Activation and repression of transcription by the gap proteins *hunchback* and *Krüppel* in cultured *Drosophila* cells

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We have studied the ability of the *Drosophila* gap proteins *Krüppel* and *hunchback* to function as transcriptional regulators in cultured cells. Both proteins bind to specific sites in a 100-bp DNA fragment located upstream of the segment polarity gene *engrailed*, which also contains functional binding sites for a number of homeo box proteins. The *hunchback* protein is a strikingly concentration-dependent activator of transcription, capable of functioning both by itself and also synergistically with the pair-rule proteins *fushi tarazu* and *paired*. In contrast, *Krüppel* is a transcriptional repressor that can block transcription induced either by *hunchback* or by several different homeo box proteins. While repression of the homeo box protein activators requires a *Krüppel*-binding site on the DNA, repression of *hunchback* can occur efficiently in the absence of a *Krüppel*-binding site. We discuss the possible molecular mechanisms underlying these activities, as well as the potential significance of these results with respect to segmentation in *Drosophila*.

[Key Words: *Drosophila* gap proteins; segment polarity gene; pair-rule proteins; homeo box protein]

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The segmentation process in *Drosophila* is controlled by a set of ~25 regulatory genes that are active in the early embryo (for review, see Akam 1987; Ingham 1988). Localized expression of the segmentation genes depends on a hierarchy of regulatory interactions (for review, see Scott and O'Farrell 1986). The first step is the establishment of broadly distributed gradients of maternal morphogens during oogenesis. The best understood of these is the homeo box protein *bicoid* (*bcd*), which plays a key role in localized expression of the next class of genes in the hierarchy, the gap genes (Frigerio et al. 1986; Frohnhofer and Nüsslein-Volhard 1986; Driever and Nüsslein-Volhard 1988a,b). Each gap gene is expressed in one or two broad domains that span several adjacent segment primordia. They influence the segmentation process by regulating the expression of the pair-rule genes, which are each expressed as seven stripes in alternating segment primordia along the length of the embryo (Carroll and Scott 1986; Ingham et al. 1986; Frasch et al. 1987). The pair-rule genes implement segmentation by controlling the expression of the segment polarity genes, which are expressed within specific subsets of cells in all 14 segment primordium (Kornberg et al. 1985; DiNardo and O'Farrell 1987).

A recurring theme of this regulatory hierarchy is the progressive refinement in the patterns of gene expression, whereby a given segmentation gene displays a relatively sharp "on/off" pattern of expression in response

to cruder gradients of factors upstream in the hierarchy. This process transduces the broad gradients of maternal morphogens (e.g., *bcd*) into the precise, single-cell limits of expression seen for the segment polarity genes. Perhaps the most dramatic increase in the complexity of these patterns involves the transition from the broad domains of the gap genes to the periodic expression of the pair-rule genes. A central problem in understanding the molecular basis of segmentation is how relatively few, broadly expressed gap genes are able to specify periodic patterns of stripes. The domains of gap gene expression are now known to result in overlapping concentration gradients of each of the different gap proteins (Gaul and Jäckle 1989; Stanojevic et al. 1989). Consistent with the view that these expression patterns are functionally significant, genetic studies suggest that both the concentrations and combinations of gap proteins are in some way important for determining the on/off periodicity of pair-rule promoters (for review, see Carroll 1990).

The gap genes characterized to date all encode DNA-binding proteins, and the most thoroughly studied contain DNA-binding Zn²⁺ fingers (Rosenberg et al. 1986; Tautz et al. 1987; Nauber et al. 1988; Redemann et al. 1988). *Krüppel* (*Kr*) and *hunchback* (*hb*) proteins have both been shown to bind to specific DNA sequences, each recognizing a distinct 10-bp consensus. Binding sites have been detected upstream of several potential target genes (Pankratz et al. 1989, 1990; Stanojevic et al.

1989; Treisman and Desplan 1989), the most striking example being the localization of >20 *hb*-binding sites in a 1.7-kb DNA segment situated upstream of the pair-rule gene *even-skipped* (*eve*) (Stanojevic et al. 1989). A number of genetic studies are consistent with the idea that *hb* can be an activator and *Kr* a repressor of gene expression (Jäckle et al. 1986; Frasch and Levine 1987; Carroll and Vavra 1989; Pankratz et al. 1990). However, it is difficult from genetic studies alone to distinguish between direct and indirect effects, and it has also been suggested that *hb* can act negatively and *Kr* positively (e.g., Pankratz et al. 1989, 1990; Struhl 1989).

Most of the other segmentation genes that have been cloned and characterized also encode sequence-specific DNA-binding proteins (for review, see Levine and Manley 1990). Mechanistically, the best understood are the homeo box-containing proteins, including the *bcd* protein, the pair-rule proteins *eve*, *fushi tarazu* (*ftz*), and *paired* (*prd*), and the protein encoded by the segment polarity gene *engrailed* (*en*). With the exception of *bcd* (Driever and Nüsslein-Volhard 1989), all recognize a common 10-bp consensus sequence (Desplan et al. 1988; Hoey and Levine 1988). Furthermore, all five are able to modulate transcription, as demonstrated by transient cotransfection (Jaynes and O'Farrell 1988; Han et al. 1989; Winslow et al. 1989), embryo microinjection (Driever and Nüsslein-Volhard 1989), expression studies in yeast (Fitzpatrick and Ingles 1989; Struhl et al. 1989), and in vitro transcription assays (Biggin and Tjian 1989; Ohkuma et al. 1990). These types of experiments have also provided evidence that the concentration gradient of *bcd* in the early embryo is functionally significant, as activation of the *hb* promoter by *bcd* was found to be markedly dependent on the concentration of the activator (Driever et al. 1989; Struhl et al. 1989). Likewise, the notion that combinatorial interactions between different segmentation gene products are important for correct pattern formation was strengthened by the observations that the *ftz* and *prd* proteins can function cooperatively to bring about strong synergistic activation of transcription (Han et al. 1989), while *eve* and *en* proteins are able to block the activation induced by *ftz* and/or *prd* (Jaynes and O'Farrell 1988; Han et al. 1989).

