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Mechanism and Bicoid-dependent control of *hairy* stripe 7 expression in the posterior region of the *Drosophila* embryo

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Pair-rule gene *hairy* (*h*) expression in seven evenly spaced stripes, along the longitudinal axis of the *Drosophila* blastoderm embryo, is mediated by a modular array of separate stripe enhancer elements. The minimal enhancer element, which generates reporter gene expression in place of the most posterior *h* stripe 7 (*h*7-element), contains a dense array of binding sites for factors providing the *trans*-acting control of *h* stripe 7 expression as revealed by genetic analyses. The *h*7-element mediates position-dependent gene expression by sensing region-specific combinations and concentrations of both the maternal homeodomain transcriptional activators, Caudal and Bicoid, and of transcriptional repressors encoded by locally expressed zygotic gap genes. Caudal and Bicoid, which form complementing concentration gradients along the longitudinal axis of the embryo, function as redundant activators, indicating that the anterior determinant Bicoid is able to activate gene expression in the most posterior region of the embryo. The spatial limits of the *h* stripe 7 domain are brought about by the local activities of repressors which prevent activation. The results suggest that the gradients of Bicoid and Caudal combine their activities to activate segmentation genes along the entire axis of the embryo.

Keywords: Bicoid-dependent posterior gene activation/ *Drosophila* segmentation/stripe 7-element/stripe expression/transcription factor

Introduction

The spatial organization of the *Drosophila* body is indicated by the expression of pair-rule genes in a series of seven sharp transverse stripes along the anterior–posterior axis of the blastoderm embryo (Ingham, 1988; Pankratz and Jäckle, 1990). Dissection of the genetic requirements leading to the proper formation and positioning of the stripe expression domains revealed that pair-rule genes, such as *even-skipped* (*eve*) and *hairy* (*h*), require multiple regulatory inputs of transcription factors encoded by the maternal anterior determinant *bicoid* (*bcd*) and the zygotic gap genes *hunchback* (*hb*), *Krüppel* (*Kr*), *knirps* (*kni*) and *giant* (*gt*) (for review, see Pankratz and Jäckle, 1993). The homeodomain protein Bicoid forms an anterior–posterior concentration gradient which arises from pre-localized maternal mRNA in the anterior pole of the egg (Berleth *et al.*, 1988; Driever and Nüsslein-Volhard, 1988). Bicoid and its posterior counterpart, the homeodomain protein encoded by *caudal* (*cad*), initiate the zygotic expression of the gap genes in spatially restricted adjacent domains along the pre-blastoderm embryo (for review, see Rivera-Pomar and Jäckle, 1996). This leads to a region-specific scenario whereby different combinations and concentrations of transcription factors provide positional information which is decoded by the *cis*-acting control regions of the subordinate pair-rule genes.

Recent studies have identified the specific *trans*-acting requirement for the generation of *eve*, *h*, *fushi tarazu* (*ftz*) and *runt* (*run*) stripe expression along the longitudinal axis of the blastoderm embryo (for review, see Pankratz and Jäckle, 1993). *ftz* expression depends on a small *cis*-acting element which directs the expression of all stripes (Hiromi *et al.*, 1985; Hiromi and Gehring, 1987) and *run* expression on a large *cis*-acting region that cannot be separated into individual stripe elements (Klingler *et al.*, 1996). In contrast, the *eve* and *h* genes contain large upstream control regions comprising autonomous regulatory modules (Howard *et al.*, 1988; Goto *et al.*, 1989; Pankratz *et al.*, 1990; Small *et al.*, 1991, 1992). Each module acts as a 'stripe element' to decode the positional information provided by the local combinations of transcription factors and to mediate gene expression in a specific stripe domain.

To date, the best studied example of pair-rule stripe expression is *eve* stripe 2, a transverse stripe which covers a band of three to four peripheral nuclei in the anterior third of the syncytial blastoderm (Frasch *et al.*, 1987; Small *et al.*, 1991, 1992). *eve* stripe 2 expression depends on two activators, Bicoid and Hunchback (Small *et al.*, 1991). The expression borders of *eve* stripe 2 are drawn by repression from the basic leucine zipper protein Giant and the zinc finger protein Krüppel expressed at each side of the *eve* stripe 2 domain (Small *et al.*, 1991; Stanojevic *et al.*, 1991). The binding sites for these four DNA-binding proteins are clustered and partially overlapping within the small regulatory element that mediates *eve* stripe 2 expression (Small *et al.*, 1992). Disruption of Bicoid- or Hunchback-binding sites causes reduced stripe expression, while the disruption of Giant or Krüppel sites results in an expansion of the stripe expression domain (Small *et al.*, 1991, 1992; Arnosti *et al.*, 1996a). These studies provided a relatively simple model of the mechanism of how crude transcription factor gradients generate a single stripe expression domain in the anterior region of the embryo: binding of Hunchback and Bicoid causes activation, and the binding of repressing factors may exclude activator binding (Small *et al.*, 1991, 1992; Stanojevic *et al.*, 1991) or prevent activator function by quenching (Arnosti *et al.*, 1996a).

Bicoid and Hunchback expression overlap in the anterior half of the embryo only. Thus, the formation of pair-rule stripes in more central and in the posterior region of the embryo is likely to involve different activators. Earlier studies on the regulation of *h* stripe 5 and 6 expression have suggested that the posterior gap gene *kni*, in combination with as yet unidentified components, are necessary for their activation and that other gap gene products such as Krüppel, Giant and posteriorly expressed Hunchback provide the flanking repressor activities that establish the sharp limits of the expression domains (Howard and Struhl, 1990; Pankratz *et al.*, 1990; Riddihough and Ish-Horowicz, 1991). As seen with the *eve* stripe 2 element, the binding sites for Knirps and Krüppel were found in overlapping clusters, suggesting that, despite the different players, the mode of activation and its spatial restriction by repression are similar to *eve* stripe 2 (Langeland *et al.*, 1994).

