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Activation of posterior pair-rule stripe expression in response to maternal *caudal* and zygotic *knirps* activities

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Abstract

Drosophila pair-rule gene expression, in an array of seven evenly spaced stripes along the anterior–posterior axis of the blastoderm embryo, is controlled by distinct cis-acting stripe elements. In the anterior region, such elements mediate transcriptional activation in response to the maternal concentration gradient of the anterior determinant BICOID and repression by spatially distinct activities of zygotic gap genes. In the posterior region, activation of *hairy* stripe 6 has been shown to depend on the activity of the gap gene *knirps*, suggesting that posterior stripe expression is exclusively controlled by zygotic regulators. Here we show that the zygotic activation of *hairy* stripe 6 expression is preceded by activation in response to maternal *caudal* activity. Thus, transcriptional activation of posterior stripe expression is likely to be controlled by maternal and zygotic factors as has been observed for anterior stripes. The results suggest that activation and the expression level mediated by the *hairy* stripe 6-element depend on the number of activator binding sites, likely to involve additive rather than synergistic interactions. We found an identical transacting factor requirement for *hairy* stripe 6 and 7 expression. The arrangement of the corresponding binding sites for the common factors involved in the control of the two stripes share a high degree of similarity, but some of the factors exert opposite regulatory functions within the two enhancer elements. © 1998 Elsevier Science Ireland Ltd.

Keywords: Pair-rule genes; *hairy*; *caudal*; *knirps*; *Drosophila*; Segmentation

1. Introduction

The formation of the segment pattern within the trunk region of the *Drosophila* embryo involves the decoding of positional information through a cascade of maternal and zygotic transcription factors (reviewed in Akam, 1987; Pankratz and Jäckle, 1993). This cascade is initiated by the asymmetric distribution of three maternal transcription factors which provide anterior–posterior polarity in the early embryo. The anterior–posterior concentration gradient of the homeodomain protein BICOID (BCD) emanates from prelocalized mRNA in the anterior pole region of the egg (Berleth et al., 1988; Driever and Nüsslein-Volhard, 1988), while the restriction of the zinc finger protein HUNCHBACK (HB) to the anterior half of the embryo (Tautz, 1988) and the posterior–anterior concentration gradient of the homeodomain protein CAUDAL (CAD) are generated by translational repression in response to *nanos* (*nos*), the key component of the posterior organizer system, and by BCD, respectively (reviewed in St Johnston and Nüsslein-Volhard, 1992; Rivera-Pomar and Jäckle, 1996). While BCD and HB cooperate for the activation of zygotic genes expressed in the anterior region of the embryo during the syncytial blastoderm stage (Simpson-Brose et al., 1994), the combined activities of CAD and BCD were shown to initiate zygotic gene expression in the posterior region (reviewed in Rivera-Pomar and Jäckle, 1996) if HB, a repressor of posterior genes, is restrained by *nos* (Hülskamp et al., 1989; Irish et al., 1989; Struhl, 1989).

The first zygotic segmentation genes that are activated by

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the maternally derived transcription factors are the gap genes (reviewed in St Johnston and Nüsslein-Volhard, 1992; Pankratz and Jäckle, 1993), and their distinct expression patterns are restricted mainly through repression by the adjacently expressed gap genes (Pankratz and Jäckle, 1993). Since the locally restricted gap gene transcripts are present already during the syncytial blastoderm stage, the gap proteins can diffuse to form a series of short-range transcription factor concentration gradients adding to the maternal long-range gradients along the anterior–posterior axis of the embryo. The combined maternal and gap gene-encoded factors are necessary for the regulation of pair-rule gene expression in series of seven evenly spaced stripes, representing a molecular prepattern of the segmented trunk of the embryo (Akam, 1987; Frasch et al., 1987; Howard, 1988; reviewed in Small and Levine, 1991).

Pair-rule genes can be grouped into two classes. The activities of primary pair-rule genes, such as *hairy* (*h*), *runt* (*run*) and *even-skipped* (*eve*), are controlled mainly by maternal and gap genes, while the regulation of the expression patterns of secondary pair-rule genes such as *fushi tarazu* (*ftz*) also depends on the preceding activities of the primary pair rule genes (reviewed in Pankratz and Jäckle, 1993). The different control of pair-rule genes is reflected by various types of cis-acting control elements. In the case of *ftz*, stripe expression is controlled by a single, small cis-acting element, called the 'zebra element', which mediates the expression of the seven *ftz* stripes (Hiromi et al., 1985; Hiromi and Gehring, 1987). Analogous *run* expression depends on a roughly 10-times larger enhancer element (Klingler et al., 1996). In contrast, the stripe patterns of *h* or *eve* are controlled by modular arrays of separate enhancer elements, termed 'stripe-elements', which mediate gene expression in single stripe domains in response to the maternal and gap gene activities (Howard and Struhl, 1990; Riddihough and Ish-Horowicz, 1991; reviewed in Pankratz and Jäckle, 1993).

The enhancer of *eve* stripe 2 expression is the most detailed pair-rule stripe enhancer studied. It mediates expression in a transverse stripe in the anterior region of the early blastoderm (Frasch et al., 1987; Small et al., 1991; Small et al., 1992). *eve* stripe 2 activation depends on the synergistic action of BCD and HB (Small et al., 1991; Small et al., 1992; Simpson-Brose et al., 1994), and the borders of *eve* stripe 2 expression are drawn by repression in response to the gap genes *giant* (*gt*) and *Krüppel* (*Kr*) which are expressed at each side of the stripe domain (Small et al., 1991; Stanojevic et al., 1991; Small et al., 1992). The binding sites for the four transcription factors are clustered and partially overlapping in the small regulatory element that mediates *eve* stripe 2 expression in vivo (Stanojevic et al., 1991). Disruption of BCD or HB binding sites causes reduced stripe expression while the disruption of GT or KR sites result in an expansion of the stripe expression domain (Small et al., 1991, 1992; Arnosti et al., 1996). These studies provided a model for the mechanism of how transcription factor gradients control single stripe expression domains in the anterior region of the embryo: binding of broadly distributed HB and BCD to the stripe element causes activation, while the binding of gap gene factors restricts the spatial limit of expression and sharpens up its boundaries by repression.

