt* domain by the *hb-bcd*3′UTR construct is therefore highly unexpected. Interestingly, we found that this activation occurs not directly by *hb*, but is due to the activation of another activator of *gt* and is thus the consequence of a complex interaction between different concentrations of different factors (Schulz and Tautz, unpublished data). However, setting the complication with the ectopic activation of *gt* aside, we can conclude at this point that the *hb-bcd*3′UTR construct does provide rescue activity both in *hb* and in *bcd* mutant background and that therefore it does at least partially mimick the normal zygotic *hb* expression.

Effects in *bcd,tsl* embryos

Embryos coming from mothers double mutant for *bcd* and *tsl* (called *bcd,tsl* mutant embryos in the following) lack all terminally derived structures, the head segments and the first abdominal segment (Fig. 2C). Placing the *hb-bcd*3′UTR construct into this background leads to a partial rescue of this phenotype. The anterior abdominal segments now look normal and one thoracic denticle belt can be seen (Fig. 2D). In these embryos, it is possible to directly study the regulatory capabilities of *hb* on *Kr*. *Kr* is normally expressed in the whole anterior half of *bcd,tsl* mutant embryos (Fig. 4B), since all anterior repressing factors are excluded. Putting the *hb-bcd*3′UTR construct into this background leads to a repression