Here we show the *trans*- and *cis*-acting requirement for the formation and positioning of the most posterior *h* stripe 7. Our results indicate that *h* stripe 7 activation involves several factors including Caudal and the anterior morphogen Bicoid. The spatial limit of the expression domain is set by repression from gap gene products expressed in the flanking domains. Gene activation depends on the number of activator-binding sites present on the enhancer element. Deletions of such sites resulted in a *h* stripe 7 sub-element which is not sufficient for gene activation in the wild-type embryo, but which conducts gene expression in embryos deficient for repressors. Addition of *in vitro* binding sites for Caudal or Bicoid to this element restored the activation, indicating that the *h* stripe 7-element integrates positional information by decoding local combinations and concentrations of activators and repressors. The results also demonstrate that the anterior morphogen Bicoid, in addition to Caudal, is capable of activating gene expression in the most posterior region of the embryo, suggesting that the two opposing transcription factor gradients of maternal origin provide the basis for segmentation gene activation throughout the embryo.

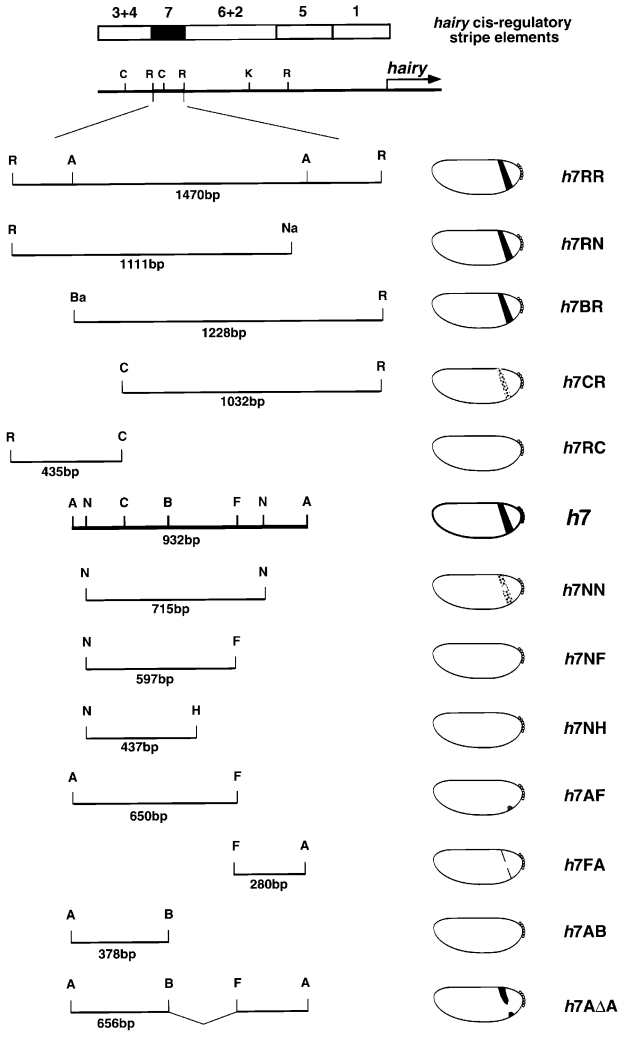

Fig. 1. Summary of *h–lacZ* fusion gene constructs (left side) and corresponding blastoderm expression patterns (right side). The numbers 1–7 (top row) refer to *cis*-acting stripe elements of the *hairy* upstream region (Pankratz *et al.*, 1990). Note the minimal *h*7-element (932 bp; '*h*7', bold) which mediates *h* stripe 7 expression in wild-type embryos (see Figure 2). Restriction enzyme sites are A, *Ava*I; B, *Bal*I; Ba, *Ban*II; C, *Cla*I; F, *Afl*II; H, *Hin*cII; K, *Kpn*I; N, *Nla*IV; Na, *Nae*I; R, *Eco*RI.

Results

The hairy stripe 7-element

Previous studies revealed a 1.5 kb DNA fragment of the *h* upstream region which conducts *lacZ* reporter gene expression in place of the endogenous *h* stripe 7 of the transgenic blastoderm embryo (Howard and Struhl, 1990; Pankratz *et al.*, 1990; Riddihough and Ish-Horowicz, 1991). This stripe is the most posterior *h* stripe expressed. In order to monitor the *trans*-acting requirement for the activation and the spatial regulation of this stripe, we determined the minimal sequence requirement that is able to conduct reporter gene expression in the domain of *h* stripe 7 in wild-type embryos. Figure 1 summarizes various deletion constructs and the reporter gene expression patterns conducted in transgenic embryos. The results indicate that in wild-type embryos, *h* stripe 7 expression depends on a minimal 932 bp fragment which we refer to as the '*h*7-element' (Figure 2A). In accordance with earlier observations of Riddihough and Ish-Horowicz (1991), the expression domain mediated by the *h7*-element is shifted by about one cell posteriorly relatively to endogenous *h* stripe 7 expression. Shorter DNA fragments containing different portions of the *h*7-element resulted in either weak and irregular reporter gene expression patterns or, as in the case of the 597 bp *h*7NF-element, failed to mediate reporter gene activation in the wild-type embryo (Figure 1).

In order to unravel the genetic control of *h* stripe 7 expression, we placed the *h*7-element-containing *lacZ* reporter gene ('*h*7–*lacZ*') into embryos which lack the activity of either maternal or zygotic components required for normal segmentation in the embryo. In wild-type embryos, *h* expression is preceded by the expression of gap genes, the first zygotically expressed members of the segmentation gene cascade which are activated in response to the maternal organizer system. This system includes the transcriptional activators Bicoid and Caudal, the transcription factor Hunchback and the unknown transcriptional regulator at the receiving end of the *torso*-dependent