Comparable studies on stripe expression in the posterior region of the embryo suggested that the borders of posterior stripe expression are also set in response to adjacently expressed gap genes, but that the activation of stripe expression depends on different activators. *eve* stripe 7 activation, for example, depends on the maternal Jak/STAT system (Hou et al., 1996; Small et al., 1996; Yan et al., 1996), *h* stripe 6 activation on the zygotic activity of the posterior gap gene *knirps* (*kni*) (Pankratz et al., 1990; Langeland et al., 1994) and *h* stripe 7 activation on the maternal and zygotic activity of *cad* acting in concert with the zygotic activity of *Kr* (La Rosée et al., 1997). Thus, it appeared unlikely that activation of stripe expression by two synergistically interacting maternal activators, as shown for *eve* stripe 2 expression, represents a paradigm of how stripe gene expression is activated along the anterior–posterior axis of the preblastoderm stage *Drosophila* embryo.

Here we show that the activation of *h* stripe 6 expression is initiated in response to maternal *cad* activity, and that its activation does not depend exclusively on the zygotic activity of the gap gene *kni* as thought previously (Pankratz et al., 1990; Langeland et al., 1994). *cad* and *kni* activities cooperate in a non-synergistic manner to activate *h* stripe 6 transcription. The results indicate that the absence of KNI does not cause the lack of *h* stripe 6 activation but delays the appearance of the stripe significantly. The activation of the stripe depends on a minimal number of activator binding sites which are scattered throughout the *h* stripe 6-element. The tardy appearance of *h* stripe 6 expression in the posterior region of the embryo is therefore not the result of its gap gene-dependence but due to the coordinate function of a maternal component along with *kni* which, in contrast to *hb* in the anterior region, has no maternal complement to co-initiate transcription.

2. Results

2.1. caudal is necessary for the expression of posterior h stripes

The pattern of *h* stripe expression at blastoderm is activated in a non-uniform fashion with *h* stripe 6 being the last of the seven stripes to appear. *h* stripe 6 expression can be detected when all other stripes appeared and when stripes 3 and 4 are expressed as a coherent stripe domain (Fig. 1A,B). During blastoderm cellularization, when the *h* stripes 3 and 4 domains become separated, the level of *h* stripe 6 expression increases to the level of the others (Fig. 1B,C). The late

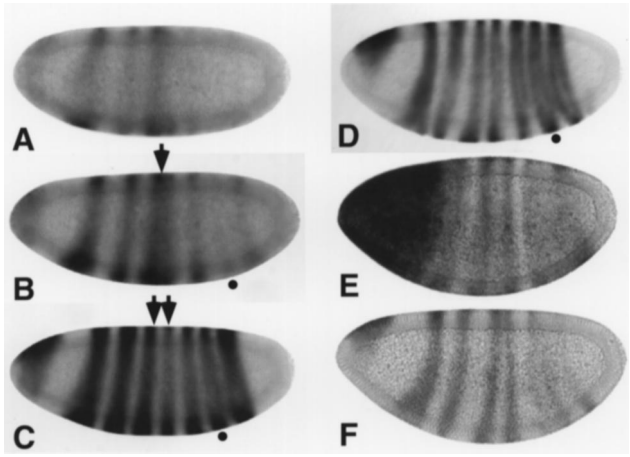

Fig. 1. Expression of *hairy* in wild-type and mutant embryos at blastoderm stages as detected by RNA in situ hybridization. (A–C) *h* expression in a wild-type embryo. (A) The anterior stripes 1–4 are expressed earlier than posterior stripes 5–7 (the orientation of the embryos is anterior to the left, dorsal up; stripe 1 is the left most transverse stripe). (B) Initial *h* stripe 6 expression (indicated by a dot) when *h* stripes 3 and 4 (arrow) are still fused. (C) At beginning of cellularization, stripes 3 and 4 separate (arrows) and the expression level of *h* stripe 6 reaches the level of the other stripes. (D–F) *h* expression in a *cad* mutant embryos (compare to the wild-type pattern in C). (D) *h* stripes 5–7 expression is not affected in an embryo lacking zygotic *cad* activity. (E) *h* stripes 5–7 are disarrayed and their level of expression is reduced in an embryo lacking maternal *cad* activity. (F) The pattern and level of *h* stripes 5–7 expression in mutants lacking both maternal and zygotic *cad* are indistinguishable from embryos only defective for maternal *cad*. Note that the anterior expression domain seen in E is due to *hb-lacZ*, a chromosome marker used to distinguish embryos lacking maternal *cad* activity from those that lack both maternal and zygotic *cad* activities (for details see Rivera-Pomar et al., 1995).

activation of *h* stripe 6 expression had been previously attributed to its activation by zygotic *kni* activity (Pankratz et al., 1990; Langeland et al., 1994). Consistent with this proposal, both the expression of *h* stripe 6 and reporter gene expression mediated by the *h* stripe 6 enhancer (Pankratz et al., 1990; Langeland et al., 1994) was below detection limits in *kni*-deficient embryos during the syncytial blastoderm and at the early phase of blastoderm cellularization. However, re-examination of *h* expression revealed *h* stripe 6 expression during blastoderm cellularization of *kni* mutant embryos (not shown). This suggested that *h* expression in the stripe 6 domain is delayed but not significantly reduced in embryos lacking *kni* activity.

In search for a factor that provides the *kni*-independent activation of *h* stripe 6 expression, we examined the *h* expression pattern in embryos lacking *cad* activity. The lack of zygotic *cad* activity had no discernable effect on the expression of posterior stripes including stripe 6 (Fig. 1D). In contrast, the absence of maternal *cad* activity variably disrupts the posterior stripe pattern of *h* (Fig. 1E). Using the separation of the *h* stripes 3 and 4 expression domains as an internal reference, *h* stripes 5–7 were found to be disarrayed and expressed at low levels when compared to the anterior stripes that appear normally. The same effects on *h* expression were observed in embryos lacking both maternal and zygotic *cad* activities (Fig. 1F). This indicates that maternal *cad* activity is required for the proper activation of posterior *h* stripe expression, while the zygotic complement of *cad* activity is not required.

