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hairy stripe 7 element mediates activation and repression in response to different domains and levels of Krüppel in the *Drosophila* embryo

Anna La Rosée-Borggreve[1], Thomas Häder[1,2], David Wainwright[1], Frank Sauer[3], Herbert Jäckle[1,2,*]

Abteilung Molekulare Entwicklungsbiologie, Max-Planck-Institut für biophysikalische Chemie, Am Fassberg, D-37077 Göttingen, Germany

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Abstract

The *Drosophila* gap gene *Krüppel* (*Kr*) encodes a zinc finger-type transcription factor required for controlling the spatial expression of other segmentation genes during early blastoderm stage. Here we show that two independent and transferable repressor domains of Krüppel act to control expression of the pair-rule gene *hairy*, and that the minimal cis-acting element of *hairy* stripe7 (*h*7) mediates either Krüppel-dependent activation or repression in different regions of the blastoderm embryo. The C-terminal region of Krüppel which encompasses the predominant repressor domain is not essential for activation, but is required to fully suppress *h*7-mediated transcription in response to high levels of Krüppel activity. This domain contains an interaction motif for dCtBP, a homologue of the human co-repressor CtBP. dCtBP activity is, however, dispensable for Krüppel-mediated repression in the embryo since Krüppel-mediated repression functions in the absence of dCtBP. Possible modes of *h*7-mediated gene regulation in response to the different domains and levels of Krüppel are discussed.

Keywords: *Drosophila* embryo; Krüppel; Trans-activation domain; Trans-repression domains; Stripe formation

1. Introduction

The *Drosophila* gene *Krüppel* (*Kr*) was initially identified as a member of the gap class of segmentation genes. It encodes a zinc finger-containing transcription factor which binds to the consensus sequence AAAA$^{C}/_{G}$GGGTTAA (Rosenberg et al., 1986; Pankratz et al., 1989). The zinc finger domain of the *Kr* protein (Krüppel) is framed by two evolutionarily conserved transrepressor domains (TR1 and C64, see Fig. 1a) and a single, weak transactivator domain (TA1, see Fig. 1a; Licht et al., 1990; 1994; Sauer and Jäckle, 1991). C64 was initially identified when transferred to the DNA-binding domain of the yeast transcriptional activator GAL4 (Sauer and Jäckle, 1993) and all three transacting domains, TR1, TA1 and C64 (see Fig. 1a) have been shown to confer their activities onto the bacterial LacI protein (Licht et al., 1990; Hanna-Rose et al., 1997).

In mammalian tissue-culture studies, Krüppel functions as a transcriptional repressor when acting from a single binding site located in close proximity to a basal promoter (Sauer and Jäckle, 1991; Licht et al., 1993). However, in *Drosophila* cell culture and in cell-free reactions, Krüppel can function either as a transcriptional activator at low concentrations or as a transcriptional repressor at high concentrations. The switch between repressor or activator activity is governed by the formation of Krüppel homodimers which forces the latter function (Sauer and Jäckle, 1993). Nevertheless, in vivo gene expression studies have indicated that Krüppel functions principally as a repressor of adjacently expressed gap genes and subordinate pair-rule genes within the segmentation gene cascade (Ingham, 1988; Hoch and Jäckle, 1993; Pankratz and Jäckle, 1993) by acting through far-upstream enhancer elements. A detailed analysis of Krüppel function on the *even-skipped* (*eve*) stripe2 element, which mediates reporter gene expression in a stripe located in the anterior third of the blastoderm embryo (Goto et al., 1989), showed that Krüppel functions exclusively as a repressor and acts via quenching and competitive binding likely to cause the displacement of activators (Small et al., 1991; Stanojevic et al., 1989, 1991). Thus, the available in vivo evidence suggests that Krüppel acts as a transcriptional repressor whilst conclusive in vivo evidence demonstrating that Krüppel can addition-

* Corresponding author.

[1] These authors contributed equally to this work.

[2] Present address: DeveloGen, Rudolf-Wissell-Str. 28, D37079 Göttingen, Germany.