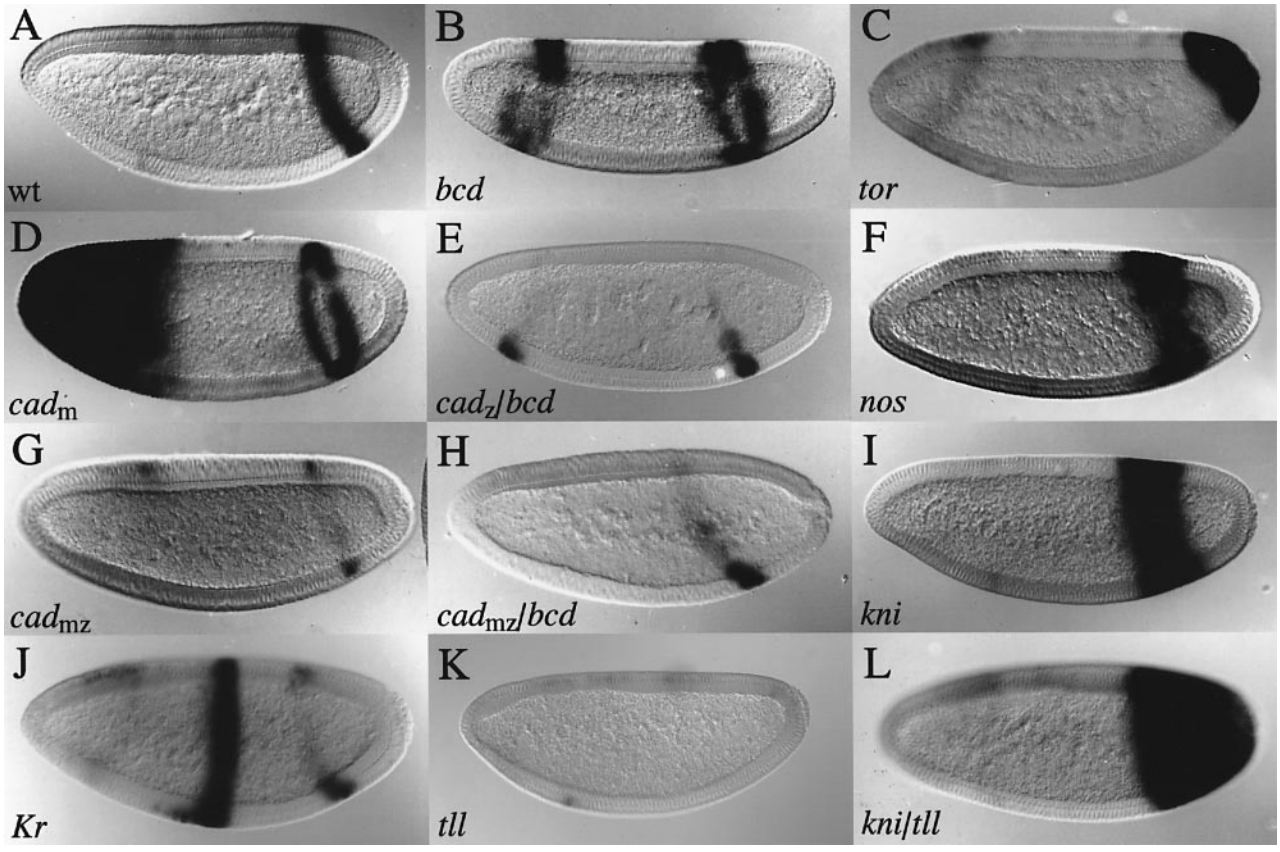

Fig. 2. β-Gal reporter gene expression of the *h*7–*lacZ* fusion gene in maternal effect and gap mutant embryos. Gene abbreviations refer to embryos which are deficient for the respective gene activity. (**A**) *h*7–*lacZ* transgene-bearing wild-type embryo showing β-gal expression in the position of *h* stripe 7. (**B**) In the absence of *bcd* activity, the expression domain is duplicated. The posterior domain appears irregular and is shifted anteriorly. (**C**) In the absence of *tor* activity, the expression domain is shifted posteriorly and expands to the posterior pole. (**D**) In the absence of maternal *cad* activity, expression is slightly reduced. The anterior expression domain is due to the balancer chromosome, which carries a *hb–lacZ* marker gene construct to identify the genotype of the embryos (see Materials and methods). (**E**) In the absence of zygotic *cad* and *bcd* activity, the expression domain is duplicated and strongly reduced. (**F**) In the absence of *nos* activity, the *h*7-mediated stripe domain expands anteriorly. (**G**) In the absence of maternal and zygotic *cad* activity, expression is strongly reduced. (**H**) In the absence of *cad* (maternal and zygotic) and *bcd* activity, the expression domain is reduced and shifted anteriorly. (**I**) *kni* mutant embryo showing that the expression domain is expanded anteriorly. (**J**) *Kr* mutant embryo showing two expression domains. Note that the posterior expression is reduced. (**K**) In *tll* mutant embryos expression is absent. (**L**) In *kni, tll* double mutant embryos, the expression domain expands into the posterior pole region.

Ras/Raf signal transduction pathway (for reviews, see St Johnston and Nüsslein-Volhard, 1992; Rivera-Pomar and Jäckle, 1996).

The hairy stripe 7-element mediates maternal gene activities

Activation of *h*7–*lacZ* expression is dependent on the anterior and posterior, but not on the terminal maternal organizer systems. In embryos lacking terminal *torso* activity, *h*7–*lacZ* is activated at the posterior pole exclusively (Figure 2C). In embryos lacking *bcd* as the key component of the anterior organizer system, the *h*7–*lacZ* expression domain is duplicated. The normal posterior expression domain appears irregular and is shifted anteriorly (Figure 2B). In the absence of *nanos* activity, a key component of the posterior system, *h*7–*lacZ* expression expands anteriorly (Figure 2F). Furthermore, in the absence of *cad*, *h*7–*lacZ* expression is strongly affected (Figure 2G).

cad is expressed both maternally and zygotically (Macdonald and Struhl, 1986; Mlodzik and Gehring, 1987). In the absence of zygotic *cad* activity, *h*7–*lacZ* expression appears normal (data not shown). In the absence of maternal *cad* activity, and in the absence of both maternal and zygotic *cad* activities, *h*7–*lacZ* expression is decreased (Figure 2D and G). This suggests that maternal rather than zygotic *cad* activity is required for the normal expression level of *h*7–*lacZ*. In embryos lacking *bcd* and zygotic *cad* activities, *h*7–*lacZ* expression is decreased (Figure 2E). This observation suggests that *bcd* participates in the activation of *h*7–*lacZ* expression. Embryos which lack *bcd* and both maternal and zygotic *cad* activities show weak *h*7–*lacZ* expression (Figure 2H). This indicates that a third component, *Kr* (see below), participates in the activation of *h*7–*lacZ* expression in the posterior region of the embryo. Note that such embryos lack the anterior expression domain seen in Bicoid-deficient embryos (Figure 2B), suggesting that their anterior expression domain depends on ectopic *cad* activity in the anterior region due to the lack of *bicoid*-dependent translational repression of *caudal* mRNA (reviewed in Rivera-Pomar and Jäckle, 1996).