2.2. CAD and KNI binding sites within the h stripe 6-element

To further investigate the role of maternal *cad* activity on *h* stripe 6 expression and to elucidate the mechanism of how *h* stripe 6 activation is achieved in response to *cad* and *kni* activities, we made use of the previously identified *h* stripe 6-element (Howard and Struhl, 1990). This element drives *h* stripe 6-like reporter gene expression shifted by one cell posteriorly when compared to the endogenous *h* stripe 6 expression domain (Howard and Struhl, 1990; own observations). To establish the potential of CAD and KNI to interact with the cis-acting DNA that mediates *h* stripe 6-like expression in the embryo, we performed in vitro footprinting experiments with the 532 bp *h* stripe 6-element DNA. Bacterially produced CAD and KNI bind to 36, in part overlapping in vitro binding sites throughout the element (Fig. 2A,B). The sequence of the CAD and KNI binding sites match the consensus described for each of the two proteins (Dearolf et al., 1989; Hartmann et al., 1994; Fig. 2C,D). Most of the potential CAD and KNI binding sites are close to or are overlapped by binding sites for KR, HB and TAILLESS (TLL) (Fig. 2E; see also Langeland et al., 1994). These proteins were previously shown to act as local repressors which determine the spatial limit of the *h* stripe 6 expression domain (Pankratz et al., 1990; Langeland et al., 1994). The sequence of the *h* stripe 6-element is shown in Fig. 2F.

The finding of in vitro binding sites for both CAD and KNI suggests that the binding sites may represent targets for an interaction with these two factors in vivo. In order to determine whether a specific set of CAD and/or KNI binding sites within the 532 bp *h* stripe 6-element might be relevant for the activation of gene expression, we examined subfragments of the *h* stripe 6-element for their ability to conduct activation of reporter gene expression in transgenic blastoderm embryos irrespective of the correct spatial control of the stripe domain.

2.3. The h stripe 6-element does not contain a minimal activation element

In the 528 bp element (528-SG), which drives *h* stripe 6-like expression, 17 CAD and 19 KNI binding sites could be identified (Fig. 3A). Strong activation was also conducted by the 284-HT-element, although the number of CAD and KNI binding sites were reduced to 10 and 8 sites, respectively. In contrast to the 528-SG-element, the 284-HT-dependent expression domain was expanded posteriorly (Fig. 3B), which is likely due to a loss of TLL and HB

repressor binding sites (Langeland et al., 1994; own observations). Deletion of 3′-sequences from the 284-HT-element, which leaves 8 CAD and 7 KNI binding sites, resulted in an element that is sufficient for the activation of stripe expression, but the level of expression was strongly reduced (221-HSt; Fig. 3C). The deletion of 66 bp from the 5′-end of 221-HSt, which leaves 5 CAD and 5 KNI binding sites, resulted in an element that failed to drive reporter gene activation at detectable levels (155-HfSt; Fig. 3D). Two subelements of the 528-SG-element (204-SD, 324-DG) mediated activation, although the stripe is formed irregularly (Fig. 3E,F). 204-SD contains 7 CAD and 10 KNI binding sites, while 324-DG contains 9 CAD and 9 KNI binding sites. Taken together, the results indicate that the sequences mediating activation of reporter gene expression are not maintained within a minimal activation element but they are dispersed throughout the enhancer. A subfragment of the *h* stripe 6-element containing 10 CAD/KNI binding sites (155-HfSt) failed to mediate activation, a subfragment containing 15 CAD/KNI binding sites (221-HSt) led to weak activation and various subfragments containing more than 15 CAD/KNI binding sites (204-SD, 324-DG, 284-HT, 528-SG) caused strong activation. Thus, it appears that the *h* stripe 6-element requires a minimal number of non-clustered activator binding sites to mediate gene activation in response to the combined *cad* and *kni* activities.

2.4. kni- and cad-dependent activation mediated by the h stripe 6-element

Both 528-SG and 284-HT mediate strong gene activation in wild-type embryos (Fig. 4A,B). In the absence of zygotic *cad* activity, the expression mediated by the two elements is at best weakly reduced (Fig. 4C,D). In the absence of maternal *cad* activity, however, the expression of the stripe mediated by 528-SG was strongly reduced (Fig. 4E), and no expression was mediated by 284-HT (Fig. 4F). On the contrary, in the absence of *kni* activity, expression was not affected in a discernible manner (data not shown). In *cad*-deficient embryos, 528-SG mediates only weak stripe expression (Fig. 4G), while 284-HT did not respond to the remaining *kni* activity of such embryos (Fig. 4H). In embryos lacking both *cad* and *kni* activities, the 528-SG-

