The EMBO Journal vol.14 no.19 pp.4773–4780, 1995

Heterodimeric *Drosophila* gap gene protein complexes acting as transcriptional repressors

Frank Sauer[1] and Herbert Jäckle

Abteilung Molekulare Entwicklungsbiologie, Max-Planck-Institut für biophysikalische Chemie, Postfach 2841, D-37018 Göttingen, Germany

[1]Present address: Department of Molecular and Cell Biology, Howard Hughes Medical Institute, University of California, Berkeley, CA 94720, USA

The *Drosophila* gap gene *Krüppel* (*Kr*) encodes a transcriptional regulator. It acts both as an integral part of the *Drosophila* segmentation gene in the early blastoderm and in a variety of tissues and organs at later stages of embryogenesis. In transfected tissue culture cells, the *Kr* protein (KR) was shown to both activate and repress gene expression in a concentration-dependent manner when acting from a single binding site close to the promoter. Here we show that KR can associate with the transcription factors encoded by the gap genes *knirps* (*kni*) and *hunchback* (*hb*) which affect KR-dependent gene expression in *Drosophila* tissue culture cells. The association of DNA-bound *hb* protein or free *kni* protein with distinct but different regions of KR results in the formation of DNA-bound transcriptional repressor complexes. Our results suggest that individual transcription factors can associate to form protein complexes which act as direct repressors of transcription. The interactions shown here add an unexpected level of complexity to the control of gene expression.

Keywords: *Drosophila*/gap genes/*krüppel*/transcription regulation

Introduction

The successful execution of the genetic programme depends largely on the coordinate regulation of gene expression by mechanisms that control transcription precisely in time, space and level. In eukaryotes, this regulation operates through protein–protein interactions between transcription factors bound to *cis*-acting enhancers and components of the basal transcription machinery (for review see Lewin, 1990; Roeder, 1991; Gill and Tjian, 1992; Tjian and Maniatis, 1994). In view of multiple transcriptional repressors and activators that bind to a typical enhancer element, the regulation of gene expression in a given cell type is likely to be defined by their precise interplay which eventually determines the frequency of transcription initiation by the polymerase II (for reviews see Ptashne, 1988; Levine and Manley, 1989; Ptashne and Gann, 1990; Renkawitz, 1990; Carey, 1991; Tjian and Maniatis, 1994).

Transcriptional activators (for review see Johnson and McKnight, 1989; Mitchell and Tjian, 1989) are composed of at least two distinct domains, the DNA-binding domain and the activation domain (Frankel and Kim, 1991). While the DNA-binding domain provides the contact to specific target sites within enhancer elements, the activation domain interacts with one or more components of the general transcription machinery (Carey, 1991; Gill and Tjian, 1992) to mediate activation of gene expression. The function of the transcriptional activators can be counter-regulated in numerous ways by factors which physically associate and thereby extinguish transcription (for review see Renkawitz, 1990, 1993). Modes by which such repressors may work include competitive binding to overlapping or closely linked DNA-binding sites to cause the displacement of activators (Small *et al.*, 1991; Stanojevic *et al.*, 1991; Hoch *et al.*, 1992). Alternatively, such repressors might mask the DNA-binding domain (Diamond *et al.*, 1990; Yang-Yen *et al.*, 1990; Treacy *et al.*, 1991), the activation domains (Ma and Ptashne, 1987) or the nuclear localization signals of activators (Bäuerle and Baltimore, 1988). Other modes of repression involve factors which share the modular organization of activators but contain repressor domains instead of the activator domains (Licht *et al.*, 1990; Han and Manley, 1993). Those repressors were shown to interfere directly with the formation, the stability or the activity of the basal transcription machinery (Dostatni *et al.*, 1991; Meisterernst and Roeder, 1991; Inostroza *et al.*, 1992; Fondell *et al.*, 1993; Roy *et al.*, 1993; Sauer *et al.*, 1995).

The zinc finger-type transcription factor Krüppel (KR) (Rosenberg *et al.*, 1986) plays an essential role for several apparently unrelated morphoregulatory circuitries throughout *Drosophila* embryogenesis. During the early blastoderm stage, KR functions as an integral component of the segmentation gene cascade in the preblastoderm embryo (Ingham, 1988; Hoch and Jäckle, 1993; Pankratz and Jäckle, 1993). Subsequently, it is both expressed and required in a number of different tissues and organs (Harbecke and Janning, 1989; Hoch *et al.*, 1990; Gaul and Weigel, 1991; Schmucker *et al.*, 1992). The regulatory potential of KR was assessed previously by reporter gene expression studies involving the minimal *cis*-acting 'stripe element' of the pair-rule gene *even-skipped* (*eve*) (Stanojevic *et al.*, 1989, 1991; Small *et al.*, 1991) and by transient expression assays with tissue culture cells (Licht *et al.*, 1990; Zhuo *et al.*, 1990; Sauer and Jäckle, 1991). When acting within the *eve* stripe 2 element, KR functions exclusively as a repressor and its mode of action, at multiple sites, involves quenching as well as competetive binding likely to cause the displacement of activators (Stanojevic *et al.*, 1989, 1991; Small *et al.*, 1991). Similarly, KR exerts repressor function through an alanine-rich N-terminal repressor domain in transfected mammalian cells (Licht *et al.*, 1990). These findings establish the function of KR as a transcriptional repressor.

In *Drosophila* tissue culture cells, KR acts as a repressor (Zhuo *et al.*, 1990), but it can also function as a transcriptional activator when acting from a single binding site in