The hairy stripe 7-element mediates gap gene activities

The spatial limit of *h*7–*lacZ* expression is significantly altered in the gap mutants *tll*, *kni* and *Kr*, but not in

embryos lacking either *hb*, *gt* or the activity of the terminal gap gene *huckebein* (*hkb*). In *kni* mutant embryos, *h*7–*lacZ* expression expands anteriorly (Figure 2I). Thus, *kni* is required to repress *h*7–*lacZ* expression and thereby establishes the anterior border of the expression domain. In *tll* mutant embryos, *h*7–*lacZ* expression is absent (Figure 2K), implying that *tll* activity is necessary for the activation of the transgene. However, *h*7–*lacZ* expression is observed in embryos lacking *torso* activity (see above) which fail to express *tll* (Klingler *et al.*, 1988). This suggests that *tll* does not act as an activator but provides activation indirectly by derepression. In fact, the repressing activity of *kni* expands posteriorly in embryos deficient for *tll* (Pankratz *et al.*, 1989).

In order to determine whether posteriorly expanded *kni* activity is the cause of repressed *h*7–*lacZ* expression in *tll* mutant embryos, we monitored *h*7–*lacZ* expression in embryos lacking both *tll* and *kni* activities. Figure 2L shows that *h*7–*lacZ* expression in *kni*, *tll* double mutant embryos expands into the posterior pole region. *tll* is therefore not only required to prevent *kni*-dependent repression of *h*7–*lacZ* activity but serves also to prevent terminal *h*7–*lacZ* expression in wild-type embryos. In summary, these results suggest that the anterior border of the *h*7–*lacZ* expression domain is established by repression in response to *kni*, while the posterior border of *h*7–*lacZ* expression is set in response to the repressor activity of *tll*.

A surprising result was obtained with *Kr* mutants. *Kr* is expressed in the central region of the embryo, anteriorly adjacent to the *kni* expression domain. In the absence of *Kr* activity, two *h*7–*lacZ* expression domains were observed (Figure 2J). *h*7–*lacZ* expression in the normal stripe 7 position was reduced, while a second and stronger activity of *h*7–*lacZ* expression appeared in the *Kr* expression domain found in wild-type embryos, covering the area of *h* stripes 3 and 4. The expression of these stripes was shown to be dependent on *cis*-acting sequences 5′ to the *h*7-element, which give rise to only a single stripe in *Kr* mutant embryos (Hartmann *et al.*, 1994). The finding of a corresponding stripe of *h*7–*lacZ* expression suggests that the *h*7-element hosts a second element which is silent in the presence of *Kr* activity. The results also suggest that Krüppel functions as a repressor in the context of the *h* stripe 3,4 sub-element, while it is likely to represent the third activator required for *h*7–*lacZ* expression in embryos which lack *bcd* and *cad* activities (see above).

In vitro binding of trans-acting factors

The genetic analysis of *h*7 expression revealed the maternal and gap gene requirement for the activation and proper spatial expression of the *h*7–*lacZ* gene. The six genes identified encode transcription factors. Caudal and Bicoid are homeodomain proteins, Krüppel and Hunchback are C_2H_2 zinc finger proteins, and Knirps and Tailless are orphan receptor-type zinc finger proteins (for review, see Jäckle and Sauer, 1993). We next asked whether the *h*7-element contains *in vitro* binding sites for these proteins. As summarized in Figure 3, DNase footprinting detected multiple sites for each factor which are scattered throughout the element. Many of the putative activator-binding sites (Caudal, Bicoid and Krüppel) are overlapping with putative repressor-binding sites for Knirps and Tailless, suggesting that part of the suppression could be provided

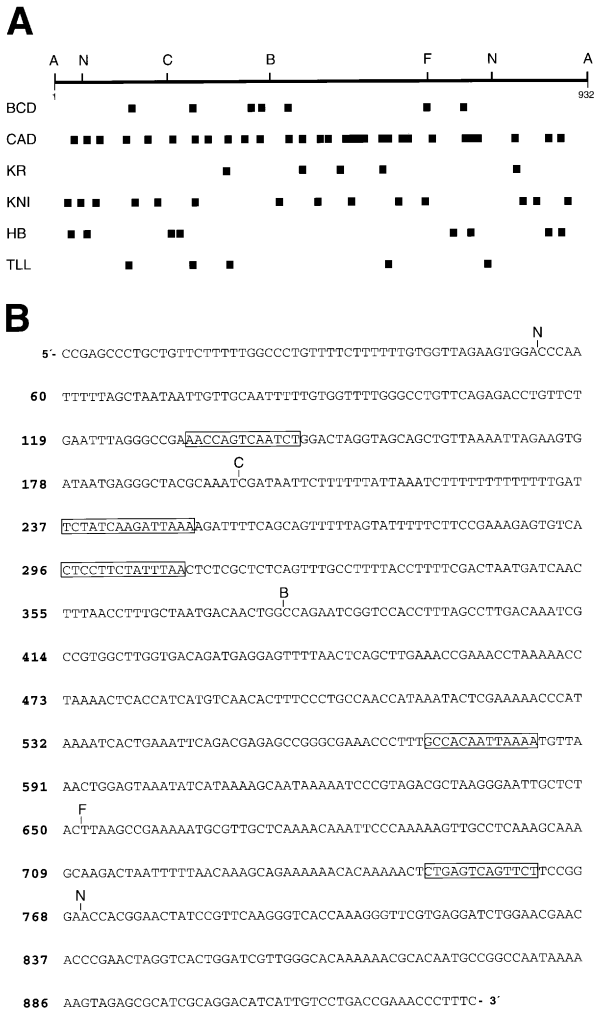

Fig. 3. Schematic representation of *in vitro* binding sites within the 932 bp *h*7-element. (**A**) Physical map of the *h*7-element, showing diagnostic restriction sites: A, *Ava*I; B, *Bal*I; C, *Cla*I; F, *Afl*II; N, *Nla*IV. Regions protected by Bicoid (BCD), Caudal (CAD), Krüppel (KR), Knirps (KNI), Hunchback (HB) and Tailless (TLL) are shown as boxes. (**B**) Sequence of the *h*7-element. For orientation, note the diagnostic restriction sites N, C, B and F (see A) and the five TLL-binding sites (boxed).