[3] Present address: ZMBH, Im Neuenheimer Feld 282, D69120 Heidelberg, Germany.

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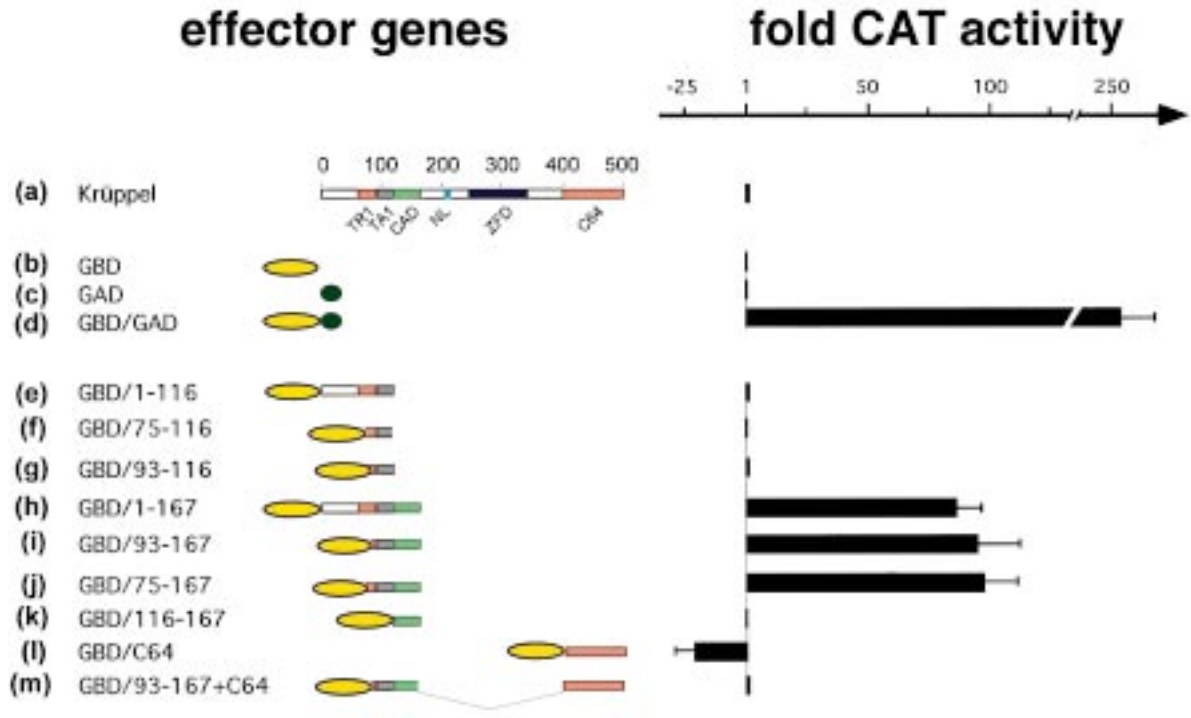

Fig. 1. Mapping of transregulator domains of Krüppel and their activity in co-transfected *Drosophila* Schneider cells. (a) Schematic representation of Krüppel and (b–m) effector genes (left panel). Numbers refer to amino acids, abbreviations are TR1: transrepressor domain 1 (Licht et al., 1990, 1994); TA1: transactivator domain 1 (Licht et al., 1994); CAD: co-activator domain; NL: nuclear location signal; ZFD: Zinc finger domain; C64: C-terminal repressor domain. Effector genes expressing GAL4 DNA-binding domain (GBD) (b), the the GAL4 activator domain (GAD) (c), the GBD/GAD fusion (d) or the GAL4 DNA-binding domain (GBD) fused to portions of Krüppel (e–m; sequence intervals correspond to numbers and colour code used in a). Right panel: Corresponding reporter gene activity (UAS_G-CAT; see Gerwin et al., 1994 for detailed description) in response to the effector genes listed in the left panel. Mean values obtained from at least four independent co-transfection experiments (for details see Gerwin et al., 1994) are shown.

ally function as an activator of gene expression is as yet missing.