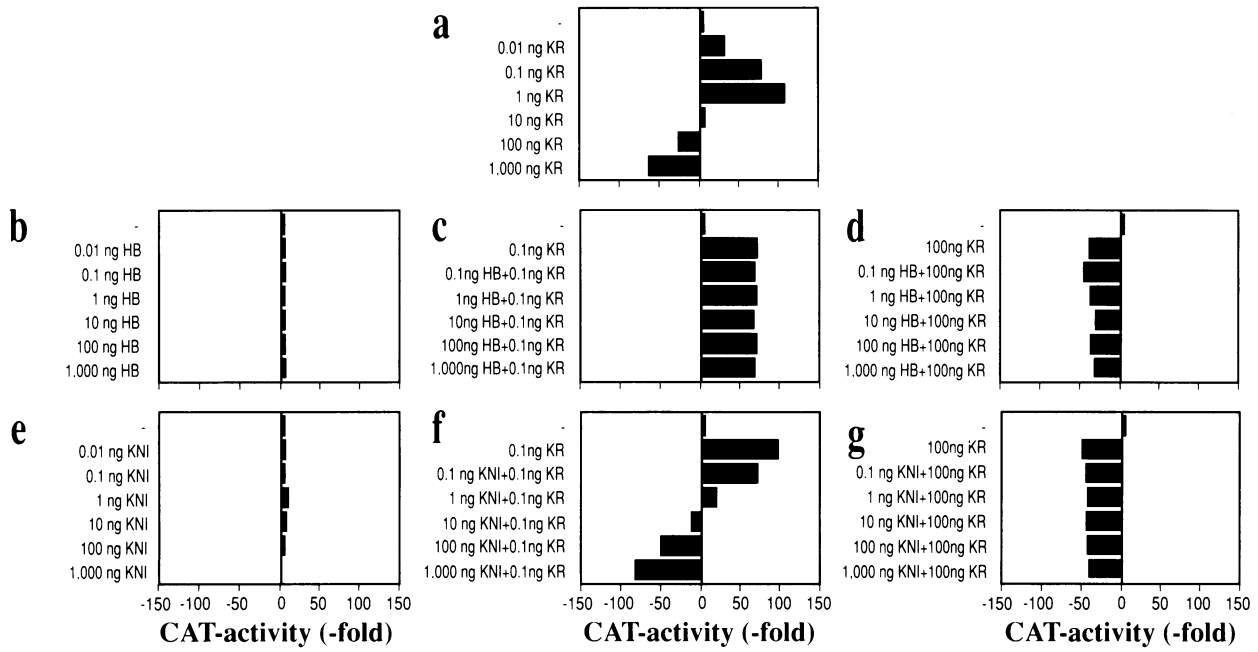

Fig. 1. KR-dependent transcriptional regulation in the presence of KNI or HB in *Drosophila* Schneider cells. Co-transfection experiments using 2 μg of the reporter plasmid pAdh86CAT-1K (1K refers to a single KR *in vitro* DNA-binding site which mediates expression) and the indicated amounts of the expression plasmids pPacKR (**a**), pPacHB (**b**) or pPacKNI (**e**). A constant amount of pPacKR leading either to activation (**c** and **f**) or repression (**d** and **g**) of reporter gene expression was co-transfected with increasing amounts of pPacHB (c and d) or pPacKNI (f and g). The basal level of pAdh86CAT-1K expression is not affected in response to HB or KNI; KR has no effect when the KR *in vitro* DNA-binding site of the pAdh86CAT-1K reporter gene is absent or replaced by KR non-binding sequences (Sauer and Jäckle, 1991; and data not shown). Reporter gene activity was determined 60 h after transfection using a CAT–ELISA. Black bars represent the mean value of CAT activity (relative to the basal activity of the pAdh86CAT-1K reporter gene; basal activity is 1) from at least six independent experiments; standard deviation was <8% in each experimental series shown.

front of the promoter (Sauer and Jäckle, 1991). Low concentrations of KR cause activation, while at high concentrations, KR forms homodimers which cause repression (Sauer and Jäckle, 1991, 1993). Repression involves the KR C-terminal region which also functions as the homodimerization domain (Sauer and Jäckle, 1991, 1993). Recent *in vitro* transcription studies involving a single binding site in front of the promoter have shown that the interaction of monomeric KR with the basal transcription factor TFIIB results in activation, while the interaction of the KR homodimer with TFIIEβ causes transcriptional repression (Sauer *et al.*, 1995). These findings suggest different modes of KR action which are different when KR is acting from a single site close to the promoter and when it acts within the context of various regulators that assemble within an upstream enhancer element. Here we describe that KR-dependent transcriptional regulation from a site close to the promoter can be modified by co-expression of transcription factors encoded by the gap genes *hunchback* (*hb*) and *knirps* (*kni*) (reviewed by Pankratz and Jäckle, 1993). Our results suggest that *hb* and *kni* proteins (HB and KNI) can associate with DNA-bound KR to form heterodimers which exert specific and novel characteristics relevant for transcriptional regulation.

Results

KR-dependent gene regulation in the presence of other gap proteins

Recent co-transfection studies on *Drosophila* Schneider cells have shown that KR can act as a concentration-dependent transcriptional activator or repressor of gene expression (Sauer and Jäckle, 1991, 1993). In this system, KR-dependent gene expression is mediated by a single KR *in vitro* binding site of the sequence -AAAAGGGTTAA- (termed 'K-element') (Sauer and Jäckle, 1991) in front of the basal *Adh* promoter which drives the expression of the bacterial chloramphenicol acetyltransferase reporter gene (CAT). Low concentrations of KR, as provided by low amounts of co-transfected pPacKR plasmid DNA cause transcriptional activation; high concentrations of KR, as provided by high amounts of pPacKR, lead to repression below the basal level of reporter gene expression (Sauer and Jäckle, 1991, 1993; see also Figure 1a). The phenomenon of the opposing regulatory effects of KR at different concentrations of the transcription factor was attributed to the concentration-dependent formation of KR homodimers by protein–protein interactions involving the C-terminus of KR (Sauer and Jäckle, 1993). The finding that KR is capable of protein–protein interactions prompted us to examine KR-dependent reporter gene regulation (Figure 1a) in the presence of other gap gene proteins such as the zinc finger protein HB (Tautz *et al.*, 1987) and the orphan receptor-type protein KNI (Nauber *et al.*, 1988).

Co-transfections of *Drosophila* Schneider cells with plasmid DNAs expressing either HB or KNI (pPacHB or pPacKNI; see Materials and methods) had no effect on the basal level of reporter gene expression in the absence of KR (Figure 1b and e). This indicates that none of these transcription factors is able to regulate transcription mediated by the K-element or by other sequences contained