through competition of activator and repressor binding, as observed in *eve* stripe 2 and the *Kr* control elements (Hoch *et al.*, 1992; Small *et al.*, 1992). Some of these putative repressors, however, bind to individual sites which are close to non-overlapping activator sites. This suggests that repression mediated by the *h*7-element also involves quenching of activator activity by close-by repressors. The finding that the *trans*-acting factors required for the control of *h*7–*lacZ* expression bind to the *h*7-element *in vitro* is consistent with the argument that their control is of a direct nature.

Mechanism of hairy stripe 7-element control

The 597 bp *h*7NF-element does not mediate reporter gene expression in wild-type embryos (Figure 4A). An anterior expression domain shown in Figure 4A is mediated by vector DNA. It appeared only when the transgenic embryos were overstained after *in situ* hybridization, and serves as an internal staining control (see also Rivera-Pomar *et al.*, 1995). The lack of *h*7NF-dependent gene activation in

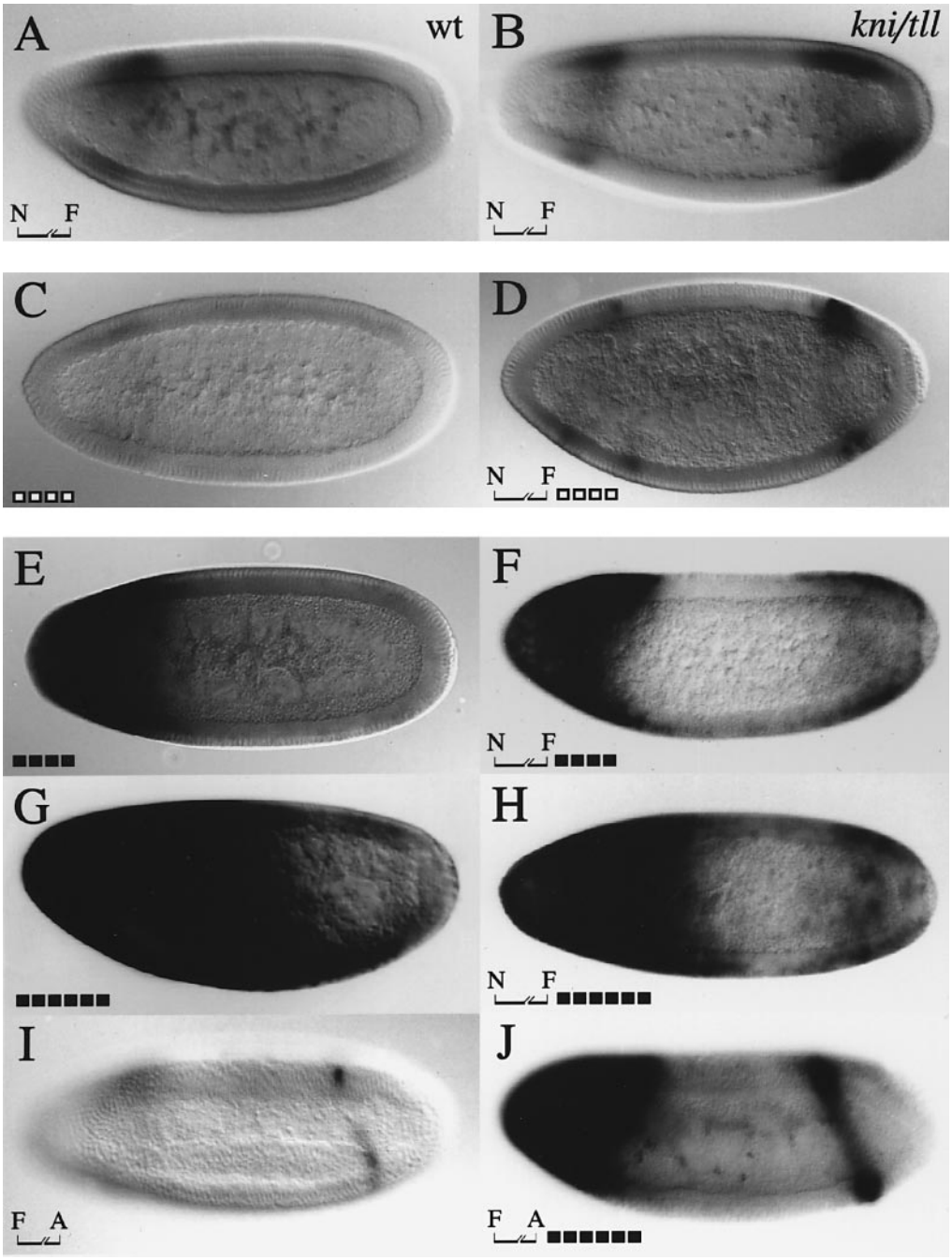

Fig. 4. *h–lacZ* fusion gene constructs and corresponding β-gal expression patterns. CAD-binding sites are indicated by open boxes and BCD-binding sites by filled boxes. For orientation of the binding sites, see Materials and methods. Gene abbreviations refer to embryos which are deficient for the respective gene activity. (**A**) The *h*7NF-element (see Figure 1) fails to mediate expression in wild-type embryos. The anterior expression is due to vector sequences and serves as an internal control demonstrating that even after a prolonged staining, no *h*7NF-mediated β-gal expression can be detected. (**B**) In *kni, tll* double mutant embryos, the *h*7NF-element mediates posterior expression, indicating that *kni*- and *tll*-dependent repression is lifted. The anterior expression domain (see A) is due to vector sequences. (**C**) Four CAD *in vitro* binding sites (see Materials and methods) fail to mediate expression. (**D**) Four CAD *in vitro* binding sites added to the *h*7NF-element rescue gene activation. (**E**) Four BCD *in vitro* binding sites (see Materials and methods) mediate expression in the anterior-most region of the embryo (see also Hoch *et al.*, 1991). (**G**) Six BCD *in vitro* binding sites mediate gene activation in an anterior-to-posterior gradient (Rivera-Pomar *et al.*, 1995). Four BCD (**F**) and six BCD (**H**) *in vitro* binding sites added to the *h*7NF-element cause gene activation in the most posterior region of the embryo. (**I**) The *h*7FA-element mediates a very weak and irregular *h* stripe 7-like expression. (**J**) The addition of six BCD *in vitro* binding sites to the *h*7FA-element restores a normal *h* stripe 7 domain.

transgenic wild-type embryos suggests that the *h*7NF-element contains either too few or lacks the essential activator-binding sites. Alternatively, the combination of activator and repressor sites present on the *h*7NF-element may be unbalanced such that bound repressors outnumber and thus overrule regulation by the activators. In order to distinguish between these possibilities, we monitored the *h*7NF-dependent reporter gene expression in *kni, tll* double mutant embryos. Figure 4B shows the expression of the *h*7NF-*lacZ* transgene in such embryos, indicating that the lack of the two repressors derepresses *h*7NF-mediated activation.

We next asked whether additional activator-binding sites on the *h*7NF-element override repression. For this, we added four Caudal-binding sites to the *h*7NF-element. While control constructs driven by the four Caudal sites alone were not expressed (Figure 4C), the addition of the Caudal-binding sites to the *h*7NF-element rescues gene activation (Figure 4D). Thus, the four added Caudal sites must act in concert with the activator-binding sites already present on the *h*7NF-element. These results establish that the lack of *h*7NF-dependent activation can be overcome by either reducing repressor activities or by increasing the number of activator-binding sites. This suggests a mechanism for gene activation that depends on a balanced ratio of enhancer-bound repressors and activators.

Bicoid activates gene expression in the posterior region directly