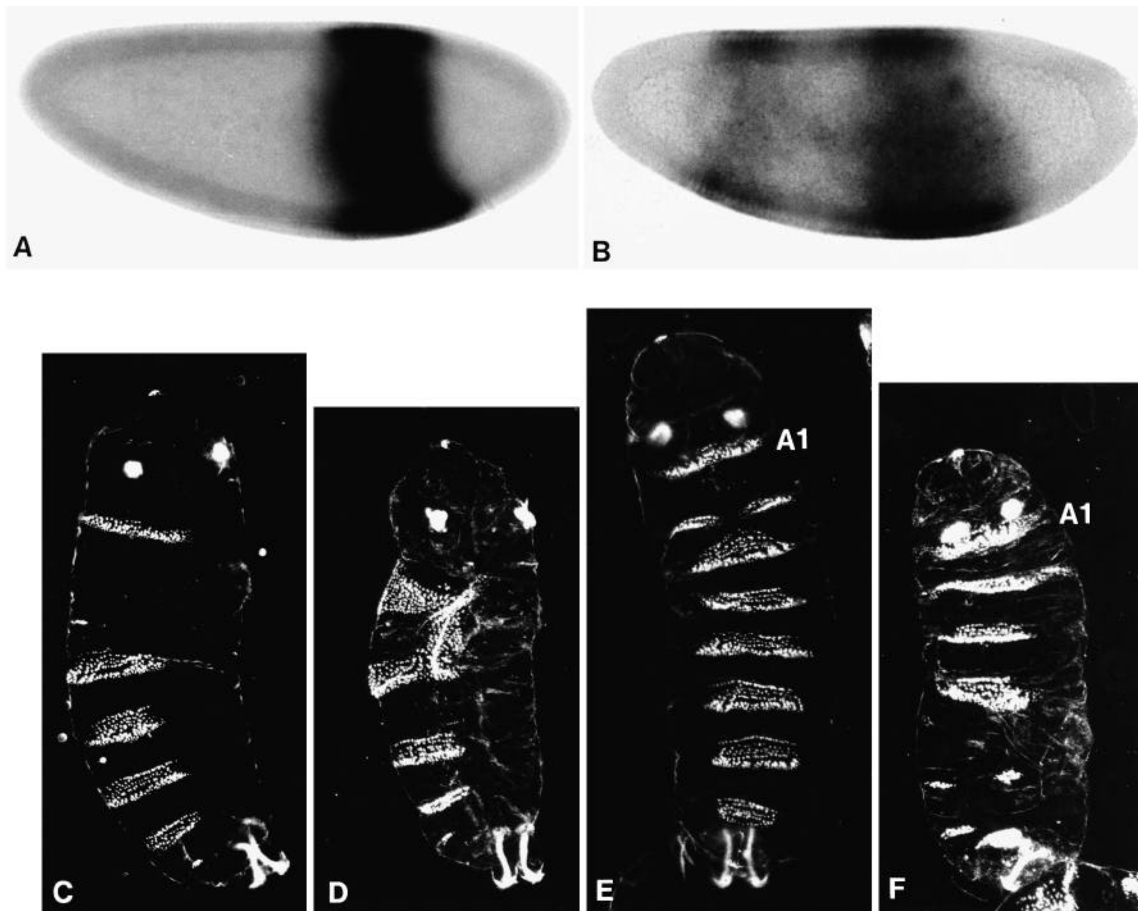

Fig. 3. Ectopic expression of *gt* in *bcd* mutant embryos caused by the *hb-bcd*3′UTR construct. (A,B) In situ hybridization with the *gt* probe; (C-F) cuticle phenotypes of the larvae. (A) *gt* expression in a *bcd* mutant embryo. No expression is seen in the anterior region. (B) *gt* expression in a *bcd* mutant embryo carrying the *hb-bcd*3′UTR construct. An ectopic anterior *gt* domain becomes visible. (C) Typical bcd^{E1} mutant phenotype. Note that the extent of the defects is temperature dependent. The experiments in this series were therefore all performed at the same temperature (18°C). (D) *bcd* mutant larva carrying the *hb-bcd*3′UTR construct. An enhancement of the normal phenotype is evident. (E,F) *bcd* mutant larva carrying the *hb-bcd*3′UTR construct and heterozygously (E) or homozygously (F) mutant for *gt*. The posterior abdominal defects seen in F are typical for the *gt* phenotype (Kraut and Levine, 1991; Eldon and Pirrotta, 1991).

of the *Kr* domain at the anterior end of the embryo (Fig. 4C). This experiment shows clearly that elevated levels of *hb* act as a repressor of *Kr* expression. To rule out the possibility that this repression effect is mediated via *gt*, we have performed a double staining with the *gt* and the *Kr* probes. Fig. 4 shows a comparison between embryos stained for *Kr* only with those stained for *gt* and *Kr*. It is evident that the anterior region remains free of *gt* (Fig. 4d), indicating that the repression effect of *hb* on *Kr* is likely to be direct.

Effects in '*Bicaudal* like' embryos

Genetic removal of both maternal *hb* and *bcd* activity leads to the formation of '*Bicaudal* like' embryos. i.e. embryos with two mirror image symmetric sets of posterior abdominal segments (Hülskamp et al., 1990; Struhl et al. 1992). The same phenotype is seen when *nos* is ectopically localized to the anterior end of the embryo via the *bcd*3′UTR (the *nos-bcd*3′UTR construct, Gavis and Lehmann, 1992) (Fig 2E), since *nos* inhibits the translation of both *bcd* and *hb* (Wharton and Struhl, 1991). We have tested the effects of the *hb-bcd*3′UTR construct in the *nos-bcd*3′UTR background, since these embryos are genetically easier to handle than embryos double mutant for hb_{mat} and *bcd*. The presence of the *hb-bcd*3′UTR construct leads to a suppression of the double abdomen phenotype, restores anterior-posterior polarity and rescues all abdominal segments (Fig. 2F). Again, this genetic background allows one to study the effects of *hb* on the *Kr* regulation directly. Embryos carrying the *nos-bcd* 3′UTR construct do not express *Kr* in the central domain (Gavis and Lehmann, 1992) (Fig. 4E). Addition of the *hb-bcd*3′UTR construct restores a central *Kr* domain (Fig. 4F). Thus, the appearance of the *Kr* domain shows that there is a direct activation effect of the *hb-bcd*3′UTR construct on *Kr* expression. To prove that *hb* can also act as a repressor of *Kr* in these embryos, we have in addition removed *tsl*. We find that *Kr* is indeed repressed from the anterior end of the embryo, as in *bcd,tsl* mutant embryos (Fig. 4G). This result shows most clearly that *hb* acts as an autonomous repressor and activator on *Kr*, since these embryos are devoid of any other known factors that are involved in anterior-posterior pattern formation.