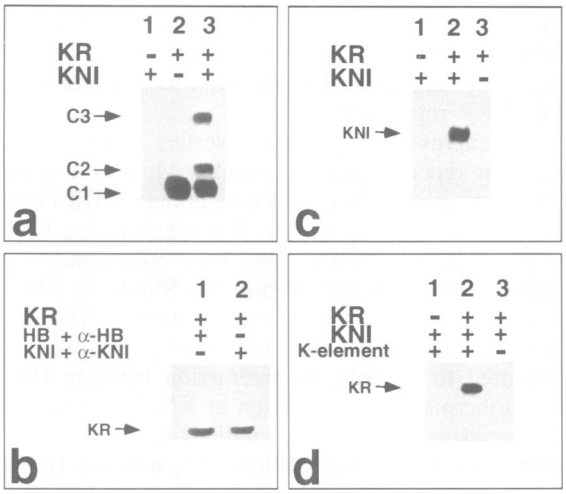

Fig. 2. Association of KR and KNI *in vitro*. Gel mobility shift assays (a) and co-immunoprecipitation experiments (b–d) involving *in vitro* translated KR, KNI and HB. (**a**) Gel mobility shift assays were performed with the ^{32}P-labelled K-element (a 22 bp oligonucleotide containing a single KR-binding site), *in vitro* translated KR (lanes 2 and 3) and *in vitro* translated KNI (lanes 1 and 3 contain 5.0 µl KNI-programmed reticulocyte lysate). C_1 indicates the position of the DNA-bound KR monomer, C_2 the position of the DNA-bound KR homodimer (Sauer and Jäckle, 1993). C_3 marks the position of an additional complex obtained in the presence of KNI. (**b**) ^{35}S-labelled KR (arrow) was co-precipitated with unlabelled HB (lane 1) or KNI (lane 2) using anti-HB (lane 1), or anti-KNI (lane 2) antibodies. Note that KR only co-precipitates with HB when both the K-element and DNA containing HB *in vitro* binding sites were present in the reaction mixture (data not shown; see also Figure 3a). (**c**) Co-immunoprecipitation experiments using anti-KR antibodies, *in vitro* translated KR and [^{35}S]methionine-labelled KNI (arrow). The K-element was included in the reactions. (**d**) Co-immunoprecipitation experiments using anti-KNI antibodies, *in vitro* translated KNI and [^{35}S]methionine-labelled KR (arrow). The K-element was present (lanes 1 and 2) or absent (lane 3) in the reaction mixture. Co-precipitates were separated by SDS–PAGE, and labelled proteins were visualized by fluorography.

within the reporter gene construct. Furthermore, co-expression of HB did not interfere with KR-dependent transcriptional regulation of the reporter gene (Figure 1c and d). In contrast, the co-expression of KNI altered the profile of KR-dependent gene expression significantly (Figure 1f and g). Increasing concentrations of KNI caused repression of KR-dependent activation below the basal level of reporter gene expression (Figure 1f), while KNI had no effect on KR-dependent repression (Figure 1e). Since KNI had no direct effect on the basal level of gene expression (see Figure 1b) and is not able to bind directly to the K-element (Figure 2a; and data not shown), it appeared likely that the specific regulatory effects of KNI are mediated by protein–protein interactions with KR.

In vitro association of KNI and KR

In order to assess the ability of KNI to associate with KR, we performed gel mobility shift assays and immunoprecipitation experiments (Figure 2). Gel mobility shift experiments were carried out with *in vitro* translated proteins and the labelled K-element. In the presence of standard amounts of KR, two DNA–protein complexes representing the KR monomer (C1) and the KR homodimer (C2) bound to the K-element (Figure 2a; see also Sauer and Jäckle, 1993). The addition of KNI to the reaction

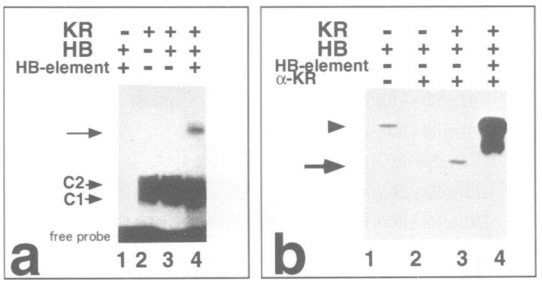

Fig. 3. Association of KR and HB *in vitro*. Gel mobility shift assays (a) and co-immunoprecipitation experiments (b) performed with *in vitro* translated KR and HB. (**a**) Gel mobility shift assays included the ^{32}P-labelled 22 bp K-element containing a single *in vitro* KR-binding site, *in vitro* translated KR and *in vitro* translated HB (lanes 3 and 4) in the absence (lane 3) or presence (lane 4) of the HB-element, an oligonucleotide containing a single HB-binding site (see Material and methods). C_1 and C_2 indicate the positions of the DNA-bound KR monomer and KR homodimer, respectively. An arrow marks an additional complex obtained in the presence of HB and the HB-element. (**b**) Co-immunoprecipitation experiments using anti-KR antibodies (lanes 2–4), ^{35}S-labelled HB (lanes 2–4), ^{35}S-labelled KR (lane 3; arrow) and unlabelled KR (lanes 2 and 4). ^{35}S-labelled HB (lane 1; arrowhead) served as a marker for HB (see lane 4). Immunoprecipitates were separated by SDS–PAGE and labelled proteins were visualized by fluorography. Note two forms of *in vitro* translated, ^{35}S-labelled HB (lane 4). Both of them are recognized by anti-HB antibodies. The higher molecular weight form is always the predominant form (see low amounts of *in vitro* translated, ^{35}S-labelled HB in reference lane 1 and the ratio of the two forms in lane 4). We do not know whether the lower molecular weight band represents a specific degradation product or a second translation initiation site within the *hb* mRNA (see also Figure 6). Note also that in the absence of HB *in vitro* binding sites, KR and HB fail to associate (compare lanes 3 and 4).

mixture resulted in the formation of a new complex (C3) which migrates more slowly than C1 and C2 (Figure 2a). Since KNI does not bind to the K-element by itself (Figure 2a), these results suggest that KNI is able to associate with KR and thereby causes the formation of a KR–KNI heterodimer complex.

To ensure that KR and KNI can associate directly, we performed co-immunoprecipitation experiments using *in vitro* translated, radiolabelled KNI together with unlabelled KR and anti-KR antibodies. Immunocomplexes were precipitated with protein A–Sepharose and analysed by SDS–PAGE (see Materials and methods). The results indicate that KNI associates with KR in the presence of the K-element (Figure 2b–d), while KNI did not co-immunoprecipitate when the K-element was absent from the reaction mixture (Figure 2d). Thus, the association between KNI and KR requires that KR is bound to target DNA.

Interaction between KR and DNA-bound HB

HB-dependent activation of gene expression can be suppressed in tissue culture cells by co-expressed KR (Zhuo *et al.*, 1990). The interesting feature of KR-dependent suppression is that this phenomenon occurred in the absence of detectable KR-binding sites, suggesting that HB and KR may associate under certain conditions only. The gel mobility shift experiments shown in Figure 3a indicate that the formation of the KR–DNA complexes C1 and C2 is not affected in the presence of HB. However, when unlabelled DNA containing a single HB-binding site ('HB-element'; see Material and methods) was added