Genetic analysis of *h*7–*lacZ* reporter gene expression led to the surprising result of *bcd*-dependent gene activation in the most posterior *h* stripe 7 expression domain of the blastoderm embryo. In view of this result, we added four or six Bicoid-binding sites to the *h*7NF-element. In isolation, the four Bicoid-binding sites cause gene activation in the most anterior region of the embryo (Figure 4E), while the four Bicoid-binding sites in combination with the *h*7NF-element mediate gene activation in the most posterior region of the wild-type embryo (Figure 4F). Similarly, six Bicoid-binding sites mediate gene activation in an anterior-to-posterior gradient (Figure 4G), while their combination with the *h*7NF-element causes gene activation in the most posterior region of the embryo (Figure 4H). We note that the six Bicoid-binding sites contain a single Caudal-binding site. However, no *h*7NF-mediated gene expression was observed in embryos lacking *bcd* activity, irrespective of whether or not the four or six Bicoid-binding sites were added (data not shown). This indicates that gene activation mediated by the modified *h*7NF-element is dependent on Bicoid and that the single Caudal-binding site did not allow for *cad*-dependent gene activation.

In the case of six Bicoid-binding sites, anterior and posterior expression domains are observed, but no expression is found in the central region of the embryo. This indicates that the repressors present on the *h*7NF-element function also in the context of the six added Bicoid sites. However, the activation of posterior gene expression by Bicoid is reduced as compared with the level of gene activation mediated by corresponding Caudal sites. To demonstrate further that Bicoid is able to regulate stripe expression in the posterior region of the embryo, we fused the six Bicoid-binding sites to a 280 bp fragment of the *h*7-element, termed the *h*7FA-element (see Figure 1). This element mediates only very weak and irregular *h* stripe 7-like expression (Figure 4I). Transgenic embryos containing the fusion gene construct show that the addition of the Bicoid-binding sites can restore a normal *h* stripe 7 domain (Figure 4J). These results establish that the anterior determinant Bicoid functions as a transcriptional activator of pair-rule gene expression in the posterior region of the embryo.

Discussion

We have presented evidence that the generation of the most posterior stripe expression domain of the pair-rule gene *h* involves at least three different activators and spatially restricted repressors which antagonize their activities. We demonstrate that the ability of non-functional *cis*-acting elements to mediate gene expression can be restored either by deleting repressors from the embryo or by adding activator-binding sites to the control region. The data suggest that the region-specific transcription in stripes depends on the combination and number of enhancer-bound activators and repressors which determine whether the *cis*-acting element mediates gene activation or fails to provide this activity.

Regulation of h7 stripe expression

In the absence of either *cad* or *bcd* activity, *h*7 expression is still activated. Even if both activities are deleted from the embryo, activation occurs. Thus, a third activator, likely to be Krüppel, must function in such embryos. The assignment of Krüppel as an activator in the context of the *h*7-element is consistent with the observation that *h*7–*lacZ* expression in *Kr* mutant embryos is considerably reduced, while a second expression domain, likely to be part of the *h* stripe 3,4-element, becomes de-repressed in *Kr*-deficient embryos. This would imply that Krüppel acts both as a repressor and as an activator within the *h*7-element, depending on its concentration. At high concentrations of Krüppel, in the central region of the embryo, it prevents activation of stripe 3,4 expression while at low concentrations, such as in the posterior region of the embryo, Krüppel can act as an activator. This conclusion is consistent with tissue culture studies showing that Krüppel is able to provide both activities at different concentrations (Sauer and Jäckle, 1991). Whether activation is provided by Krüppel monomers and repression by Krüppel dimers, as has been shown with both tissue culture and *in vitro* studies (Sauer and Jäckle, 1993), remains to be shown.