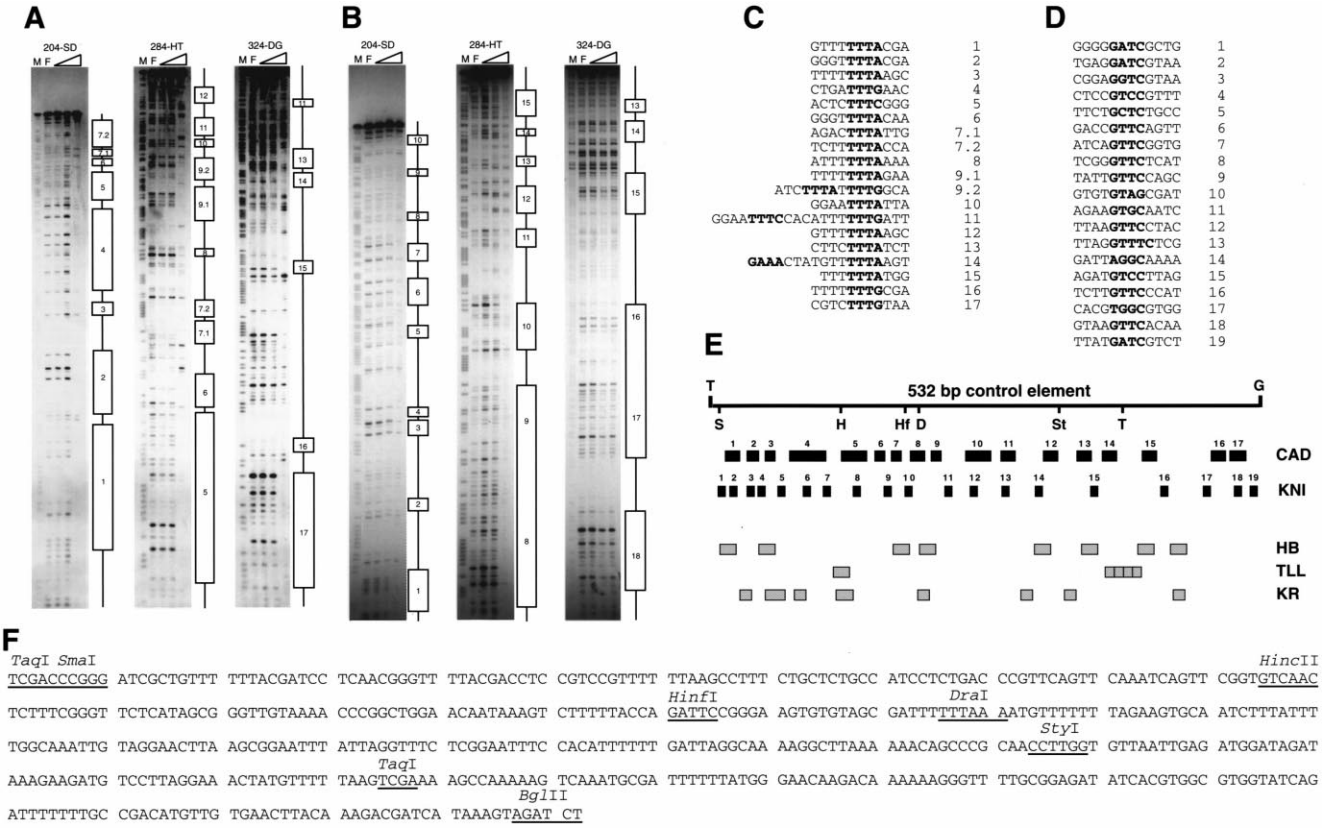

Fig. 2. Distribution of CAD and KNI in vitro binding sites within the 532 bp *h* stripe 6-element. (A,B) DNase footprinting on overlapping DNA fragments that cover the 532 bp enhancer element using CAD (A) and KNI (B). M indicates the sequence marker, F is free DNA, the triangle on top corresponds to increasing amount of protein, the open bars along the autoradiographs indicate protected sites. The DNA fragments used are named according to their size and flanking restriction sites (see E). Alignment of the consensus for CAD binding sites (C) and KNI binding sites (D) derived from the footprinting experiments. (E) Array of activator (black boxes) and repressor binding sites (grey boxes, taken from Langeland et al., 1994; own observations) in the *h* stripe 6-element. T, *Taq*I; S, *Sma*I; H, *Hinc*II; Hf, *Hin*fI; D, *Dra*I; St, *Sty*I; G, *Bgl*II. (F) Sequence and diagnostic restriction sites of the *h* stripe 6-element.

element was not able to conduct reporter gene expression (Fig. 4I). This indicates that the *h* stripe 6-enhancer does not respond to activators different from KNI and CAD or KNI- and CAD-dependent genes, but the role of KNI as an activator would depend on CAD. This would suggest a different role of KNI and CAD for the activation of *h* stripe 6 expression. Furthermore, 19 KNI binding sites that are present on the 528-SG-element are able to mediate weak gene activation in the absence of CAD, while the 8 KNI binding sites left on the 284-HT-element and the 10 activator binding sites on 155-HfSt fail to do so. In summary, the results consistently indicate that both maternal *cad* and *kni* are required to activate *h* stripe 6-element-mediated gene expression and they suggest that the number of binding sites is critical towards the activation of the reporter gene.