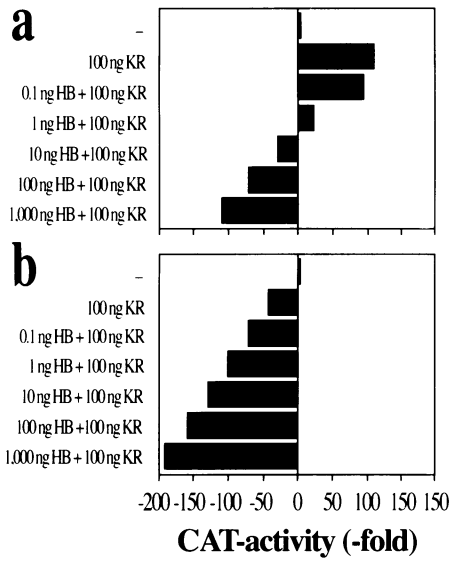

Fig. 4. Gene expression in *Drosophila* Schneider cells. Cells were co-transfected with 2 μg of the reporter plasmid pAdh86CAT-1K (a and b), with the indicated amounts of expression plasmid pPacKR leading to either to activation (**a**) or to repression (**b**), and with increasing amounts of the expression plasmid pPacHB (a and b). In contrast to the experiments shown in Figure 1b–d, 3 μg of the plasmid pBlue KNI-KX containing six HB *in vitro* binding sites were included in each transfection. The CAT activity was determined 60 h after transfection. Bars indicate the mean values of at least six independent measurements CAT gene (expressed as times the basal activity of the pAdh86CAT-1K reporter gene); standard deviation was <8% in each experimental series.

to the reaction mixture, additional complexes with reduced mobility were observed (Figure 3a). Furthermore, co-immunoprecipitation experiments revealed that KR and HB associate, provided that both the HB-element and the K-element were present in the reaction mixture (Figures 2b and 3b). These findings establish that the two proteins can associate only when they are bound to target DNA.

Based on these results we re-examined the regulatory effect of HB co-expression on KR-dependent reporter gene expression. For this we co-transfected Schneider cells with activating or repressing amounts of pPacKR and with increasing amounts of pPacHB. In contrast to the experiments described above (Figure 1c and d) plasmid DNA containing six HB-binding sites (see Materials and methods) were added together with the reporter gene-containing plasmid. In the presence of the HB-binding sites, HB causes repression of KR-dependent activation below the basal level of transcription (Figure 4a) and it enhances KR-dependent repression of reporter gene expression (Figure 4b). These findings suggest that the association of KR with HB results in a heterodimer which acts as a repressor of transcription.

HB and KNI associate with different regions of KR

To delineate the regions within KR that are necessary for the interactions with HB and KNI, we made use of truncated versions of KR which maintain the potential either to activate or to repress reporter gene expression in tissue culture cells (Figure 5). The KR derivatives KRC-187 and KRC-64, which lack different C-terminal portions of KR, act only as activators (Figure 5a and b), while KR derivatives, which lack the N-terminal 166 or 210 amino acids, act only as repressors (Figure 5c and d). Thus, the different KR truncations could be used to examine the ability of co-expressed HB and KNI to interfere with the residual KR regulatory function.

KNI expression did not interfere with KRC-64-dependent reporter gene activation, while co-expression of HB in the presence of DNA containing six HB-elements caused repression (Figure 5e). This suggests that KNI has lost the ability to interfere with KRC-64-dependent gene expression (compare with Figure 2g). Similarly, KRC-187 has lost the ability to respond to both KNI and HB (Figure 5f). Thus, the amino acid interval 279–402 of KR is required to mediate the interaction between HB and KR. Furthermore, co-expression of KNI had no effect on KR-dependent expression when the N-terminal 116 or 210 amino acids were deleted (Figure $5g_1$ and h_1). However, HB caused strong repression in both cases (Figure $5g_2$ and h_2). These results indicate that KNI and HB require distinct regions of KR to exert their effects on KR-dependent reporter gene regulation.

We asked next whether these regions of KR are also required for the association with KNI and HB. For this we performed immunoprecipitation experiments. The results shown in Figure 6 indicate that the regions required to mediate the regulatory effects of KNI or HB on KR-dependent reporter gene expression in Schneider cells (see Figure 5) are also necessary for the *in vitro* heterodimer formation with KNI and HB (Figure 6a and b). This suggests that the regulatory effects on KR-dependent reporter gene expression observed in tissue culture cells are mediated by the association of KNI and HB with KR.

Discussion

Our study provides evidence that the transcription factors HB and KNI can associate with KR *in vitro* and that they interact functionally with KR-dependent target gene expression mediated by a single KR-binding site close to the promoter in *Drosophila* Schneider cells. This finding appears to contradict *in vivo* studies which assessed the functions of KR and HB within the segmentation gene cascade through systematic deletions and replacements of binding sites within the *cis*-acting *eve* stripe 2 control element (Stanojevic *et al.*, 1989, 1991; Small *et al.*, 1991). Within the scenario of factors and multiple binding sites for HB and the fly morphogen bicoid which act as activators, KR acts as a repressor competing for the binding and/or activity of activators by a mechanism that involves weak protein–protein interactions rather than by factor association (Small *et al.*, 1991; Stanojevic *et al.*, 1991; reviewed in Hoch and Jäckle, 1993). The cultured cell assay described in the present study is therefore not valid to represent the mode of HB and KR interaction within the *eve* stripe 2 control element and vice versa. This suggests that a single assay system is not sufficient to assess all aspects of the potential gap gene functions *in vivo* but rather allows the study of one mode by which HB, KR and KNI exert their functions at different stages and cell types during development.

The observed interactions between HB or KNI and KR fall into none of the known interactions between transcriptional activators and associated proteins which block activation domains (Ptashne, 1988), DNA-binding