Here we show that the gap proteins *Kr* and *hb* function as transcriptional regulators in cultured cells. Unexpectedly, binding sites for both proteins were found in a well-characterized DNA segment located upstream of the *en* gene, which also contains functional binding sites for several of the pair-rule proteins mentioned above. We present evidence that *hb* can function as an activator of transcription and *Kr* as a repressor. We show further that these two gap proteins can functionally interact, not only with each other but also with several homeo box proteins.

Results

To determine whether the proteins encoded by the gap genes *Kr* and *hb* can function as sequence-specific transcription factors, we employed a transient expression as-

say similar to that utilized previously by us and others to examine the activities of several *Drosophila* homeo box proteins (e.g., Jaynes and O'Farrell 1988; Han et al. 1989; Krasnow et al. 1989). Specifically, Schneider line 2 cells were cotransfected with plasmids capable of expressing *Kr* or *hb* proteins, an appropriate reporter plasmid, and an internal control plasmid (see Materials and methods). Indirect immunofluorescence assays indicated that both proteins were efficiently expressed in transfected cells (results not shown). Reporter plasmids used in this study contained, except where otherwise indicated, the metallothionein "basal" promoter (i.e., TATA box plus transcriptional start site) fused to the *Escherichia coli* chloramphenicol acetyltransferase (CAT) gene, with DNA fragments containing *Kr*- and *hb*-binding sites ligated upstream (e.g., see Fig. 2). In preliminary experiments, we tested fragments from the *eve* 5'-flanking region shown previously to contain multiple *hb*- and/or *Kr*-binding sites (Stanojevic et al. 1989). Cotransfection with *hb* and/or *Kr* expression vectors failed to reveal significant effects on promoter activity with these reporter plasmids (results not shown), perhaps reflecting requirements for additional factors. However, we were surprised to observe that a plasmid nominally used as a control, k'-TATA-CAT, was, under certain conditions, responsive to both *Kr* and *hb* (see below).

The k' fragment is a 100-bp DNA segment naturally located 5' to the *en* promoter and was shown previously to contain five consensus binding sites for a number of different homeo box proteins (Desplan et al. 1988; Hoey and Levine 1988), all of which can modulate transcription in response to these sequences (Han et al. 1989). To determine whether *Kr* and/or *hb* proteins might also be able to function by directly binding to sequences within k', we analyzed the ability of *Kr* and *hb* proteins synthesized in *E. coli* to footprint on the k' fragment. The results, shown in Figure 1A, indicate that both proteins bind specifically to sequences within fragment k'. *hb* protein protects two regions from DNase I digestion, whereas *Kr* protects one site. Examination of the nucleotide sequences of the protected regions revealed in each case an excellent match to the previously determined consensus for each protein (Stanojevic et al. 1989). These findings are summarized in Figure 1B, which also indicates the previously defined homeo box protein-binding sites. Note the striking overlap of the *Kr* site with the two head-to-head homeo box-binding sites. An additional match (8/10) to the *hb* consensus overlaps the second of the three head-to-tail homeo box sites. However, we have never observed protection of this site, and its significance is unknown.

The *hb* protein is a transcriptional activator

To investigate the ability of the *hb* protein to modulate transcription in response to the *en* k' fragment, we first asked whether CAT expression from reporter plasmids containing single or multiple copies of either k' or smaller subfragments was affected by *hb* in cotransfection assays. The sequences of the kI and kII subfrag-

Figure 1. *Kr* and *hb* proteins bind to specific sites in the *k'* fragment of the *en* promoter. (A) Footprint analysis of *hb*- and *Kr*-binding sites in the *k'* fragment. A 500-bp *Hind*III–*Eco*RI fragment containing the *k'* fragment from *k'*–TATA–CAT was ³²P-labeled at the *Hind*III site and incubated with increasing amounts of *Kr* or *hb* protein extracts. Following DNase digestion, samples were resolved on an 8% polyacrylamide–7.5 M urea gel. The samples shown in lanes 3 and 4 contained 50 or 75 μ g, respectively, of *Kr* protein extract. One region was protected from digestion with DNase I (indicated by the solid rectangle, right). The samples in lanes 5–7 contained 25, 75, or 150 μ g, respectively, of *hb* protein extract. Two regions of protection were observed, hb1 and hb2 (open rectangles, right). Lane 2 (–) displays a control in which DNase I digestion of the *Hind*III–*Eco*RI fragment was performed without added protein. Lane 8 (C) shows a control done with 75 μ g of protein extract from cells containing the T7 expression vector without *hb* or *Kr* cDNA inserts. Lane 1 (GA) shows a G + A sequencing reaction of the *Hind*III–*Eco*RI DNA fragment. (B) Position of binding sites in the *k'* fragment. The numbers –949 and –849 indicate the distance (in bp) of the *k'* fragment from the *en* transcription start site. The thin arrows indicate the five binding sites for a number of different homeo box proteins. The bold arrows indicate the *hb*- and *Kr*-binding sites determined from the DNase I protection analysis shown in Fig. 1A. The match of each site to previously established *hb* and *Kr* consensus sequences (Stanojevic et al. 1989) is indicated, and these sequences are shown at the bottom. A *Pvu*II site that defines the boundary between the kI and kII subfragments is indicated.

ments are indicated in Figure 1B. The 63-bp kI fragment contains the three head-to-tail homeo box protein-binding sites and a *hb* site, while the 37-bp kII fragment contains the second, inverted *hb* site, the single *Kr* site, and the two head-to-head homeo box protein sites.