In order to test this hypothesis, we generated an artificial

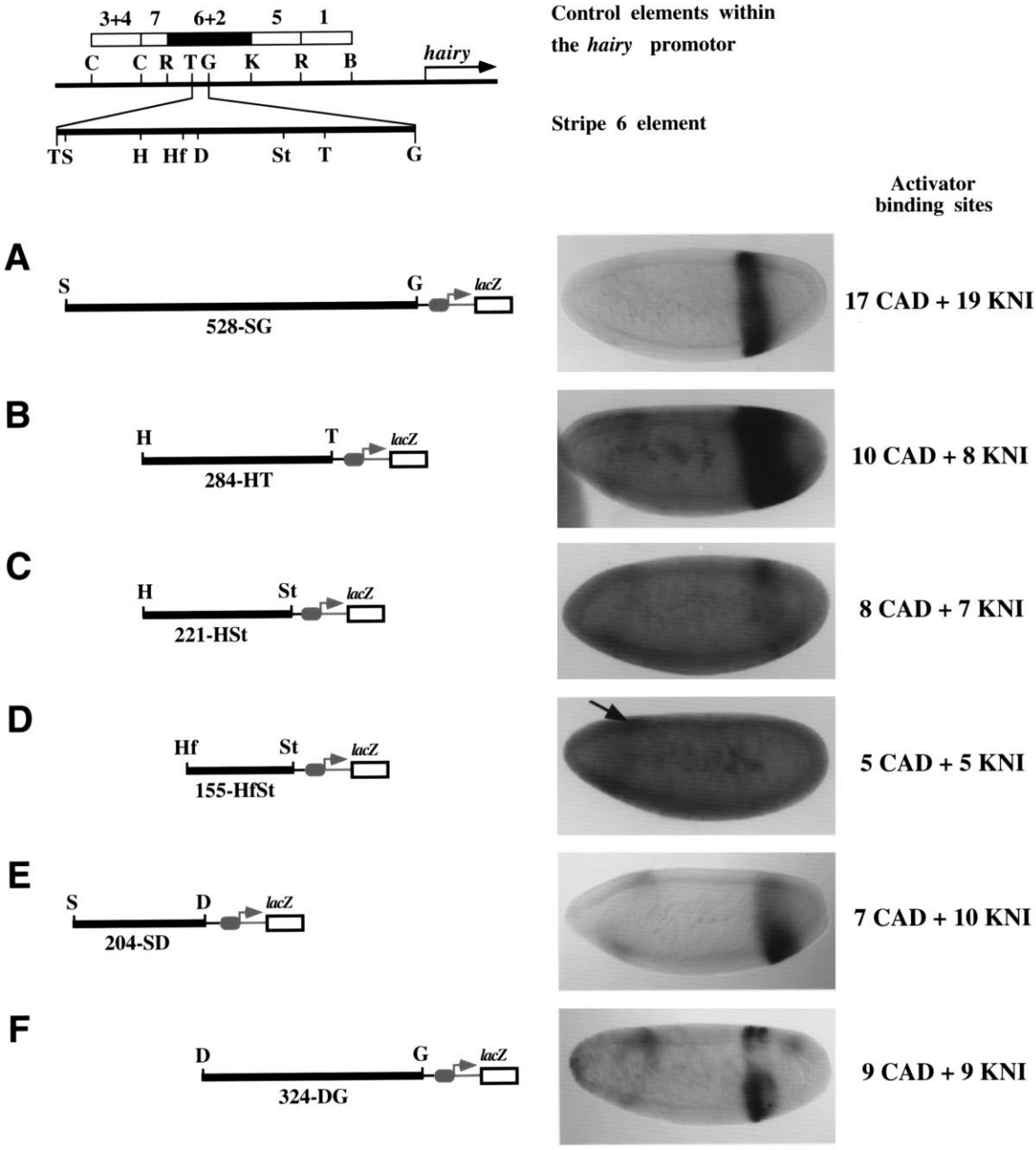

Fig. 3. Activation of reporter gene expression mediated by sequences derived from the *h* stripe 6-element. The diagram on top represents the genomic structure of the gene *h*, open bars refers to the different stripe elements, black bar corresponds to the location of the *h* stripe 2 and 6-elements (Pankratz et al., 1990; Howard and Struhl, 1990). Diagnostic restriction sites are indicated. C, *Cla*I; R, *Eco*RI; K, *Kpn*I; B, *Bam*HI; other sites are indicated in Fig. 2. (A–F) *h* stripe 6-element derived *lacZ* reporter gene constructs (left), RNA in situ hybridization pattern of the reporter gene in transgenic embryos (middle), and number of activator binding sites (CAD and KNI) within each element (right). The designation of each element is shown below each reporter gene construct. The arrow in D indicates the expression of an anterior stripe (derived from the basal promoter) serving as an internal control to evaluate the relative levels of expression. Orientation of the embryos: anterior is left, dorsal up.

enhancer element composed of 155-HfSt DNA (containing 5 CAD and 5 KNI binding sites) to which 4 CAD and 4 KNI binding sites (corresponding to the binding sites CAD-2 and KNI-4 in the *h* stripe 6-element; see Fig. 2E) were fused. Neither the 155-HfSt-element (Figs. 3D and 5A) nor the CAD/KNI binding sites alone (not shown) were able to conduct reporter gene expression in the embryo. However, 155-HfSt-4CAD/KNI that bears the 8 additional activator binding sites mediates gene expression in the posterior region of the wild-type embryo (Fig. 5B). The absence of TLL binding sites in this gene construct allowed terminal expression of the reporter gene as has been observed with the 284-HT-element (see Fig. 3B). This observation is consistent with the finding that TLL (in addition to HB) delimits the posterior border of the *h* stripe 6 expression domain (Pankratz et al., 1990; Langeland et al., 1994).

3. Discussion

Our results provide evidence that maternal *cad* activity plays an essential role for the activation of *h* stripe 6 expression in the posterior region of the *Drosophila* blastoderm embryo. Zygotic *cad* activity cannot complement the lack of the maternal *cad* function although zygotic CAD is expressed in a region of the embryo that includes the *h* stripe