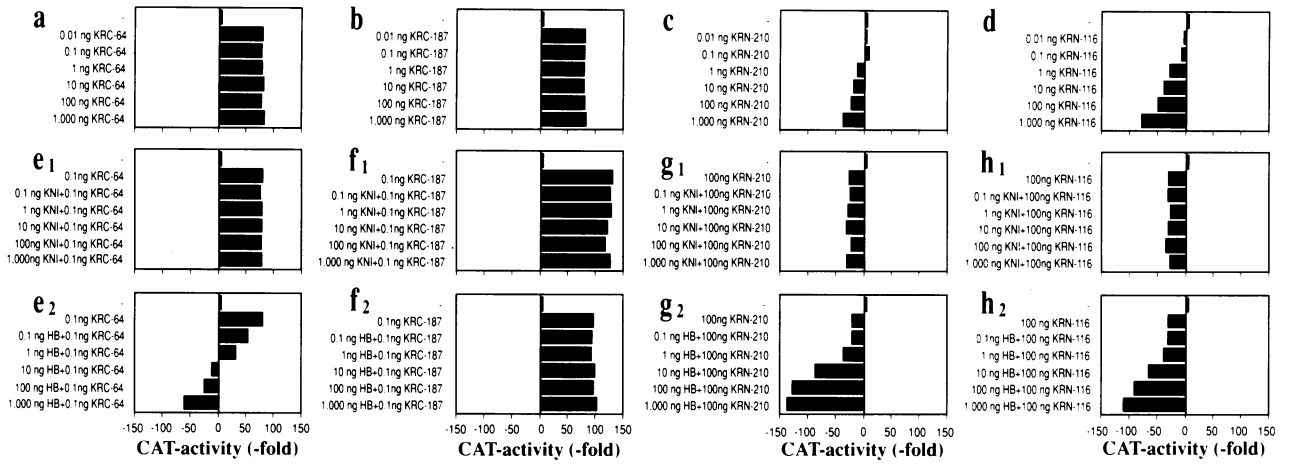

Fig. 5. Co-transfection experiments with truncated versions of KR together with HB or KNI. Schneider cells were co-transfected with 2 μg of the reporter plasmid pAdh86CAT-1K and with increasing amounts of pPacKRC-64 (**a**), pPacKRC-187 (**b**), pPacKRN-210 (**c**) or pPacKRN-116 (**d**). Constant amounts of pPacKRC-64 ($\mathbf{e_1}$ and $\mathbf{e_2}$) and pPacKRC-187 ($\mathbf{f_1}$ and $\mathbf{f_2}$) leading to activation of reporter gene expression, or constant amounts of pPacKRN-210 ($\mathbf{g_1}$ and $\mathbf{g_2}$) and pPacKRN-116 ($\mathbf{h_1}$ and $\mathbf{h_2}$) leading to repression of gene expression were co-transfected with increasing amounts of pPacKNI (e_1–h_1) or pPacHB (e_2–h_2). Transfections including pPacHB contained 3 μg of the plasmid pBlueKNI-KX containing six HB *in vitro* binding sites. Reporter gene expression was assayed 60 h after transfection using a CAT–ELISA. Bars indicate the mean values of at least six independent measurements of CAT gene (expressed as times the basal activity of the pAdh86CAT-1K reporter gene); standard deviation was <8% in each experimental series. Note that the KRN-210 protein is capable of entering the nucleus (within 60 h), although it has lost the nuclear localization signal of KR (F.Sauer, unpublished result). KRN-210 appears small enough to enter the nuclear compartment of the cell to act as a transcriptional repressor. It functions less efficiently than KRN-116 containing the nuclear location signal.

domains (Treacy *et al.*, 1991) or nuclear translocation signals (Nolan and Baltimore, 1992). Instead, HB and KNI act through DNA-bound KR and thereby generate functional transcription repressor complexes. This observation is a first view reminiscent of the interaction between the glucocorticoid receptor (GR) and transcription factor AP1 when only one of the two transcription factors is bound to DNA (for review see Renkawitz, 1993). In case AP1 is bound to DNA, co-expression of GR can silence AP1-dependent transcriptional activation and vice versa, suggesting that protein–protein interactions between AP1 and GR block the activating functions of the two transcription factors. In terms of transcription, these interactions reverse the level of transcription from activated to basal, but they do not repress transcription completely (for review see Renkawitz, 1993). In contrast to AP1–GR interactions, the interaction of HB or KNI with KR not only suppresses the activating function of KR but also generates a complex that functions as a transcriptional repressor. Another remarkable feature that distinguishes the KR–KNI and KR–HB repressor complexes from AP1–GR complex formation is that the association of HB as well as KNI with KR is conditional. That is, it requires DNA-bound HB and unbound KNI. These specificities suggest that the binding of HB and KR to DNA may generate a conformational change leading to the exposure of distinct protein domains or protein surfaces necessary for the protein associations observed. The different regions of KR required for an interaction with KNI or HB lack known protein motifs. Thus, we do not know whether only a few distinctly positioned amino acid residues are sufficient to mediate the association as has been shown for the yeast transcription factors GAL4 and GAL80 (Ma and Ptashne, 1987) or for the interaction between human transcription factor Sp1 and TAF110, a component of the basal transcription machinery (Hoey *et al.*, 1993; Gill *et al.*, 1994).

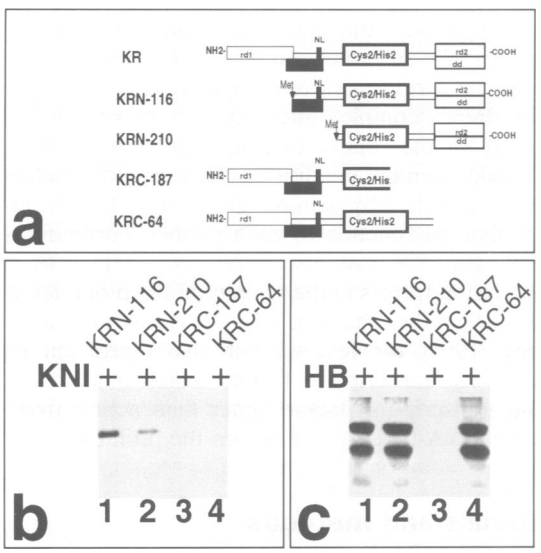

Fig. 6. Localization of the regions of KR necessary for the association with KNI and HB. (**a**) Schematic representation of KR and various KR deletion mutants. Wildtype KR is shown on top. It contains two repressor domains (rd1 and rd2), a N-terminal activator domain (black bar), the C-terminal dimerization domain (dd), the DNA-binding zinc finger region (open bar; Cys2/His2) and a nuclear translocation signal (NL). Deletion mutants KRN-116, KRN-210, KRC-187 and KRC-64 are shown below; Met refers to the experimentally induced translation start sites. For further details see Sauer *et al.* (1995) and references therein. (b and c) Fluorographs show co-immunoprecipitation experiments using *in vitro* translated [^{35}S]methionine-labelled KNI (**b**) or HB (**c**) and the truncated versions of KR KRN-116 (lanes 1), KRN-210 (lanes 2), KRC-187 (lanes 3) and KRC-64 (lanes 4). Protein complexes were precipitated using anti-KR antibodies coupled to protein A–Sepharose separated by SDS–PAGE, and labelled proteins were visualized by fluorography. Note that KNI and HB require different regions of KR to associate *in vitro*. For the appearance of two forms of *in vitro* translated, ^{35}S-labelled HB see legend of Figure 3.