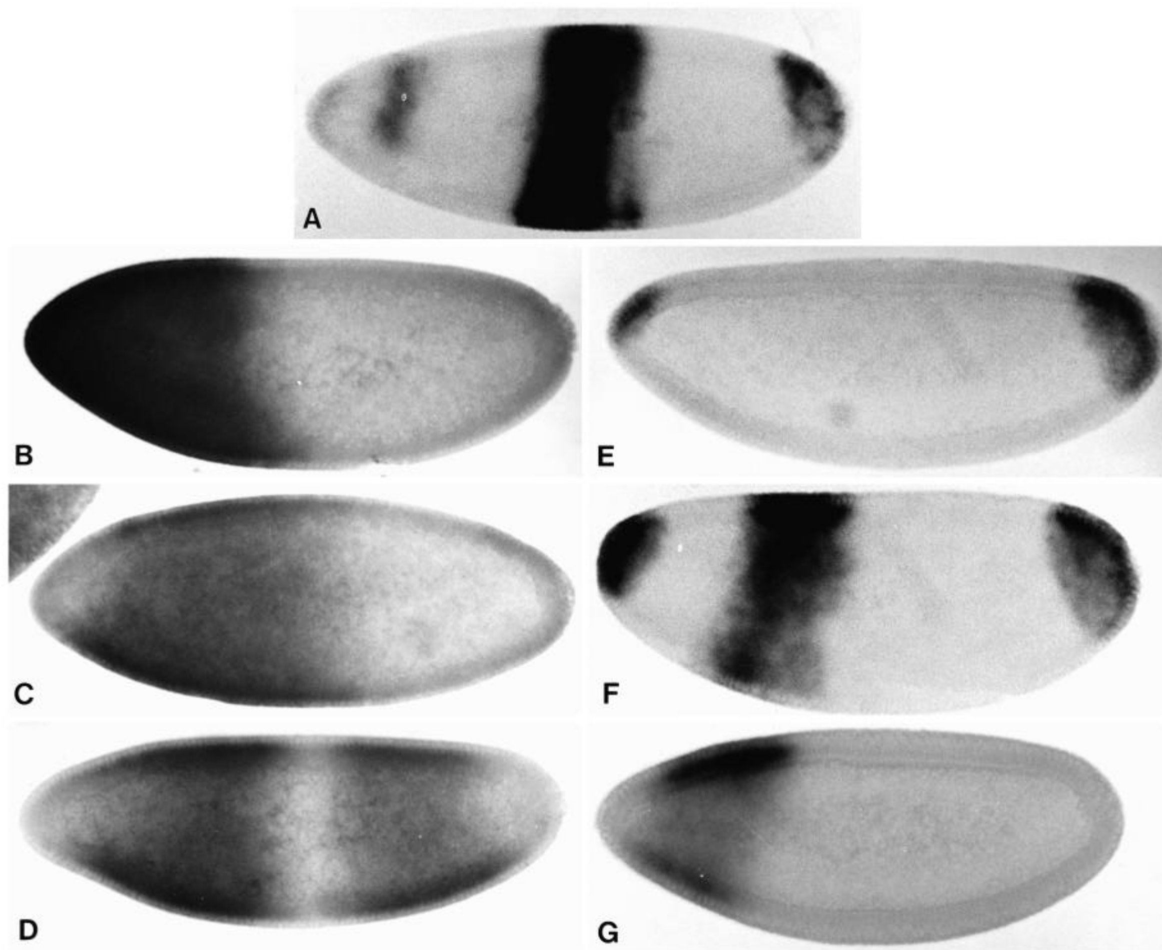

Fig. 4. Regulation of the central *Krüppel* domain by the *hb-bcd3′*UTR construct in *bcd,tsl* mutant background and in embryos carrying the *nos-bcd3′*UTR construct. Whole-mount in situ hybridizations are shown. (A) *Kr* wild-type expression. Note that *Kr* shows, in addition to the central domain, an anterior and a posterior expression domain at blastoderm stage which are regulated by different means. (B) *bcd,tsl* mutant embryo. Note that the normally central *Kr* expression domain extends to the anterior tip of the embryo. (C) *bcd,tsl* mutant embryo carrying the *hb-bcd3′*UTR construct. Note that the *Kr* expression domain is retracted from the anterior tip. (D) Embryo as in C, but double stained with the *Kr* and *gt* probes (see text). (E) Embryo carrying the *nos-bcd3′*UTR construct. *Kr* expression in the central domain is absent, while the posterior domain has become duplicated at the anterior end. (F) Embryo as in E, but carrying in addition the *hb-bcd3′*UTR construct. A central *Kr* expression domain is restored, albeit at a more anterior position. This is to be expected in view of the more anterior position of the artificial *hb* gradient. (G) Embryo as in F, but in addition mutant for *tsl*. The artifical anterior *hb* gradient is in this situation the sole regulator of the *Kr* expression domain, proving its effects as an autonomous morphogen (see text).

DISCUSSION

The segmentation of the *Drosophila* embryo requires the generation of more and more refined internal boundaries. The first boundaries to be established are those of the gap gene and primary pair-rule gene expression domains and stripes. One way to create a domain or a stripe within the embryo is to put it under the control of a morphogen which acts as a repressor at high concentrations and as an activator at low concentrations. However, there has so far been little direct evidence that a single transcription factor can achieve this autonomously in the *Drosophila* embryo. It is frequently assumed that *bcd* can act in this way, but in those cases where this has been analysed more closely, this inference was not supported. The formally inferred repression effect of *bcd* on *Kr* was found to be mediated by *gt* (Kraut and Levine, 1991; Eldon and Pirotta, 1991) and the secondary repression effect of *bcd* on the zygotic *hb* expression was found to be mediated via differential phosphorylation (Ronchi et al., 1993), rather than by differential concentrations of *bcd*. Thus, for *bcd* it has yet to be shown that it can act as a threshold-dependent activator and repressor on the same target gene.