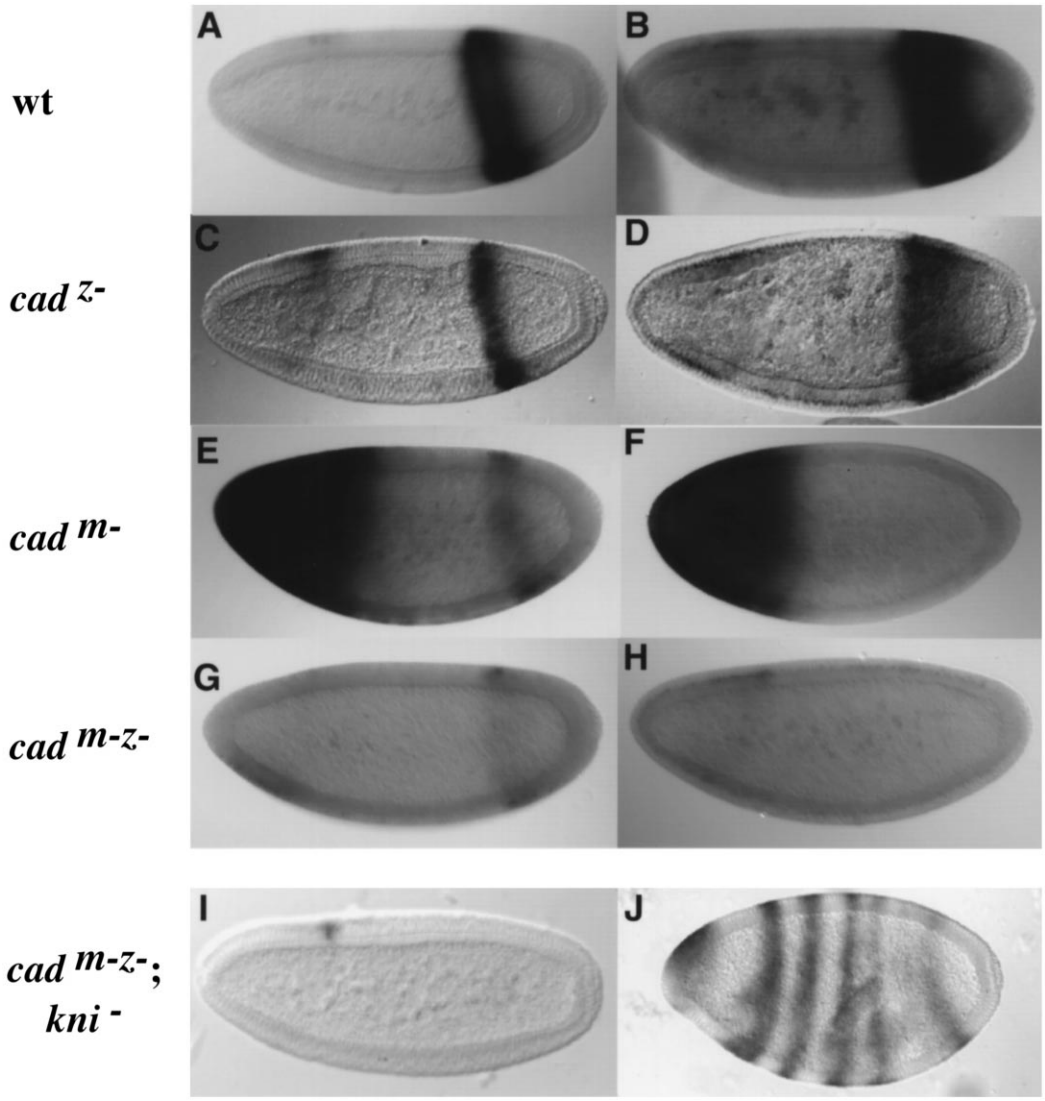

Fig. 4. 528-SG- and 284-HT-dependent reporter gene expression in *cad* and *kni* mutant embryos. The left panel (A,C,E,G,I) shows 528-SG-mediated expression (as detected by *lacZ* antisense RNA in situ hybridization). The right panel (B,D,F,H) shows the pattern conducted by the 284-HT-element. Note that the absence of zygotic *cad* activity (C,D) at best weakly affects the expression levels, while the absence of maternal *cad* activity (E,F) or both maternal and zygotic *cad* activities (G,H) affect the reporter gene expression mediated by each element in a dissimilar manner: while reporter gene expression mediated by the 528-SG-element remains at low levels, it is not detectable if driven by the 284-HT-element. Note that 528-SG-mediated reporter gene expression is abolished in embryos lacking both maternal and zygotic *cad* and *kni* activities (I) and that the endogenous expression of *h* stripes 5 and 6 is not detectable in embryos mutant for maternal and zygotic *cad* and *kni* (J). The anterior expression domain in (E,F) is the chromosome marker *hb-lacZ* (see Fig. 1). Orientation of the embryos: anterior is left, dorsal up.

6 domain (Rivera-Pomar et al., 1995; Schulz and Tautz, 1995). The activation of *h* stripe 6 expression in *kni*-deficient embryos is not significantly reduced but delayed. Thus, in the absence of *kni*, previously thought to be the activator of *h* stripe 6 expression (Pankratz et al., 1990; Langeland et al., 1994), maternal CAD is sufficient for the activation. Conversely, in *cad*-deficient embryos *h* stripe 6 activation occurs at a low level, but in time. Therefore, although maternal *cad* activity is necessary for activating *h* stripe 6 expression at high levels, it is not sufficient for a temporally correct transcriptional activity in the absence of *kni*.

The 532 bp *h* stripe 6-element does not contain distinct activation modules, one for each activator, as has been observed in the cis-acting region of *kni* (Rivera-Pomar et al., 1995). Instead, several subregions of the element were able to conduct gene activation in the embryo, provided they contain a minimal number of CAD and KNI binding sites. The idea that gene activation depends on the number of activator binding sites is supported by the finding that the addition of CAD/KNI binding sites to non-activating *h* stripe 6-subelement causes posterior gene activation. The fact that the 221-HSt-element (15 activator binding sites) activates gene expression at a low level, whereas the 155-HfSt-element (10 activator binding sites) failed to do so, indicates that the critical number of activator binding sites lays between 11 and 15 such sites. The finding that the 155-HfSt-4CAD/KNI-element (18 activator binding sites) mimics the expression mediated by a different arrangement of 18 activator binding sites on the 284-HT-element suggests that also the level of expression strongly depends on the number of activator binding sites. We found no evidence that KNI and CAD interact in a synergistic manner, leaving the possibility that the *h* stripe 6-element-mediated activation occurs in an additive fashion.

The absence of *cad* activity affects not only the expression of *h* stripe 6 but also the expression of *h* stripes 5 and 7. This event supports a major role for *cad* in activating *h* stripe expression in the posterior region of the embryo. Interestingly, the absence of BCD affects *h* expression in a complementary fashion, that is expression of stripes 1–4 is affected (Hooper et al., 1989; Hartmann et al., 1994), but stripes 5–7 appear normal. Thus, the two opposing protein gradients of BCD and CAD provide a complementing transcriptional activator system along the entire axis of the preblastoderm embryo necessary for proper *h* stripe expression. The importance of CAD as a posterior activator had been overlooked for a long time. This was because BCD can at least in part compensate for the role of CAD as an activator of posterior gap genes (Rivera-Pomar et al., 1995). The activating role of CAD in the posterior region of the embryo (Macdonald and Struhl, 1986), already suggested in the context of the *ftz* cis-acting control element (Dearolf et al., 1989), was substantiated by misexpression studies on *hb*, a regulator of zygotic *cad* expression (Schulz and Tautz, 1995). The results of the misexpression studies implied that gene activation in response to CAD involves the combined action of maternal and zygotic *cad* activities. Our results indicate, however, that zygotic *cad* activity cannot compensate for the lack of maternal *cad* activity in the case of *h*.