Ongoing studies of transcriptional regulation suggest that an interplay of transcriptional activators, indirect repressors (which modulate the activators) and direct repressors determines the regulatory input from an enhancer site on gene expression (Levine and Manley, 1983; Renkawitz, 1990; for reviews). KNI or HB cause repression of KR-dependent transcriptional activation, although they do not bind to the functional enhancer that mediates the transcriptional response. The finding that only DNA-bound KR can functionally associate with KNI and HB suggests that both HB and KNI employ DNA-bound KR as a mediator to provide their functions. This implies that heterodimer formation generates KR–HB and KR–KNI which act as direct repressors under conditions where KR would normally act as an activator. Where does the repressor activity of the complex come from if none of the components by itself exerts repressor function under the experimental conditions applied? In the case of the KR–HB heterodimer, both components may participate in repression since both possess an inherent repressor function (Licht *et al.*, 1990; Zhuo *et al.*, 1990; Sauer and Jäckle, 1991; Struhl *et al.*, 1992). One may speculate that the association of KR with KNI or HB results in a conformational change of the KR molecule which opens up one or both of the known KR repression domains (Licht *et al.*, 1990; Sauer and Jäckle, 1991) that interact with components of the basal transcription machinery to cause the repression effect observed.

Although the molecular mechanisms of repression are unknown, the phenomenon that individual transcription factors can form repressor complexes adds new variables to the already complex interplay of transcriptional factors required for the control of gene regulation. This implies that under certain conditions, transcription factors can combine and thereby gain novel functions that differ from the individual function of each partner. Furthermore, the finding that KR can also serve as a tether to recruit transcription factors in the vicinity of the promoter alludes to the possibility that not only transcriptional activation (Carey, 1991; for review) but also repression can be directed by transcription factors which bind to a DNA-bound transcription factor rather than acting from their specific DNA-binding site within the promoter.

Materials and methods

In vitro transcription and translation

The plasmids used for our *in vitro* transcription studies were based on pBluescript SK$^+$ (Stratagene, San Diego, CA). cDNAs were inserted in a way that *in vitro* transcription could be initiated by T7 RNA polymerase. KR, KRN-210 and KRC-64 mRNAs were derived from the recently described plasmids pBlueKR, pBlueKRN-210 and pBlueKRC-64, respectively (Sauer and Jäckle, 1993). KRN-116 and KRC-187 mRNA was obtained from the plasmids pBlueKRN-116 and pBlueKRC-187 which were generated as follows: a 1.5 kb *Bst*XI–*Eco*RI fragment from pBlueKR was end-filled, ligated with *Eco*RI linkers to create a new start codon at amino acid position 210, and the resulting 1.5 kb *Eco*RI fragment was inserted into the corresponding restriction site of pBluescript to generate pBlueKRN-116. To create pBlueKRC-187, a 1.2 kb *Nde*I–*Pvu*II fragment from pBlueKR was blunt-ended ligated with *Eco*RI linkers and inserted into the *Eco*RI restriction site of pBluescript. pBlueHB was generated by inserting a 2.8 kb *Xba*I full-length *hb* cDNA (Tautz *et al.*, 1987) into the *Xba*I restriction site of pBluescript. pBlueKNI was created by inserting the end-filled 2.1 kb full-length *kni* cDNA fragment from pcEH2 (Gerwin *et al.*, 1994) into the *Eco*RV restriction site of pBluescript. *In vitro* transcription reactions were performed as described before (Sauer and Jäckle, 1993). For *in vitro* translation reactions we used a nuclease-treated reticulocyte lysate (Promega) which was programmed in the absence or presence of [^{35}S]methionine with 2 μg capped mRNA. KR and KNI had to be co-translated to detect a protein–protein interaction between these proteins. Reticulocyte lysate was programmed with 2 μg *kni* mRNA in the presence of [^{35}S]methionine, and the reaction mixture was incubated for 1 h at room temperature. The $CaCl_2$ concentration of the mixture was increased to 150 mM to activate the internal nuclease activity for destroying the added *kni* mRNA. After 15 min at 30°C, 25 mM EDTA was added to inactivate the nuclease, and the reaction mixture was dialysed four times against buffer FS4 (25 mM Tris, 66 mM Kacetate, 1 mM EDTA, pH 7.9) to remove the [^{35}S]methionine. One quarter of the resulting mixture was mixed with three quarters of new reticulocyte lysate, and this hybrid lysate was programmed with *Kr* mRNA.

Co-immunoprecipitation

All steps of the immunoprecipitation experiments were performed on ice, unless otherwise mentioned. Aliquots (5–10 μl) of a reticulocyte lysate programmed with *Kr* or *Kr*-derived mRNA, mixed with equal amounts of HB-, KNI- or GT-programmed lysate and 10 μl 5× binding buffer (125 mM Tris–HCl, pH 7.9, 500 mM NaCl, 50 mM KCl, 0.5% NP-40, 50% glycerol, 10 mg/ml spermidine, 5 mg/ml BSA, 10 mM $ZnSO_4$, 100 ng/ml salmon sperm DNA) and variable volumes of water were added to make up a total volume of 49 μl. After preincubation for 15 min, 200 ng of K-element DNA were added. If target DNA for HB was included, we used a synthetic oligonucleotide ('HB-element') which contains a single HB-binding site (underlined) of the sequence 5′-GGATAGCGGCCAAAAAAAGCG-3′ of the *eve* stripe 2 enhancer (Stanojevic *et al.*, 1989). The reaction mixture was incubated for another 30 min. The volume of the probe was then increased to 500 μl by adding ice-cold 1× binding buffer, and the antibody was added. We used the polyclonal antibodies anti-KR (Gaul and Weigel, 1991), anti-KNI (Gerwin *et al.*, 1994) and anti-HB (Tautz, 1988) produced in rabbits. The probe was incubated for at least 2 h on a rotating wheel. The total volume of the reaction mixture was increased to 1.5 ml by adding 1× binding buffer and 25 μl protein A–agarose beads (Sigma) prewashed with 1× binding buffer. This reaction mixture was incubated for 2 h. Immunocomplexes were precipitated and washed eight times with 2.5× binding buffer. Proteins were analysed by SDS–PAGE. Gels were fixed and treated with Enhancer (Amersham) and exposed for 24 h against a Kodak X-ray film to detect [^{35}S]methionine-labelled proteins.

Gel mobility shift assays

Gel mobility shift assays were performed with *in vitro* translated, unlabelled proteins. Aliquots (0.1–8.0 μl) of reticulocyte lysates programmed with mRNA of *Kr*, *Kr* derivatives or target proteins (HB, KNI, GT) were incubated in 1× binding buffer (20 mM Tris–HCl, 35% glycerol, 25 mM NaCl, 10 μM $ZnSO_4$, 10 mg/ml spermidine, 1 mg/ml BSA, 100 ng/ml salmon DNA) together with 0.01 ng [^{32}P]CTP-labelled K-element DNA in a total volume of 30 μl for 30 min at room temperature. One third of each reaction was loaded onto a native 6% polyacrylamide (29:1) gel. The probes were separated at 15 V/cm gel length. Gels were dried and exposed for 16 h against a Kodak X-ray film. When HB was present in the reaction mixtures, 100 ng/reaction of the HB-element DNA (see above) was included, and the probes were analysed on a native 4% polyacrylamide (29:1)/ 7.5% glycerol gel.