Figure 2 displays the relative CAT activities obtained when the indicated reporter plasmids were cotransfected with the actin 5C promoter-containing expression vector either lacking an insert (Act–PPA) or with *hb*-coding sequences inserted (Act–*hb*). To facilitate comparisons of the effects of *hb* on the different reporter constructs, all values were normalized so that in each case the CAT activities from the cotransfections with Act–PPA were set at 1.0 (see legend to Fig. 2). The results indicate that the *hb* protein is a sequence-specific activator of transcription, giving rise to a 20-fold activation with the reporter construct containing a single copy of the kI fragment. However, the behavior of *hb* was somewhat unusual. Although activation was observed with both the intact *k'* fragment and the kI subfragment, increasing the number of either of these fragments resulted in significantly decreased *hb*-dependent promoter activity. This is in contrast to many previous studies showing that multimerization of transcription factor-binding sites, including the homeo box-binding sites in the *k'* fragment itself, frequently leads to substantial increases in promoter activity (e.g., Herr and Clarke 1986; Zenke et al. 1986; Jaynes and O'Farrell 1988; Han et al. 1989). A decrease in activity has not, to our knowledge, been observed previously. Consistent with the fact that increasing the num-

ber of potential binding sites decreased activation by *hb*, the *k'* fragment, which contains two binding sites, was only half as active as the kI fragment, which contains just one. Indeed, the only detectable effect of the kII subfragment was to reduce activation mediated through kI, as a reporter plasmid containing only the kII fragment (kII–TATA–CAT) was unresponsive to *hb*. Although we do not know why the *hb*-binding site in kII was not functional, it does not appear to reflect the inverted orientation of this site, as reversing the orientation of kI resulted in only a slight reduction in *hb*-induced activation (cf. 2kI–TATA–CAT and 2kI–TATA–CAT–rr).

One possible explanation of the above results is that increasing the local concentration of the *hb* protein might by some mechanism result in reduced activity. An implication of such a model is that the activity of *hb* might also be reduced at higher concentrations of protein. To address this possibility, we performed a dose-response analysis over a wide range of concentrations of *hb* expression vector, using a constant amount of kI–TATA–CAT. The results, shown in Figure 3, reveal a somewhat skewed, bell-shaped activation curve. Significant (fourfold) enhancement of CAT expression was achieved with only 10 ng of Act–*hb*, and optimal (20-fold) activation was observed with 200 ng. Above this amount, significant decreases in activation were detected, with CAT activity dropping to only threefold above the base line with 1.5 μ g of Act–*hb*. That increasing amounts of Act–*hb* resulted in elevated concentrations of *hb* protein in the transfected cells was verified

Figure 2. *hb* is a sequence-specific transcription activator. The schematic at the top displays features of the parental reporter plasmid used in these experiments, k'-TATA-CAT. The arrows denote the *hb* consensus-binding sites present in the two regions of fragment k', referred to as kI and kII. The bottom displays the results of cotransfection experiments containing 0.2 μ g Act-PPA or Act-*hb* and 5.0 μ g of the indicated reporter plasmids (see Materials and methods). To compare the effects of *hb* on the different reporter constructs, all CAT activities were normalized so that in each case the values from the cotransfections with Act-PPA were set at 1.0. The actual basal CAT activities from the different reporter plasmids varied by a factor of 3 or less.

by Western blotting (Fig. 3, insert). This dose-response curve differs from those observed in similar previous experiments with other transcription factors, including our own studies with a number of different homeo box proteins (Han et al. 1989; K. Han and P. Zuo, results not shown). In these cases, activity plateaued and remained essentially constant with as much as 2–5 μ g of expression vector. Furthermore, the observed inhibition appears to be relatively specific for *hb*-responsive promoters, because expression from several different promoters lacking *hb*-binding sites was not affected by any concentration of Act-*hb* (results not shown).

hb protein can function cooperatively with pair-rule proteins *ftz* and *prd*

The fact that *hb* can interact with the *en* k' fragment to activate transcription raises the question of whether the protein can function synergistically with other proteins that bind this DNA. Specifically, we showed previously that several homeo box proteins that recognize the multiple binding sites within k' can, by themselves, enhance transcription only weakly, but together result in much

Figure 3. Activation by *hb* is concentration-dependent. A dose-response analysis was performed over a wide range of concentrations of Act-*hb* (0.01–1.5 μ g) with a constant amount of kI-TATA-CAT (5 μ g). The amount of expression vector in each sample was adjusted to 1.5 μ g by addition of Act-PPA [see Materials and methods]. CAT activities are expressed relative to the activity from kI-TATA-CAT plus 1.5 μ g of Act-PPA, which was taken as 1.0. The insert shows the *hb* protein, detected by Western blotting, in extracts of cells transfected with the indicated amounts of Act-*hb*.

greater synergistic increases in activity (Han et al. 1989). These include the products of the pair-rule genes *prd* and *ftz*, and the dorsal-ventral gene *zerknüllt* (*zen*). To test whether these proteins can function synergistically with *hb*, we performed cotransfection experiments with plasmids expressing each of the proteins, alone or in combinations, along with k'-TATA-CAT (Table 1). Although each of these proteins can give rise to significant activation with reporter plasmids containing multiple copies

Table 1. *hb* functions cooperatively with *ftz* or *prd* but not *zen*

Expression vectors	k'-TATA-CAT
Act-PPA	1.0
Act- <i>hb</i>	6.3
Act- <i>ftz</i>	1.1
Act- <i>hb</i> + Act- <i>ftz</i>	53
Act- <i>prd</i>	1.8
Act- <i>hb</i> + Act- <i>prd</i>	19
Act- <i>zen</i>	1.6
Act- <i>hb</i> + Act- <i>zen</i>	8.2

Cotransfections were carried out as described in Materials and methods. In each case, 0.5 μ g of the indicated expression vector was used, together with an appropriate amount of Act-PPA so that all transfections contained 1.0 μ g of expression vector. The numbers shown are the ratios of the CAT activity obtained from the indicated cotransfection with the CAT activity obtained when k'-TATA-CAT was cotransfected with Act-PPA, which was set at 1.0.

of *k'* (Han et al. 1989), they were almost inactive with a single copy of *k'*. However, when cotransfected with Act-*hb*, synergistic activation of transcription was observed with both *ftz* and *prd*. In contrast, the *zen* protein, although able to function synergistically with *ftz* and/or *prd*, was essentially inactive with *hb*. These results indicate that not only can *hb* interact synergistically with certain pair-rule proteins but that this synergism is not entirely promiscuous with respect to the nature of the second activator.