The generation of the sharp *h*7 expression borders depends on at least two repressors, one on each side of the expression domain. The posterior border is set in response to *tll* activity under the control of the terminal maternal organizer system. The anterior border of the expression domain is due to repression in response to *kni* activity. In embryos lacking both *bcd* and *cad* activities, where *kni* activity is absent (Rivera-Pomar *et al.*, 1995), an anterior border of the *h*7–*lacZ* expression is established in a position slightly more anterior than in wild-type. This indicates that a repressor other than Knirps is involved in setting this anterior border. This repressor could be maternal *hb* activity, consistent with the finding of multiple Hunchback-binding sites within the *h*7-element. Repression by Hunchback could also contribute to the generation of the posterior expression border through zygotic *hb* expression in a posterior stripe (Tautz *et al.*, 1987). Our results do not rule out or support this possibility, since *hb* expression is absent in *tll* embryos (Casanova, 1990) and, thus, its contribution to posterior repression cannot be assessed directly by genetic means.

Modes of regulation

We generated the *h*7NF-element which failed to mediate gene expression in wild-type embryos. It contains only a subset of binding sites for all repressors and activators. The rescued gene expression in embryos which lack *tll*

and *kni* activities indicates that the absence of these repressors, that would normally bind to the *h*7NF-element, allows for activators to initiate gene expression. Also, when the number of activator-binding sites was increased, activation was obtained. In the case of the added Caudal-binding sites, which are by themselves insufficient to activate gene expression, it is clear that they act in the context of the other activator sites which by themselves are also insufficient to act when the full complement of repressors is present. This repression can be overcome by additional binding sites for activators such as Bicoid or Caudal. These findings strongly argue that the ability of the *cis*-acting element to conduct gene activation is strictly dependent on the balance of bound repressors and activators, which in turn is a function of the distribution and concentration of the *trans*-acting factors in a given position along the anterior–posterior axis of the embryo.

Repressors recently were shown to act by different means, which are reflected by their position relative to the activator-binding sites. In the case of the *Kr* promoter, a set of redundant repressors was found close to or overlapping activator-binding sites, suggesting that repression can be provided by preventing the activator from binding to its site (Hoch *et al.*, 1992). In addition, Knirps was shown recently to extinguish activation by a number of unrelated activators, such as Bicoid, Hunchback, Dorsal or basic helix–loop–helix activators, when acting over short distances (Arnosti *et al.*, 1996b). Although the mechanism of repression by quenching has not yet been shown, it almost certainly involves protein–protein interactions either between the enhancer-bound factors, the repressors and recruited co-repressors, or repressors with components of the basal transcription machinery (for a detailed discussion see Arnosti *et al.*, 1996b). The arrangement of overlapping and separated binding sites within the *h*7-element allows for both mechanisms.

Bicoid-dependent activation throughout the embryo

Recent studies revealed activation of *kni* expression by Caudal and Bicoid, which act upon two distinct *cis*-acting elements (Rivera-Pomar *et al.*, 1995). Each activator element conducts *kni* expression in the posterior half of the embryo. This observation showed that Bicoid, previously shown and thought to act as the anterior determinant of the *Drosophila* embryo exclusively (for review, see Driever, 1993), also contributes to the expression of posteriorly acting gap genes.

Our finding of Bicoid-dependent *h* stripe gene expression in the most posterior region demonstrates that Bicoid can activate gene expression throughout the embryo, a phenomenon which is not apparent in the *bcd* mutant phenotype. Our results also argue that gene activation in the posterior region depends more or even exclusively on activators other than Bicoid, suggesting that Caudal, in biological terms, plays an essential role in the activation while Bicoid only partially contributes, in a redundant fashion, to posterior gene activation. A minor contribution of Bicoid to posterior segmentation is consistent with the phenotype of Caudal-deficient embryos. They show reduced levels of posterior *h* stripe expression (Häder *et al.*, in preparation) and develop significant posterior segmentation defects (Macdonald and Struhl, 1986), indicating that Bicoid cannot fully compensate for the lack of Caudal. Also, minor and variable posterior segmentation defects are observed in embryos which lack Bicoid, although such defects only occur at low frequency. This observation argues then that Bicoid is indeed required to generate the posterior segment pattern reliably, possibly by supporting the major role of Caudal in activating posterior segmentation genes.

A common strategy for activation of stripe expression

The demonstration that repression mediated by gap proteins forms the *h* stripe 7 expression is reminiscent of the situation previously described for the *eve* stripes 2 (Small *et al.*, 1992), 3 and 7 (Small *et al.*, 1996) and for the *h* stripes 3, 4 (Hartmann *et al.*, 1994), 5 and 6 (Langeland *et al.*, 1994). Activation of anterior stripes has been studied in detail with *eve* stripe 2, indicating that Bicoid and Hunchback are the primary activators likely to act in a synergistic fashion (Simpson-Brose *et al.*, 1994; Sauer *et al.*, 1995a,b). In the central region of the embryo, the identities of activators that regulate *eve* stripe 3 or *h* stripes 3 and 4 have been elusive.

Recent studies suggest that a Jak/Stat system is required for the optimal activation of *eve* stripe 3, which acts in concert with one or more ubiquitously distributed activators (Hou *et al.*, 1996; Small *et al.*, 1996). In the posterior region, Caudal and Bicoid have been shown jointly to activate gap gene *kni* expression, and Tailless was discussed to activate *eve* stripe 7 (Small *et al.*, 1996). The genetic evidence presented here rules out the possibility that Tailless acts as an activator by demonstrating that in *tll*-deficient embryos, the repressor Knirps expands posteriorly and thereby extinguishes activation. In the case of *h* stripe 7, this leaves three proteins to act as activators, i.e. Caudal, Bicoid and Krüppel, with the latter playing a dual role as it also represses activation in its high concentration domain in the central region of the embryo (see above).