Expression plasmids and reporter genes

Expression plasmids are based on pPac which contains the constitutive actin 5C promoter and the actin polyadenylation signal (Krasnow *et al.*, 1989). pPacKR, pPacKRC-64, pPacKRN-116 and pPacKNI were described recently (Sauer and Jäckle, 1991, 1993; Hoch *et al.*, 1992). pPacKRN-210 and pPacKRC-187 were generated by inserting the 1.3 kb end-filled *Eco*RI fragment from pBlue KRN-210 (Sauer and Jäckle, 1993) or the 1.2 kb *Eco*RI fragment from pBlueKRC-187 into the blunt-ended *Bam*HI restriction site of pPac. For generating pPacHB and PacGT full-length *hb* or *gt* cDNA (see *in vitro* transcription) was inserted into the *Bam*HI restriction site of pPac. The reporter plasmid pAdh86CAT-1K was described recently (Sauer and Jäckle, 1991, 1993). pBluekniKE was generated by inserting the 0.6 kb *Kpn*I–*Eco*RI fragment out of the *knirps* upstream region (Pankratz *et al.*, 1992) into the corresponding restriction sites of pBluescript. Plasmids used for transfections were purified using Qiagen columns and subsequent CsCl gradients.

Transfections

Drosophila Schneider cells were maintained as described by Gerwin *et al.* (1994), except that the cells were raised in a medium containing

12% fetal calf serum. Transfections were done as described recently (Sauer and Jäckle, 1993). For the expression of all proteins of this study the expression vector pPac (Krasnow *et al.*, 1989) was used. Cells were transfected with a constant amount of DNA (20 μg) which consists of variable amounts of expression plasmids, 2 μg reporter plasmid pAdh33-1K (Sauer and Jäckle, 1993), 2 μg of the reference gene plasmid pPaclacZ (Driever and Nüsslein-Volhard, 1989) and variable amounts of pBluescript (Stratagene, San Diego). Cells were harvested 60 h after transfection. Reporter gene activity was determined as described (Sauer and Jäckle, 1991) using a commercial CAT-ELISA (5-prime/3-prime, Boulder, USA). The reporter gene activity was standardized against the reference gene activity. In each case, the results shown represent the mean values of at least eight independent experiments. The standard deviation in each experimental series shown was <10%.

References

Bäuerle,P.A. and Baltimore,D. (1988) IkB: a specific inhibitor of the NF-kB transcription factor. *Science*, **242**, 540–545.

Beato,M. (1989) Gene regulation by steroid hormones. *Cell*, **56**, 335–344.

Carey,M. (1991) Mechanistic advanced in eukaryotic gene activation. *Curr. Opin. Cell Biol.*, **3**, 452–460.

Diamond,M., Miner,J.N., Yoshigana,S.K. and Yamamoto,K.R. (1990) Transcription factor interactions: Selectors of positive or negative regulation from a single DNA element. *Science*, **429**, 1266–1272.

Dostatni,N., Lambert,P.F., Sousa,R., Ham,J., Howley,P.M. and Yaniv,M. (1991) The functional BPV-1 E2 trans-activating protein can act as a repressor by preventing formation of the initiation complex. *Genes Dev.*, **5**, 1657–1671.

Driever,W. and Nüsslein-Volhard,C. (1989) The *bicoid* protein is a positive regulator of *hunchback* transcription in the early *Drosophila* embryo. *Nature*, **337**,138–143.

Fondell,J.D., Roy,A.L. and Roeder,R.G. (1993) Unliganded thyroid hormone receptor inhibits formation of a functional preinitiaition complex: implications for active repression. *Genes Dev.*, **7**, 1400–1410.

Frankel,A.D. and Kim,P.S. (1991) Modular structure of transcription factors: implications for gene regulation. *Cell*, **65**, 717–719.

Gaul,U. (1987) Funktionelle Charakterisierung des Transkriptionsfaktors KRÜPPEL von *Drosophila melanogaster*. Ph.D. thesis. University of Tübingen, Germany

Gaul,U. and Weigel,D. (1991) Regulation of *Kr* expression in the anlage of the Malpighian tubules in the *Drosophila* embryo. *Mech. Dev.*, **33**, 57–68.

Gerwin,N., La Rosée,A., Halbritter,H.-P., Neumann,M., Jäckle,H. and Nauber,U. (1994) Functional and conserved domains of the *Drosophila* transcription factor encoded by the segmentation gene *knirps*. (1994) *Mol. Cell. Biol.*, **14**, 7899–7908

Gill,G. and Tjian,R. (1992) Eukaryotic coactivators associated with the TATA box binding protein. *Curr. Opin. Genet. Dev.*, **3**, 234–241.

Gill,G., Pascal,E., Tseng,Z. and Tjian,R. (1994) A glutamine-rich, hydrophobic patch in transcription factor Sp1 contacts the $TAF_{II}110$ component of the *Drosophila* TFIID complex and mediates transcriptional activation. *Proc. Natl Acad. Sci. USA*, **91**, 192–196.

Han,K. and Manley,J. (1993) Transcriptional repression by the *Drosophila* even-skipped protein: definition of a minimal repression domain. *Genes Dev.*, **7**, 491–503.

Harbecke,R. and Janning,W. (1989) The segmentation gene *Krüppel* of *Drosophila melanogaster* has homeotic properties. *Genes Dev.*, **3**, 114–122.

Hoch,M., Schröder,C., Seifert,E. and Jäckle,H. (1990) *Cis*-acting control elements for *Krüppel* expression in the *Drosophila* embryo. *EMBO J.*, **9**, 2587–2595.

Hoch,M., Gerwin,N., Taubert,H. and Jäckle,H. (1992) Competition for overlapping sites in the regulatory region of the *Drosophila* gene *Krüppel*. *Science*, **256**, 94–97.

Hoch,M. and Jäckle,H. (1993) Transcriptional regulation and spatial patterning in *Drosophila*. *Curr. Opin. Genet. Dev.*, **3**, 566–573.

Hoey,T., Weinzierl,R.O.J., Gill,G., Chen,J.L., Dynlacht,B.D. and Tjian,R. (1993) Molecular cloning and functional analysis of *Drosophila* TAF110 reveal properties expected of coactivators. *Cell*, **72**, 247–260.

Ingham,P.W. (1988) The molecular genetics of embryonic pattern formation in *Drosophila*. *Nature*, **335**, 25–34.