The above experiments indicate that *hb* functions more efficiently with either *prd* or *ftz* than it does by itself. An interesting question then becomes whether higher concentrations of *hb* expression vector, which alone give rise to relatively poor activation, can function effectively with *prd* or *ftz*. We therefore carried out a dose-response analysis similar to that shown in Figure 3, except that 0.5 μ g of Act-*prd* vector was also included in the transfections and *k'*-TATA-CAT was used as the reporter plasmid. The results, shown graphically in Figure 4, reveal that the concentration of *hb* that gave optimal activation with *prd* was significantly higher than when *hb* was transfected alone. Specifically, 1.2 μ g of Act-*hb* gave rise to maximal CAT activity with *prd*, and activation was only slightly lower with 1.8 μ g of *hb* expression vector. This contrasts markedly with the results observed with Act-*hb* alone, which again showed maximal activation at 200 ng. These findings are not likely to reflect differences in *hb* expression in the cotransfected cells, because expression from an Act-

Figure 4. High levels of *hb* function effectively in the presence of a second activator. A dose-response analysis with the indicated amounts of Act-*hb*, similar to that shown in Fig. 3, was performed, except that 0.5 μ g of Act-*prd* (solid line) or Act-PPA (dashed line) was included in the transfections, and *k'*-TATA-CAT (5 μ g) was used as the reporter plasmid. The total amount of expression vector in each sample was adjusted to 2.2 μ g with Act-PPA. The graph displays the percentage of the maximal activation observed with each concentration of Act-*hb*. Maximal activations were observed with 0.2 μ g of Act-*hb* plus Act-PPA and 1.2 μ g when Act-*prd* was present.

CAT vector was not influenced by cotransfection with Act-*prd* (results not shown). These results suggest that the high levels of *hb* protein that alone lead to reduced promoter activation function effectively in the presence of a second activator protein.

Kr protein is a transcriptional repressor

To examine the ability of *Kr* to function as a transcriptional regulator, we carried out a series of experiments with an Act-*Kr* expression vector similar to those shown in Figures 2 and 3 with Act-*hb*. Under no conditions tested was any evidence obtained suggesting that *Kr* could activate CAT expression. Rather, reductions of approximately twofold in CAT activity produced from plasmids containing the *k'* fragment were frequently observed (results not shown). However, because the magnitude of these effects was low and they were not seen reproducibly, we have not pursued further the possibility that *Kr* can function as a direct repressor (Levine and Manley 1989; see Discussion). Instead, we have studied the ability of *Kr* to affect the activation induced by other proteins that interact with the *k'* fragment.

Table 2 displays the relative CAT activities from cotransfections with several expression vectors encoding homeo box proteins plus or minus a similar amount of the *Kr* expression plasmid, along with *k'*-TATA-CAT. In one experiment, we examined the ability of *Kr* to influence the activity of the homeo box protein *z2*. The *z2* gene, which is related to the dorsal-ventral gene *zen* (Rushlow et al. 1987; Kaufman et al. 1990), encodes a protein that is the strongest *k'*-dependent activator in the cotransfection assays that we have performed (Han et al. 1989). In the experiment shown, *z2* induced a 70-fold activation of *k'*-TATA-CAT in the absence of *Kr* but only a 2.5-fold activation in its presence. In a second experiment, we asked whether *Kr* could function through the 37-bp *kil* fragment, which contains the *Kr*-binding site centered over two head-to-head homeo box protein sites (see Fig. 1B). Cotransfection of expression vectors encoding *ftz* and *prd*, together with *kil*-TATA-CAT, resulted in a 15-fold increase in relative CAT activity (Table 2). When Act-*Kr* was included in the cotransfection, however, CAT activity was reduced to near basal levels. In contrast, CAT activity was reduced only slightly when a *Kr* expression vector encoding a mutant, DNA binding-defective (results not shown) protein deleted for 183 carboxy-terminal amino acid residues (*Kr*-2), including two of the four putative Zn^{2+} fingers, was cotransfected with either *z2*- or *ftz*- plus *prd*-encoding plasmids. These results together provide strong support for the notion that the *Kr* protein can function as a transcriptional repressor to block activation induced by several different positive transcription factors.

Kr specifically blocks activation by *hb* independent of *Kr* DNA-binding sites

The possibility that *Kr* and *hb* proteins might functionally interact during embryogenesis has been raised by

Table 2. *Kr* blocks activation by homeo box proteins

Promotor–CAT fusions	– <i>Kr</i>			+ <i>Kr</i>			+ <i>Kr-2</i>		
	Expression vectors								
	Act–PPA	<i>ftz</i> + <i>prd</i>	<i>z2</i>	Act–PPA	<i>ftz</i> + <i>prd</i>	<i>z2</i>	Act–PPA	<i>ftz</i> + <i>prd</i>	<i>z2</i>
kII–TATA–CAT	1.0	15		0.9	1.5		0.9	9.5	
k'–TATA–CAT	1.0		70	0.8		2.5	0.9		62

Cotransfections with the indicated plasmids were performed as described in Materials and methods. Transfections contained 0.5 μ g each of Act-*ftz* and Act-*prd*, 0.2 μ g of Act-*z2*, and/or 0.8 μ g of Act-*Kr*, as indicated. Act-PPA was added where appropriate so that all transfections contained equivalent amounts of expression vector. Blank spaces indicate combinations that were not tested.