The lack of maternal *cad* activity disrupts but does not extinguish posterior *h* stripe expression. This finding is consistent with the observation that the lack of maternal *cad* activity can be partially substituted for by other activators as shown for *kni* (Rivera-Pomar et al., 1995), *h* stripe 7 (La Rosée et al., 1997) and *h* stripe 6 expression. Furthermore, *h* stripe 5 was previously thought to depend on KNI and more 'general factors' (Langeland et al., 1994). We observed that *h* stripe 5 expression is strongly reduced in the absence of maternal *cad* activity and noted that the *h* stripe 5-element (Fig. 8C in Langeland et al., 1994) contains at least 5 bona

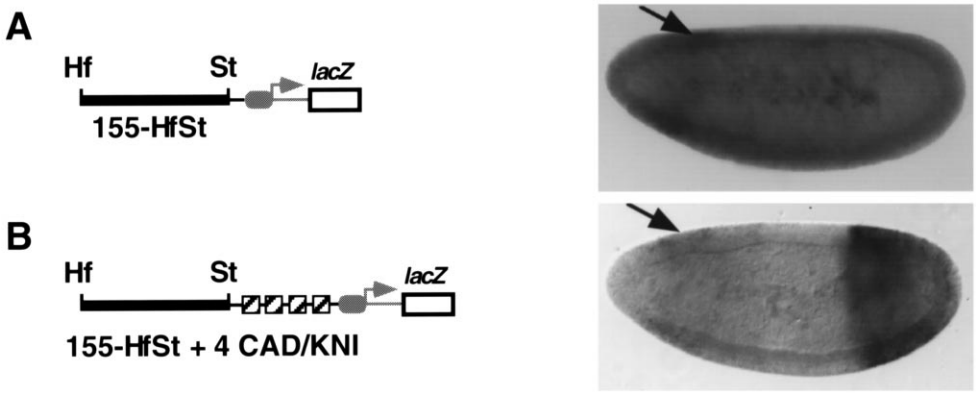

Fig. 5. Addition of CAD and KNI binding sites to the 155-HfSt-element rescues reporter gene expression. The arrow indicates the expression of an anterior stripe (derived from the basal promoter) serving as an internal control to evaluate the relative levels of reporter gene expression. (A) Absence of *lacZ* expression mediated by the 155-HfSt-element. (B) The addition of 8 activator binding sites (4 CAD and 4 KNI) downstream to the 155-HfSt-element results in reporter gene expression in the posterior region of the embryo in a manner similar to the 284-HT-element (compare to Fig. 3 for a description of the enhancer elements used). The hatched boxes indicate the CAD/KNI sites corresponding to CAD-2 and KNI-4 in Fig. 2C–E. The orientation of the embryos is anterior to the left, dorsal up.

fide CAD binding sites. Activation of *h* stripes 5 and 6 might therefore be similar with respect to the requirement for CAD and KNI as is seen in embryos lacking both activities (Fig. 4J). Moreover, recent studies on the regulation of *eve* stripe 3 and 7 expression showed a requirement for the Jak-STAT signalling system for gene activation mediated by the *eve* stripe 3,7-element (Hou et al., 1996; Small et al., 1996; Yan et al., 1996), likely to function as a ubiquitous activation system. We also noted a large number of consensus CAD binding sites within the *eve* stripe 3,7-element (Fig. 7A in Small et al., 1996). This suggests that CAD could participate in *eve* activation representing the proposed factor acting in combination with the Jak-STAT system (Small et al., 1996). Taken together, studies on pair-rule gene stripe expression are consistent with the argument that activation of posterior genes is dependent on CAD acting in concert with other transcriptional activators to provide proper expression of the target genes (summarized in Fig. 6A,B).

This proposal and the absence of significant activation of *h* expression in embryos lacking both *bcd* and *cad* activities (see below) suggest that two maternal homeodomain transcription factors provide a basis for the activation of pair-rule genes along the entire embryo. While the anterior stripes are dependent on BCD, CAD takes over the activation function in more posterior positions. This suggests that the basic scenario of transcriptional activators required for the expression of gap and primary pair rule genes might be identical: head gap genes, zygotic *hb* and *Kr*, are activated in response to BCD and HB (Hoch et al., 1991; Simpson-Brose et al., 1994; Wimmer et al., 1995), while the gap genes expressed in the abdominal region of the blastoderm embryo depend on *cad* and *bcd* activities (in the case of *kni*) and become more strongly dependent on *cad* activity in more posterior positions (in the case of *gt*) (Rivera-Pomar et al., 1995). However, BCD and CAD act by different means. While BCD is both necessary and sufficient for the activation of anterior genes and may require co-activators such as HB for establishing proper expression domains (Simpson-Brose et al., 1994), maternal CAD seems to act by providing a basal level of activation upon which other factors, such as BCD, KNI or KR, act to set the biologically relevant time and level of gene expression. The observation that the absence of BCD abolishes anterior gene expression, while posterior genes are activated in embryos lacking maternal CAD activity, although at a low level, is consistent with this proposal and it also supports the argument that CAD acts in an additive fashion rather than synergistically. Furthermore, the lack-of-function phenotypes of *bcd* embryos and *cad* embryos indicate that BCD can, at least in part, compensate for the lack of both maternal and zygotic *cad* activities, but not vice versa. In embryos which lack both BCD and zygotic CAD, but express maternal CAD throughout the embryo, the gap genes (with the possible exception of *Kr*) fail to be activated and no segments are formed. Consistently, pair-rule genes are only weakly activated, forming a coherent rather than a repetitive expression pattern in the embryo (Rivera-Pomar and Jäckle, 1996). Thus, maternal *cad* activity by itself does not seem to be sufficient to properly activate zygotic segmentation gene expression in the absence of the co-activators.

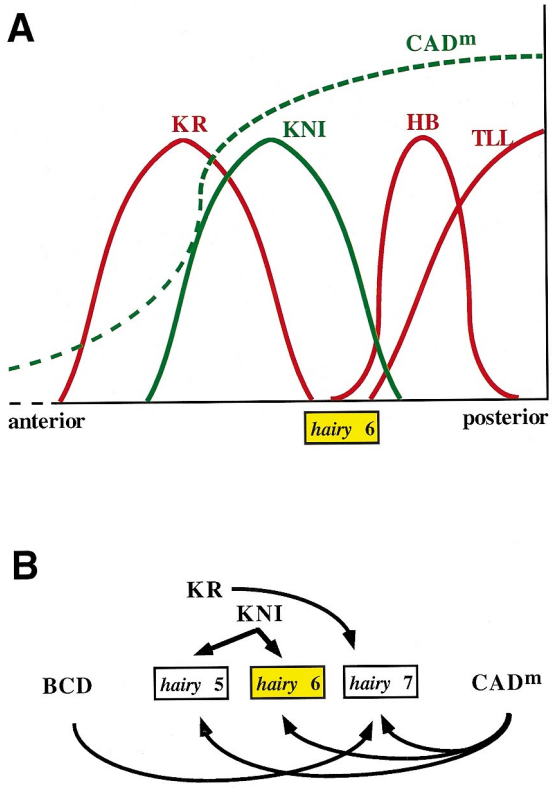

Fig. 6. Spatial distribution of transacting factors (A) and their regulatory input (B) on posterior *h* stripe activation. (A) Distribution of activators and repressors required for the proper spatial expression of *h* stripe 6 (see also Pankratz et al., 1990; Langeland et al., 1994). Activators are shown in green and repressors in red. (B) Posterior *h* stripe activation depends on maternal CAD, acting as a general activator, which functions in combination with different zygotic co-factors such as KR and KNI.