Another well-studied case is *Kr*. Using co-transfection experiments in cell cultures, Sauer and Jäckle (1991) were able to show that *Kr* can mediate concentration-dependent activation and repression via a single consensus DNA-binding site. However, it is not yet clear whether *Kr* also plays such a role in the embryo. Though *Kr* is directly involved in the regulation of neighbouring gap gene domains as well as pair-rule expression stripes (reviewed in Pankratz and Jäckle, 1990), all of these interactions are controlled by multiple factors and an autonomous effect of *Kr* on the formation of such a domain was not yet shown.

Similar cell culture co-transfection experiments were also performed with *hb* acting on a promotor fragment of the *engrailed* gene (Zuo et al., 1991). In these experiments, it was found that an increase of the *hb* dose led to an increase of the transcriptional response of the target gene. However, this effect was only seen up to a certain threshold. A further increase in *hb* dose beyond this concentration led then to a decrease of the transcriptional response. Thus, the results of these cell culture experiments are similar to the effects that we have observed with the regulation of *Kr* by *hb* in vivo.

The enhancer elements that drive the *Kr* expression in the central domain have been identified with the help of reporter gene constructs. Two such elements were found (CD1 and CD2, Hoch et al., 1990) and one of them (CD1) has been studied in detail. This element was shown to contain multiple binding sites for *bcd*, *hb* (Hoch et al., 1991) and *gt* (Capovilla et al., 1992). The CD1 element alone can drive the expression of a reporter gene in a manner reminiscent of the *Kr* expression, but does not fully mimick all wild-type regulatory effects. In particular, a repression effect of *hb* was seen at much lower concentrations than would normally have been expected and the activating effect of *hb* on this element was very poor (Hoch et al., 1991). It seems therefore likely that both elements contribute to the full regulatory effects of *hb* on *Kr*.

Judged from the above results, the autonomus effects of *hb* on *Kr* that are revealed by our experiments are likely to be direct, since we have excluded a potential interference of all other known early genes that could play a role. This does not, of course, exclude the remote possibility that there might be an as yet unknown gene that is differentially activated by *hb* and that could then act on *Kr*. Furthermore, our experiments do not exclude the possibility that regulatory co-factors may play a role. It is known that the Polycomb group genes are required for the maintenance of the regulatory effects of *hb* on *gt* and *kni* (Pelegri and Lehmann, 1994) and it is possible that they may also play a role in the *Kr* regulation.

Struhl et al. (1992) have also performed experiments with an elevated level of maternal HB protein in the embryo, though they did this simply by providing additional copies of the normal *hb* gene in the maternal background, rather then by creating an ectopic gradient. Interestingly, they also found a rescue of thoracic structures in a *bcd,tsl* mutant background. However, these authors did not observe a repression effect on *Kr* expression under these conditions. Thus, the fact that we could observe a repression of *Kr* in the equivalent experiment with the *hb-bcd*3′UTR construct suggests that this effect depends on the artificial gradient that we have provided.

The morphogenetic effect of the artificial *hb* gradient becomes most apparent in the situation where all endogenous *bcd* and *hb* activities are lacking. This is the case in the experiments with the combination of the *nos-bcd*3′UTR and the *hb-bcd*3′UTR constructs. While embryos carrying the *nos-bcd*3′UTR construct alone show a mirror-image symmetry around abdominal segment A6, the addition of the *hb-bcd*3′UTR construct restores anterior-posterior polarity, as well as the whole abdominal segment pattern. It should be made clear that in these embryos the ectopic anterior presence of the NOS protein results in an inactivation of both the anterior and the posterior maternal organizing system. The anterior system is removed because the *bcd* mRNA is directly repressed in its translation. The posterior system is rendered ineffective, because its function depends on the differential posterior inhibition of the translation of the maternal *hb* RNA (Hülskamp et al., 1989; Irish et al., 1989; Struhl, 1989), which is not possible when NOS protein is present at both ends of the embryo (Gavis and Lehmann, 1992). Thus, the rescue seen when the *hb-bcd*3′UTR construct is added to these embryos is exclusively due to the artificial *hb* gradient and does not depend on any other maternal positional information. Most interestingly, this gradient has a different shape and a different location than the normal *hb* gradient (Tautz, 1988). Yet the abdominal segment pattern looks rather normal in the larvae, indicating that their formation depends more on the correct relative order of the activation and repression of target genes, rather than on the expression of these genes at a defined location within the embryo. Thus, in this respect, the artificial *hb* gradient acts very similar as it was noted for the *bcd* gradient (Driever and Nüsslein-Volhard, 1988).

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