previous studies (e.g., Gaul and Jäckle 1989; Stanojevic et al. 1989; Treisman and Desplan 1989). To address the possibility that one of these two proteins might directly influence the activity of the other, we performed cotransfection experiments in which expression vectors encoding each protein were introduced into Schneider cells, along with one of several different reporter plasmids, and the resulting CAT activities were measured. The results, shown in Figure 5, indicate that *Kr* effectively blocked activation induced by *hb*. Unexpectedly, this was true not only with k'-TATA-CAT, which contains binding sites for both proteins (see diagram at bottom of Fig. 5), but also with kI- and 4kI-TATA-CAT, which contain functional *hb*-binding sites but no detectable *Kr* sites, as assayed by both DNase protection (Fig. 1) and gel retention assays (data not shown). These results not only indicate that *Kr* can repress the activity of *hb* very efficiently but also suggest that this repression can occur in the absence of detectable sequence-specific

DNA binding by *Kr*. The *Kr-2* mutant was unable to repress *hb*-mediated activation from either k'- or kI-containing plasmids (Fig. 5). Thus, the sequences deleted in *Kr-2* appear to be necessary for both binding site-dependent and binding site-independent repression.

To investigate the binding site-independent repression further, we tested whether the ability of *Kr* to block the activation induced by several homeo box proteins was dependent on the presence of a *Kr* DNA-binding site. We first tested the strong activator protein *z2* (Fig. 5). As described above, when the *z2* expression vector was cotransfected with k'-TATA-CAT, a strong activation was observed, which was almost totally repressed by *Kr*. In contrast, when Act-*z2* was cotransfected with kI-TATA-CAT, an even greater increase in CAT expression was observed, but this activation was almost unaffected by coexpression of *Kr*. We also tested whether a *Kr* DNA-binding site was required for repression of the synergistic activation induced by two homeo box proteins. Figure 5 shows that with k'-TATA-CAT, the combination of the *ftz* and *zen* expression vectors brought about a 25-fold increase in CAT activity. As with the other activator proteins tested, this increase was effectively repressed by *Kr*. However, when the *ftz* and *zen* expression vectors were cotransfected with the 4kI-TATA-CAT reporter plasmid, the resulting 80-fold induction of CAT activity was almost totally resistant to *Kr*-mediated repression. These results together indicate that *Kr* can block activation by at least two distinct mechanisms, one requiring a *Kr*-binding site and the other independent of such a site.

Promotor CAT fusions	Expression vectors							
	Act-PPA	<i>hb</i>	<i>hb</i> + <i>Kr</i>	<i>hb</i> + <i>Kr-2</i>	<i>z2</i>	<i>z2</i> + <i>Kr</i>	<i>ftz</i> + <i>zen</i>	<i>ftz</i> + <i>zen</i> + <i>Kr</i>
k'-TATA-CAT	1.0	7.4	0.7	8.1	76	2.7	25	0.9
kI-TATA-CAT	1.0	14	1.1	13.6	140	120		
4kI-TATA-CAT	1.0	7.8	1.4	7.3			80	70

Figure 5. *Kr* specifically blocks activation by *hb* independent of DNA-binding sites. (Top) The relative CAT activities obtained from cotransfections with the indicated combinations of expression vectors and reporter constructs. Transfections contained 0.2 μ g of Act-*hb* or Act-*z2*, 0.5 μ g each of Act-*ftz* and Act-*zen*, and/or 0.8 μ g of Act-*Kr*. Empty squares represent combinations not tested. The schematic at the bottom indicates the homeo box protein *Kr* and *hb* DNA-binding sites in the kI and kII fragments. Arrows indicate the position and orientation of the sites.

Repression of *z2*-dependent activation by *Kr* is not dependent on binding site orientation or position

The above results indicate that *Kr*, presumably by binding to its recognition site in the kII fragment, can block activation induced by homeo box proteins interacting with the upstream kI fragment. This observation allowed us to determine whether the spacing or orientation of the *Kr* and homeo box protein-binding sites influences *Kr* function. In one experiment, the orientation of the k' fragment was inverted. This changes the orientation of all the binding sites in k' relative to the basal promoter and also places the *Kr*-binding site upstream of the homeo box-binding sites in the kI fragment. In addition,

tion, another basal promoter, from the heat shock protein 70 gene, was used in this experiment (see Materials and methods), which allowed us to determine whether *Kr* repression is influenced by the identity of the basal promoter. As shown in Table 3, when *k'*-hsp-TATA-CAT and *rk'*-hsp-TATA-CAT were cotransfected with Act-z2 alone, significant activation, similar to that with *k'*-TATA-CAT, was observed with each. However, when Act-*Kr* was included in the transfections, activation was nearly abolished, indicating that the *Kr* protein can function efficiently in this context of binding sites and basal promoter. To determine whether the spacing between binding sites is important for *Kr* function, *kII(90)kI*-TATA-CAT was constructed and analyzed (Table 3). In this plasmid the *Kr*-binding site (*kII*) is again located upstream of the homeo box sites, but the spacing between them is increased by 90 bp (from 35 to 125 bp). CAT expression from this construct was activated by z2 to roughly the same level as with *k'*-TATA-CAT, and this activation was again greatly reduced by *Kr*. These findings indicate that the ability of *Kr* to block activation induced by the z2 protein is not dependent on the orientation or spacing of the binding sites.