An interesting although puzzling aspect of *h* stripe 6-element mediated gene expression is the observation that the transacting factor requirement as well as the arrangement of the corresponding in vitro binding sites are the same as those found for the *h* stripe 7-element. The two cis-acting elements contain a high density of CAD and KNI binding sites, interspersed with binding sites for KR, HB and TLL. In both cases, TLL and HB cause repression. However, in the case of the *h* stripe 6-element, KNI acts as a weak activator, while in the case of the *h* stripe 7-element, KNI acts as a repressor that delimits the anterior border of the expression domain (La Rosée et al., 1997). Conversely, KR functions as a repressor that delimits the anterior border of the gene expression domain in the case of the *h* stripe 6-element, but acts as an activator on the *h* stripe 7-element (La Rosée et al., 1997). Thus, the two proteins provide an opposite and enhancer-specific regulatory input which is not reflected in the number or in the arrangement of binding

sites, which are strikingly similar in the two cases (compare Fig. 2E here and Fig. 3A in La Rosée et al., 1997). This leaves the question of how the transcription factors distinguish an enhancer and how they can play different roles in a similar context. Neither genetics nor recent molecular studies of enhancers (Gray et al., 1995; Gray and Levine, 1996) have yet deciphered these questions. The similar scenarios of transacting factors and binding sites within the two stripe enhancers which mediate gene expression in adjacent stripes provide an entrypoint from which questions concerning the enhancer-specific function of transcription factors can be addressed systematically by swapping binding sites and enhancer subfragments.

4. Experimental procedures

4.1. Reporter constructs and P-mediated transformation

Genomic fragments of the *h* stripe 6 promoter (Pankratz et al., 1990; Howard and Struhl, 1990) were subcloned into pBluescript KS(+) (Stratagene, USA) and subsequently cloned into pCaSpeR-*hs*43 (Thummel and Pirrotta, 1992). The fragments were ligated into the shuttle vector in their original orientation with respect to the promoter. P-element-mediated germ line transformation was done as described (Rubin and Spradling, 1982). The selectable marker white was used for insertion detection in either *w* or *yw* flies. At least 3 independent homozygous viable lines representing P-insertions were analyzed. The identity of the inserted reported gene construct was determined by PCR amplification of the enhancer region using DNA from transgenic flies and specific primers for the pCaSpeR vector. After balancing over CyO, TM3 or TM2 chromosomes the lines were used for genetic analysis. A P-insertion in the third chromosome was recombined with kni^{301} to generate embryos that were double mutants for *kni* and *cad*. For the artificial enhancer 155-HfSt-4CAD/KNI we used an oligo of the sequence AACGGGTTTTACGACGACCTCCGTCCGTT containing the binding sites number 2 and number 4 for CAD and KNI, respectively (see Fig. 2C,D).

4.2. Mutant stocks and genetic analysis

The mutant stocks used for genetic analysis kni^{301}, cad^{2}, cad^{3} are described in Fly Base (http://www.cbbridges.harvard.edu/). To assess the correct mutant identity and avoid spurious phenotypes due to the P-insertion, cuticle preparations of the transgenic lines were examined for segmentation defects. In the case of using P-insertions to generate recombinant chromosomes also bearing a mutation, the P-insertions were mapped by chromosome in situ hybridization, balanced for embryonic detection of an appropriate chromosome marker and examined for segmental defects of the mutants by cuticle preparation.

4.3. RNA in situ hybridization

The pattern of *hairy* or reporter gene (*lacZ*) expression were examined by in situ hybridization to whole mounted embryos using antisense RNA probes according to Klingler and Gergen (1993). The endogenous expression of the *lacZ* reporter gene under *hs*43 promoter (an anterior stripe-like expression) was used as internal reference that allowed us to assess the level of *h* stripe 6 expression.

4.4. DNase footprinting experiments

Proteins were produced in bacteria using T7 polymerase upon induction with IPTG (Strain BL21 (DE3), Studier and Moffat, 1986). The CAD expression construct (Rivera-Pomar et al., 1995) corresponds to the amino acids 58–377 and bears a tag of six histidines at the amino-terminus. A similar fusion construct was made for KNI and is described elsewhere (La Rosée et al., 1997). The induced proteins were purified by Ni^{2+}-columns, analyzed by SDS-PAGE and quantitated by Bradford assay using bovine serum albumin as a reference. DNA labeling reaction were performed by filling-in of recessed 3′-end using the Klenow fragment of DNA polymerase (Sambrook et al., 1989).

Footprinting reaction were performed as described (Kadonaga et al., 1987). Briefly, 1 to 10 pmol of protein (purity >90% assessed by SDS-PAGE and silver staining) were incubated with 1–10 ng of labeled DNA for 5 min on ice in reaction buffer (Z-buffer: 25 mM Hepes (K^+), pH 7.5, 100 mM KCl, 12.5 mM $MgCl_2$, 0.01 mM $ZnCl_2$, 20% glycerol, 0.1% Nonidet P-40, 1 mM DTT). DNase I (Worthington, UK) was added (variable amounts according to a previous titration using free probe) for 1 min and the reaction stopped, phenol extracted, ethanol precipitated and loaded onto a 6% polyacrylamide-8 M urea gel. The precise assessment of the binding site was done by quantitation using either scanning of autoradiographs or by using a Phosphorimager (Molecular Dynamics). Footprinting was done using both strands of partially overlapped fragments covering the entire *h* stripe 6-element. The resulting protected sequences were determined according to a sequence ladder corresponding to the G + A reaction of the Maxam and Gilbert sequencing procedure. The consensus was established by alignment.

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