The previous findings and the results reported here are consistent with a model of how zygotic segmentation genes, both gap and pair-rule genes, are activated. This model depends on the two complementing maternal gradients of Bicoid and Caudal along the anterior–posterior axis of the embryo to provide the basal activation of the segmentation genes: Bicoid activates the anterior- and Caudal the posterior-acting genes. Both activators require co-activators such as Hunchback (Simpson-Brose *et al.*, 1994). Caudal activates *kni* expression in combination with Bicoid (Rivera-Pomar *et al.*, 1995). Similarly, *h* stripe 7 is activated by both Caudal and Bicoid, with possible support from Krüppel (see above). We also noted a large number of Caudal-binding sites present in both the *eve* stripe 3,7-element, and in the *h* stripe 6-element (Häder *et al.*, in preparation). This suggests that Caudal serves as a general activator of posterior genes which acts in concert with the Jak/Stat system to mediate activation through the *eve* stripe 3,7-element, and in combination with Knirps (Pankratz *et al.*, 1990; Langeland *et al.*, 1994) to activate *h* stripe 6 expression (Häder *et al.*, in preparation). In addition to these observations, we propose that the activation of segmentation genes required for setting up the metamerization of the trunk region of the embryo is

strictly dependent on either Bicoid or Caudal or both. This model implies, in a testable way, that other factors are required mainly to adjust the levels of gene expression in the domains which are shaped by the repressors which play the decisive role in defining the stripe borders.

Materials and methods

Drosophila strains and mutant embryos

Drosophila strains were kept under standard conditions. Mutant alleles have been described previously (Lindsley and Zimm, 1992): bcd^{E1}, cad^{2}, cad^{3}, hb^{9Q}, kni^{301}, $kni^{301}tll^{1}$, Kr^{2}, nos^{L7}, tll^{g}, tor^{PM}. Embryos were collected from stocks balanced with CyO or TM3. The balancer chromosomes carried a *lacZ* reporter gene containing the *fushi tarazu* or the *hunchback* promoter, which allow homozygous mutant embryos to be identified unambiguously on the basis of the lack of *hunchback* or *fushi tarazu* staining patterns. Embryos that were produced by bcd^{E1} homozygous females or by cad^{-} germline cloned females were obtained as described by Rivera-Pomar *et al.* (1995).

In situ hybridization of embryos

Whole-mount *in situ* hybridizations with *lacZ* antisense RNA were performed as described previously (Tautz and Pfeifle, 1989), using modifications for RNA probes (Klingler and Gergen, 1993).

Construction of h7–lacZ fusion genes

All *h*7–*lacZ* fusion genes were generated by cloning subfragments of the 1.5 kb *Eco*RI–*Eco*RI fragment (Pankratz *et al.*, 1990; Figure 1) into the vector pCaSpeR hs43 lacZ (Thummel and Pirrotta, 1992). For constructs carrying four Caudal-binding sites, a double-stranded oligonucleotide of the sequence 5′-CTAGAACGGGTTTTACGACCTCCGT-CCGTT-3′ was synthesized. It contains an *Xba*I site on either side and a Caudal-binding site derived from the *h*6-element (underlined; Häder *et al.*, in preparation). The binding site was self-ligated to generate tandem copies and subcloned into the *Xba*I site of pBluescript II KS+ (Stratagene). The resulting plasmid was digested with *Not*I (end-filled)–*Hin*dIII and the 169 bp fragment containing three Caudal-binding sites followed by one Caudal-binding site in reverse orientation was inserted into *Sal*I (end-filled)–*Hin*dIII plasmid *h*7NF. The Caudal-binding sites were not recognized by Bicoid as established by *in vitro* footprinting (T.Häder, unpublished). The four Bicoid-binding sites were obtained from 4bcd5KrZ (Hoch *et al.*, 1991) by digesting with *Xba*I (end-filled)–*Hin*dIII, and the fragment containing four Bicoid-binding sites in reverse orientation was inserted into *Sal*I (end-filled)–*Hin*dIII plasmid *h*7NF. The six Bicoid-binding sites were obtained from the *kni*64-element (Rivera-Pomar *et al.*, 1995) by digesting with *Bam*HI (end-filled)–*Hin*dIII, and the fragment containing the six Bicoid-binding sites was inserted into *Sal*I (end-filled)–*Hin*dIII plasmids *h*7NF and *h*7FA respectively. In those constructs, a single Caudal-binding site was overlapping a Bicoid-binding site, as established by *in vitro* footprinting (Rivera-Pomar *et al.*, 1995). No expression was observed in embryos lacking *bcd* activity (results not shown), indicating that the single Caudal-binding site is not able to mediate *cad*-dependent activation. For orientation of the Bicoid-binding sites, see Rivera-Pomar *et al.* (1995). All recombinant plasmids were introduced into the genome by P-element-mediated germline transformation (Rubin and Spradling, 1982). Several independent transformant lines were established for each construct, and expression of the *lacZ* gene was analysed by *in situ* hybridization using an RNA probe.

Footprinting experiments

The bacterial expression vector pRS*cad*XP encodes amino acids 1–156 of Caudal, pRS*bcd* 89–154 amino acids 89–154 of Bicoid (Rivera-Pomar *et al.*, 1995), pET*tll* amino acids 1–112 of Tailless (Hoch *et al.*, 1992), pPET*hb* full-length Hunchback (Hoch *et al.*, 1991) and pRSET*Kr* amino acids 29–466 of Krüppel (Hartmann, 1996). For pRS*kni*F, an *Nde*I–*Nde*I end-filled fragment from pET*kni* (Pankratz *et al.*, 1990) encoding amino acids 1–156 of Knirps was cloned in the end-filled *Nhe*I and *Pvu*II restriction sites of pRSETa (Studier and Moffat, 1986). The expression of the proteins was induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and continued for 2 h at 37°C. Caudal, Bicoid, Knirps, Krüppel and Hunchback extracts were purified by affinity chromatography on a nickel trinitriloacetic acid matrix as described (Kadonaga *et al.*, 1987). Tailless extracts were prepared as described (Kadonaga *et al.*, 1987). Footprinting experiments were done as described (Kadonaga *et al.*, 1987) except that our fragments were end-filled by the Klenow fragment of DNA polymerase (Sambrook *et al.*, 1989). For this, we generated four subfragments of the 1.5 kb *Eco*RI–*Eco*RI fragment (Pankratz *et al.*, 1990): the 455 bp *Cla*I–*Afl*II, the 395 bp *Bsp*HI–*Bal*I, the 443 bp *Dpn*I–*Bst*EII and the 555 bp *Bal*I–*Ava*I fragments.

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