Discussion

The experiments described above have provided evidence that the products of the gap genes *hb* and *Kr* can function as transcriptional regulators in *Drosophila* tissue culture cells. The *hb* protein is a strongly concentration-dependent activator of expression, functioning relatively poorly at high concentrations or with reporter plasmids containing multiple *hb*-binding sites. *hb* can also function synergistically with the pair-rule proteins *ftz* and *prd*. In contrast, *Kr* is a repressor, capable of blocking activation induced by a number of homeo box proteins, as well as by *hb*. Interestingly a *Kr* DNA-bind-

ing site is apparently not required for repression of *hb* but is required for repressing the homeo box proteins.

hb is a concentration-dependent transcriptional activator

The properties of the *hb* protein described here are consistent with several previous genetic studies and also provide some insights into the mechanisms by which it might function in the early embryo. The activity of *hb* has been shown to be required for proper expression of three of the seven *eve* stripes (2, 3, and 7; Frasch and Levine 1987; Goto et al. 1989; Harding et al. 1989). Consistent with the findings described here that *hb* is a transcriptional activator, the *cis*-acting elements required for expression of these stripes contain multiple *hb*-binding sites, suggesting a direct positive role for *hb* in *eve* regulation. Although we have not detected an effect of *hb* on *eve* expression in transfection assays, the function of *hb* in the generation of stripe 3 is nonetheless intriguing in light of the data presented here. The *cis*-acting sequences required for stripe 3 expression contain ~20 *hb*-binding sites in a 1.7-kb DNA segment (Stanojevic et al. 1989) and binding sites for other potential regulators have not been detected (D. Stanojevic and M. Levine, unpubl.). However, the concentration of *hb* in stripe 3 is low, and it decreases from anterior to posterior. While the posterior boundary of the stripe probably arises simply when a subthreshold level of *hb* protein is reached, it may be that the anterior boundary is established at the point where *hb* rises above a maximum level. The large number of binding sites may be able to respond to low concentrations of *hb* but would then be repressed at higher levels of protein. This possibility is suggested by the bell-shaped dose-response curve for *hb* activation, which indicates that high concentrations of *hb* are inactive, and offers an explanation for the otherwise paradoxical observation that the highest concentrations of *hb* protein in the early embryo are in cells between those constituting *eve* stripes 2 and 3 (Stanojevic et al. 1989).

What is the molecular mechanism responsible for the unusual behavior of the *hb* activator? We suggest two possible models. In one, a limiting factor required to mediate *hb* function [e.g., a "coactivator" (Pugh and Tjian 1990) or "adaptor" (Martin et al. 1990)] is titrated out by high concentrations of *hb*. This would result in "self-squelching" of *hb* activity, as has been observed recently with derivatives of the adenovirus E1a protein (Martin et al. 1990; for discussion of squelching, see Ptashne 1988). Note that the hypothetical coactivator is unlikely to be a general transcription factor, because high levels of *hb* were found not to reduce transcription from a number of different basal promoters. A second model offers an explanation consistent with all the properties of *hb* described here. Specifically, it suggests that *hb* activity is controlled by multimerization, with the protein being maximally active as a monomer. That the *hb*-binding site lacks dyad symmetry is consistent with the idea that the protein binds DNA in this form. However, by this model, *hb* is able to form multimers, either free in solu-

Table 3. Repression by *Kr* is not dependent on binding site position or orientation

Promotor-CAT fusions	Expression vectors		
	Act-PPA	Act-z2	Act-z2 + Act-Kr
<i>k'</i> -hsp-TATA-CAT	1.0	45	3.2
<i>rk'</i> -hsp-TATA-CAT	1.0	38	1.1
<i>k'</i> -TATA-CAT	1.0	69	2.6
<i>kII(90)kI</i> -TATA-CAT	1.0	95	7.9

Cotransfections with the indicated plasmids were performed as described in Materials and methods, except that the amount of the hsp-TATA-CAT reporter plasmids was only 0.1 µg. This was because the basal level of CAT expression from this promoter was significantly higher than from the TATA-CAT plasmids used elsewhere in this study. The amounts of expression vectors used were the same as in Table 2. CAT activities were normalized so that in each case the values from the cotransfections with Act-PPA were set at 1.0.

tion or when bound to DNA, and such forms of the protein are less efficient or nonfunctional as transcriptional activators. In keeping with the notion that *hb* molecules can interact, some evidence suggests that *hb* can bind DNA cooperatively (Stanojevic et al. 1989). This model offers an explanation for the lower activity observed with reporter plasmids containing multiple *hb*-binding sites, as this could facilitate formation of *hb* multimers. In addition, the *prd* protein, or other proteins with which *hb* can functionally interact, might interfere with the formation of such inactive complexes by binding to a nearby DNA site, thereby increasing the concentration required of *hb* required to form multimers on the DNA. Whatever the mechanism, these properties of *hb* could play an important role in the ability of the protein to bring about the proper spatially and temporally regulated activation of target genes during embryogenesis.

Kr is a transcriptional repressor

The data presented here and in a recent study employing mammalian tissue culture cells (Licht et al. 1990) indicate that *Kr* can function as a repressor. This conclusion is consistent with several previous genetic studies. For example, in *Kr*[−] embryos the domain of *hb* expression undergoes a posterior expansion into a region of the embryo that normally expresses high levels of *Kr* protein (Jäckle et al. 1986). Similarly, *eve* expression stripe 2 also expands posteriorly in *Kr*[−] embryos (Frasch and Levine 1987), while the sixth expression stripe of another primary pair-rule gene, *hairy* (*h*), expands anteriorly (Ingham et al. 1986). The 5′-flanking regions of both of these genes contain *Kr*-binding sites that are within the *cis*-acting elements required for stripe 2 (*eve*) and stripe 6 (*h*) expression (Stanojevic et al. 1989; Pankratz et al. 1990). It is therefore likely that *Kr* can act as a transcriptional repressor in embryos as it does in cultured cells and that this activity helps define the boundaries of expression of other segmentation genes. As mentioned in the introductory section, there are also genetic experiments suggesting that *Kr* can act positively (and *hb* negatively). Whether these findings reflect indirect effects (e.g., *Kr*-repressing expression of a second repressor) or the ability of these proteins to function as both activators and repressors is not known.