Inostroza,J.A., Memelstein,F.H., Ha,I., Lane,W.S. and Reinberg,D. (1992) Dr1, a TATA-binding protein-associated phosphoprotein and inhibitor of class II gene transcription. *Cell*, **70**, 477–489.

Johnson,P.F. and McKnight,S.L. (1989) Eukaryotic transcriptional regulatory proteins. *Annu. Rev. Biochem.*, **58**, 799–839.

Krasnow,M.A., Saffman,E.E., Kornfeld,K. and Hogness,D.S. (1989) Transcriptional activation and repression by *Ultrabithorax* proteins in cultured *Drosophila* cells. *Cell*, **57**, 1031–1043.

Levine,M. and Manley,J.L. (1989) Transcriptional repression of eukaryotic promoters. *Cell*, **59**, 405–408.

Lewin,B. (1990) Commitment and activation at polII promoters: a tail of protein–protein interactions. *Cell*, **61**, 1161–1164.

Licht,J.D., Grossel,M.J., Figge,J. and Hansen,U. (1990) *Krüppel* is a transcriptional repressor. *Nature*, **346**, 76–79.

Ma,J. and Ptashne,M. (1987) The carboxy-terminal 30 amino acids of GAL4 are recognized by GAL80. *Cell*, **50**, 137–142.

Meisterernst,M. and Roeder,R.G. (1991) Family of proteins that interact wih TSFIID and regulate promoter activity. *Cell*, **67**, 667–567.

Mitchell,P.J. and Tjian,R. (1989) Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science*, **245**, 371–378.

Nauber,U., Pankratz,M.J., Kienlin,A., Seifert,E., Klemm,U. and Jäckle,H. (1988) Abdominal segmentation of the *Drosophila* embryo requires a hormone receptor-like protein encoded by the gap gene *knirps*. *Nature*, **336**, 489–492.

Nolan,G.P. and Baltimore,D. (1992) The inhibitory ankyrin and activator Rel proteins. *Curr. Opin. Gen. Dev.*, **2**, 211–220.

Pankratz,M.J. and Jäckle,H. (1993) Blastoderm segmentation. In Bate,M. and Martinez Arias,A. (eds), *The Development of Drosophila melanogaster. Vol. 1*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 467–516.

Pankratz,M.J., Busch,M., Hoch,M., Seifert,E. and Jäckle,H. (1992) Spatial control of the gap gene *knirps* in the *Drosophila* embryo by posterior morphogen system. *Science*, **255**, 986–989.

Ptashne,M. (1988) How eukaryotic transcriptional activators work. *Nature*, **335**, 683–689.

Ptashne,M. and Gann,A.A.F. (1990) Activators and targets. *Nature*, **346**, 329–331.

Renkawitz,R. (1990) Transcriptional repression in eukaryotes. *Trends Genet. Sci.*, **6**, 192–197.

Renkawitz,R. (1993) Repression mechanism of v-Erb A and other members of the steroid receptor family. Zinc-finger proteins in oncogenesis: DNA-binding and gene regulation. *Ann. N. Y. Acad. Sci.*, **684**, 1–10.

Roeder,R.G. (1991) The complexities of eukaryotic transcription initiation: regulation of preinitiation complex assembly. *Trends Biochem. Sci.*, **16**, 402–407.

Rosenberg,U., Schröder,C., Preiss,A., Kienlin,A., Coté,S., Riede,I. and Jäckle,H. (1986) Structural homology of the product of the *Drosophila Krüppel* gene with *Xenopus* transcription factor IIIA. *Nature*, **319**, 336–339.

Roy,A.L. Garruthers,C., Gutjahr,T. and Roeder,R.G. (1993) Direct role for Myc in transcription initiation mediated by interactions with TFII-I. *Nature*, **365**, 359–361.

Sauer,F. and Jäckle,H. (1991) Concentration-dependent transcriptional activation or repression by KRÜPPEL from a single binding site. *Nature*, **353**, 563–565.

Sauer,F. and Jäckle,H. (1993) Dimerization and the control of transcription by *Krüppel*. *Nature*, **364**, 454–457.

Sauer,F., Fondell,J.D., Ohkuma,Y., Roeder,R.G. and Jäckle,H. (1995) Transcriptional control by *Krüppel* through interactions with TFIIB and TFIIEβ. *Nature*, **375**, 162–164

Schmucker,D., Taubert,H. and Jäckle,H. (1992) Formation of the *Drosophila* larval photoreceptor organ and its neuronal differentiation require continous *Krüppel* gene activity. *Neuron*, **5**, 1025–1039.

Small,S., Kraut,R., Hoey,T., Warrior,R. and Levine,M. (1991) Transcriptional regulation of a pair-rule stripe in *Drosophila*. *Genes Dev.*, **5**, 827–839.

Stanojevic,D., Hoey,T. and Levine,M. (1989) Sequence-specific DNA-binding activities of the gap proteins encoded by *hunchback* and *Krüppel* in *Drosophila*. *Nature*, **341**, 331–335.

Stanojevic,D., Small,S. and Levine,M. (1991) Regulation of a segmentation stripe by overlapping activators and repressors in the *Drosophila* embryo. *Science*, **254**, 1385–1387.

Struhl,G., Johnston,P. and Lawrence,P.A. (1992) Control of *Drosophila* body pattern by the hunchback morphogen gradient. *Cell*, **69**, 237–249.

Tautz,D. (1988) Regulation of the *Drosophila* segmentation gene *hunchback* by two maternal morphogenetic centers. *Nature*, **332**, 281–284.

Tautz,D., Lehmann,R., Schnürch,H., Schuh,R., Seifert,E., Kienlin,A., Jones,K. and Jäckle,H. (1987) Finger protein of novel structure

encoded by *hunchback*, a second member of the gap class of *Drosophila* segmentation genes. *Nature*, **327**, 383–389.

Tjian,R. and Maniatis,T. (1994) Transcriptional activation: a complex puzzle with few easy pieces. *Cell*, **77**, 5–9.

Treacy,M.N., He,X. and Rosenfeld,M.G. (1991) I-POU: a POU domain protein that inhibits neuron-specific gene activation. *Nature*, **350**, 577–584.

Yang-Yen,H.F., Chambard,J.C., Sun,Y.L., Smeal,T., Schmidt,T.J., Drouin,J. and Karin,M. (1990) Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein–protein interaction. *Cell*, **62**, 1205–1215.

Zhuo,P., Stanojevic,D., Colgan,J., Han,K., Levine,M. and Manley,J.L. (1990) Activation and repression of transcription by the gap proteins *hunchback* and *Krüppel* in cultured *Drosophila* cells. *Genes Dev.*, **5**, 254–264.

Received on May 24, 1995; revised on July 30, 1995