There are several ways by which DNA-binding transcriptional repressors can function to block gene expression (for review, see Levine and Manley 1989), and *Kr* may exploit at least two of them. In principle, the simplest mechanism is competitive DNA binding, where binding of the repressor prevents or interferes with binding of an activator. It is highly likely that *Kr* can function in this manner. Specifically, given the positioning of binding sites in the *kil* fragment, where the *Kr* site directly overlaps two head-to-head homeo box sites, it is probable that the repression of *prd* and *ftz* is mediated by competitive DNA binding. A second type of repression, termed quenching, occurs when binding of the repressor does not block the binding of an activator but interferes with its function by some other mechanism, for exam-

ple, by “masking” its activating domain. It is likely that *Kr* can also function in this manner, as suggested by the following two examples. First, activation induced by *hb*, which occurs through a binding site ~50 bp from the *Kr*-binding site in *k*′, is efficiently repressed by *Kr*. Given the size of the DNA footprints, and the fact that *Kr* and *hb* proteins can simultaneously occupy their respective DNA-binding sites (P. Zuo and D. Stanojević, unpubl.), we consider it unlikely that *Kr* interferes with the binding of *hb*. Furthermore, the fact that a *Kr*-binding site is not required for repression of *hb* is not consistent with DNA-binding competition. Second, *Kr* can repress *z2*-mediated activation even when the *Kr*-binding site is situated >120 bp upstream of the *z2*-binding sites [*kil*(90)*kil*–TATA–CAT]. Although it has not been shown directly that *Kr* and *z2* can simultaneously bind to this fragment, it is very likely that they do, given the distance separating the two sites, and a simple binding site competition is again unlikely. Note that these two examples themselves most likely reflect distinct mechanisms, as the latter is dependent on a *Kr* DNA-binding site while the former is not. In addition, because *Kr* can function when its binding site is situated 5′ as well as 3′ of the activators, repression must require more than a simple steric “roadblock” between the activator and the basal transcription machinery. Together, these observations suggest strongly that *Kr* can repress by mechanisms other than binding-site competition and that specific protein–protein interactions are probably involved in this type of repression.

What is the molecular mechanism underlying the ability of *Kr* to quench activation induced by several different positive transcription factors? While it is clear that additional studies will be required to answer this question fully, several points merit discussion. It has recently been shown by Licht et al. (1990) that *Kr* can function as a sequence-specific repressor in mammalian cells and, in the context of fusion proteins with a heterologous DNA-binding domain, that the amino-terminal alanine-rich region of the protein is sufficient to bring about repression. This region may well be necessary for at least some of the effects of *Kr* described here, and we have found that an alanine-rich region in the *eve* protein plays an important role in its function as a transcriptional repressor (K. Han and J.L. Manley, unpubl.). The data presented here, however, indicate that the carboxy-terminal third of *Kr* is necessary for its function in both DNA-binding site-dependent and -independent repression. In the former case, this is readily explained by the inability of the *Kr*-2 mutant to bind DNA. However, in the latter case, the deleted sequences must play another role, and a likely possibility is that they are involved in protein–protein interactions. Indeed, recent studies have implicated sequences in the DNA-binding domains and carboxyl termini of several Zn²⁺ finger steroid receptors as critical for heterodimer formation [thyroid and retinoid acid receptors (Glass et al. 1989); glucocorticoid receptor and AP-1 (Jonat et al. 1990; Schüle et al. 1990; Yang-Yen et al. 1990)]. Although our preliminary experiments have failed to detect such a complex, it is conceivable that *Kr*

and *hb* can form a heterodimer that is unable to activate transcription. Alternatively, *Kr* might interact with another protein required for *hb* function, such as the hypothetical coactivator mentioned above, thereby preventing activation.

Kr and hb can functionally interact with homeo box proteins

The demonstration that *hb* can function synergistically with the homeo box proteins *ftz* and *prd*, while *Kr* can block their activity, raises the possibility that direct competitive and/or cooperative interactions between gap and pair-rule gene products are important for the regulation of gene expression during embryogenesis. In particular, it is conceivable that the segment polarity gene *en* is regulated by combinatorial interactions between gap and pair-rule products. Genetic studies have provided strong evidence that *en* expression is controlled by a number of pair-rule genes (DiNardo and O'Farrell 1987; Ingham et al. 1988, and references therein). Because these genes are themselves regulated by the gap genes, it has been assumed that any effects of the gap genes on expression of *en* would be indirect, mediated through the pair-rule genes. Indeed, there is no evidence that gap genes directly participate in regulation of segment polarity gene expression. However, several observations are consistent with the possible involvement of *hb* and *Kr* in the regulation of *en*. First, it is clear that expression of gap genes and segment polarity genes overlap temporally and spatially (e.g., see Ingham 1988), an obvious prerequisite if the gap genes indeed regulate *en* directly. Second, the earliest patterns of *en* expression in the syncytial blastoderm are region specific and not segment specific (Karr et al. 1989), which could reflect the distribution and, hence, the direct involvement, of gap proteins such as *hb* and *Kr* in the control of *en* expression. Third, DNA sequence analysis of the *k'* region of the *en* promoter from the distantly related species *Drosophila virilis* (Kassis et al. 1989) reveals that both the *Kr*-binding site and the functional (although, intriguingly, not the nonfunctional) *hb* site are conserved, consistent with the idea that these sites are physiologically significant. Finally, it is noteworthy that pair-rule and gap proteins appear to act in concert to initiate stripes of homeotic gene expression in early embryos (Ingham and Martinez-Arias 1986; Ingham et al. 1986; Harding and Levine 1988). In any event, our results indicate that *Kr* and *hb* can functionally interact not only with each other but also with several homeo box proteins. Such combinatorial interactions may be important for defining precise patterns of gene expression in the early embryo.

Materials and methods

Recombinant plasmids

All expression vectors were derived from a plasmid containing the *Drosophila* actin 5C promoter and poly(A) site, Act-PPA, which is described in Han et al. (1989). The expression vector Act-*hb* was constructed from a cDNA clone pEMBL8-*hb* (pro-

vided by G. Struhl). pEMBL8-*hb* was cleaved with *Xba*I, the ends were filled in, and the 2.4-kb *Xba*I fragment was inserted into Act-PPA that had been cleaved with *Bam*HI, filled in, and digested with *Eco*RV. The 5'-untranslated leader of Act-*hb* contains 88 nucleotides from Act 5C and 13 from *hb*. Act-*Kr* was constructed from *Kr* cDNA clone K405 (provided by G. Struhl), hsp-CAT, and Act-PPA. hsp-CAT, which contains the heat shock protein 70 promoter-3' leader fused to the CAT gene, was used as an intermediate to facilitate cloning. Both K405 and hsp-CAT were digested with *Eco*RI and *Pst*I, and a 3.1-kb fragment from hsp-CAT was ligated with a 2-kb fragment of K405. The resulting plasmid, hsp-*Kr* was digested with *Pvu*II, and the 1.9-kb fragment was inserted into Act-PPA, which had been cleaved with *Bam*HI and filled in. The resulting Act-*Kr* plasmid encodes a *Kr* mRNA with a 5'-untranslated leader composed of 88 nucleotides from Act 5C, 104 from hsp70, and 69 from *Kr*. Act-*Kr-2*, which encodes a protein deleted for 183 carboxy-terminal amino acid residues, was constructed by digesting Act-*Kr* with *Pvu*I, digesting 3' overhangs with T4 polymerase, and then digesting with *Nco*I. The resulting 1.4-kb fragment was reinserted into Act-PPA, which had been cleaved with *Bgl*II, filled in, and digested with *Nco*I. The resulting *Kr-2* protein contains 284 amino acids from the *Kr* protein plus 4 extraneous carboxy-terminal amino acid residues.

All reporter plasmids were constructed from *k'*-TATA-CAT (described by Han et al. 1989). *k*I-TATA-CAT was constructed by digesting *k'*-TATA-CAT with *Pvu*II and *Hind*III. The 80-bp fragment containing *k*I was inserted into a metallothionein promoter-CAT plasmid (TATA-CAT), which had been digested with *Sal*I, filled in, and cleaved with *Hind*III. *k*II-TATA-CAT was constructed by cleaving *k'*-TATA-CAT with *Eco*RI and partially with *Pvu*II. The 380-bp fragment containing *k*II was isolated and inserted into TATA-CAT, which had been digested with *Sal*I, filled in, and partially cleaved with *Eco*RI. 2*k*I-TATA-CAT, 2*k*I-TATA-CAT-rr, and 4*k*I-TATA-CAT were constructed by digesting pGEM *k*I (which contains the *k*I fragment in the *Sal*I site of pGEM3) with *Sal*I. The 70-bp *k*I-containing fragment was inserted into TATA-CAT, which had been cleaved by *Sal*I. The resulting recombinant plasmids were sequenced to identify the above three plasmids. *k*II(90)*k*I-TATA-CAT was constructed by digesting pGEM *k*II (which contains the *k*II fragment in the *Sal*I site of pGEM3) with *Hind*III and *Pvu*II. The resulting 130-bp fragment containing *k*II was inserted into *k*I-TATA-CAT, which had been partially digested with *Pst*I, filled in, and cut with *Hind*III. In the resulting recombinant plasmid, the *k*II fragment is located 90 bp upstream from the *k*I fragment.

DNA transfection and transient expression assays

Transient cotransfection assays were performed essentially as described previously (Han et al. 1989). *Drosophila* Schneider L2 cells (Schneider 1972) were grown in M3 medium plus 10% fetal calf serum, plated at 2×10^6 to 4×10^6 cells/60-mm tissue culture dish 1 day before transfection, and transfected using calcium phosphate coprecipitation. Except where indicated, each transfection contained 0.2–0.8 μ g of expression vector(s), 5 μ g of promoter-CAT fusion, and either 2 μ g of *copia-lacZ* plasmid or 0.1 μ g of Act-*lacZ* plasmid as an internal control. Expression from *copia-lacZ* was observed to be reduced by a factor of ~3 by Act-*Kr*; therefore, Act-*lacZ* (which was not affected by either Act-*Kr* or any of the other expression vectors) was used in transfections containing Act-*Kr*. In a given experiment, the amount of actin promoter was kept constant in each sample by the addition of Act-PPA as required. The total amount of DNA was adjusted to 10 μ g by the addition of pGEM3 as carrier. All ex-

periments were performed in duplicate and repeated at least twice. Transfected cells were incubated at 24°C for 48–50 hr before harvesting. Cells were washed twice with PBS, resuspended in 0.1 ml of 0.25 M Tris-HCl (pH 7.8), and extracts were prepared by sonicating for 2 min in an ultrasonic sonifier (Branson), followed by centrifugation for 10 min in a microfuge. Transfection efficiencies were determined by β -galactosidase activity assays as described by Han et al. (1989). Variations between samples within a given experiment were typically less than twofold. CAT assays were also performed as described previously (Han et al. 1989).

DNase I protection assays

DNA footprinting assays were carried out as described previously (Stanojevic et al. 1989). k'-TATA-CAT was digested with *Hind*III, end-labeled with [γ ³²P]ATP and polynucleotide kinase, and then cleaved with *Eco*RI. A labeled fragment of ~500 bp containing the k' segment and the TATA box region was purified. Bacterial extracts of strains expressing *hb* or *Kr* were prepared, and increasing amounts of protein were used in DNA binding assays. Binding conditions, DNase digestion, and gel electrophoresis were carried out exactly as described (Stanojevic et al. 1989).

Immunofluorescence and immunoblotting

Indirect immunofluorescence assays of transfected cells were performed as described (Han et al. 1989). Rabbit anti-*hb* or anti-*Kr* antibodies, raised against SDS gel-purified proteins made in *E. coli*, were used as the first antibody, and rhodamine-conjugated goat anti-rabbit as the second. For immunoblot analysis, nuclear extracts of transfected cells were prepared essentially as described (Dignam et al. 1983), except that the final NaCl concentration during extraction of the nuclei was ~0.7 M. Samples containing ~50 μ g of total nuclear protein were resolved by electrophoresis through a 12% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose essentially as described (Towbin et al. 1979). Rabbit anti-*hb* antibodies were used as the primary antibody, and these were detected using a Vectastain ABC kit (Vector Laboratories) as described by the manufacturer.

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