Recent studies on two unrelated short-range repressors, the nuclear receptor protein Knirps, which controls segmentation in the abdominal region of the embryo (Nauber et al., 1988), and the zinc finger protein Snail (Boulay et al., 1987; Gray and Levine, 1996), which functions to establish a boundary between the presumptive mesoderm and neuroectoderm (Kosman et al., 1991; Leptin, 1991), have shown that both of these transcription factors contain a short protein interaction motif, Pro-X-Asp-Leu-Ser-X-Lys (PDLS-K) (Nibu et al., 1998). This motif was first described in the adenovirus transcriptional regulator E1A and shown to be requisite for its physical interaction with human CtBP. The interaction between E1A and CtBP attenuates E1A-mediated transcriptional activation and resultant tumorgenesis (Boyd et al., 1993; Schaeper et al., 1995). Nibu et al. (1998) identified *Drosophila* CtBP (dCtBP) and demonstrated that the protein can interact with the P-DLS-K motif of both Knirps and Snail and thereby mediate repression in vivo. The authors noted a PDLS-K-related motif of the sequence P-DLS-H in the C-terminal repressor domain of Krüppel, and suggested that the short-range repressor activity of Krüppel may also involve recruitment of the co-repressor dCtBP.

Here we have analysed various domains of the Krüppel protein by assaying their transregulatory potential in transfected tissue culture cells. The results indicate that depending on their spacing and/or folding, the transferable transacting domains of Krüppel act in a context-dependent manner. We have also examined the regulatory control of Krüppel on a minimal cis-acting stripe element, the stripe7 element of the pair-rule gene *hairy* (La Rosée et al., 1997), which mediates gene expression in a stripe domain in the posterior region of the blastoderm embryo. We show that this element is able to mediate either activation or repression in response to different levels of Krüppel. Truncation studies combined with functional in vivo expression assays indicate that, although the P-DLS-H motif-containing C-terminal repressor domain is required to mediate repression at increased levels of Krüppel in vivo, this regulatory effect of Krüppel is not dependent on dCtBP.

2. Results

2.1. Mapping of functional Krüppel domains in Drosophila tissue culture cells

In order to examine the transacting regulatory potential of

combinations of TR1, TA1 and C64, we fused various portions of Krüppel to the DNA-binding domain of the yeast transcription factor GAL4 (''GBD''). These fusion constructs (Fig. 1) were co-transfected into *Drosophila* Schneider cells (Schneider, 1972) together with a GAL4/UAS_G-dependent chloramphenicol acetyl transferase (CAT) reporter gene construct (described in Gerwin et al., 1994). This construct showed a basal level of CAT reporter gene activity in the absence of co-transfected effector genes (Gerwin et al., 1994) or in the presence of plasmids expressing either Krüppel (Fig. 1a), the GAL4 DNA-binding domain (Fig. 1b) or the GAL4 activator domain (Fig. 1c). This basal level of CAT activity was not altered when the fusion proteins GBD/1–116 (which contains TR1 and TA1), GBD/75–116 (which contains a non-functional portion of TR1 but a functional TA1) or GBD/93–116 (which lacks the complete TR1 but contains the TA1) were co-expressed with the reporter plasmid (Fig. 1). In contrast, GBD/1–167 and the TR1-deficient fusion genes GBD/93–167 and GBD/75–167 which contain a stretch of 51 amino acid residues (117–167) not present in GBD/1–116 were able to activate CAT activity (Fig. 1h-j). A fusion protein containing just these 51 amino acid residues (GBD/116–167) was unable to activate CAT expression (Fig. 1k). Taken together, these data indicate that in *Drosophila* cells, TA1 alone is incapable of acting as a weak transactivator domain as has been observed in mammalian cells (Licht et al., 1994). TA1 is however, activation-competent in the presence of the adjacent stretch of 51 amino acid residues. This 51 amino acid region contains sequence motifs similar to those observed in the transactivation domains of CTF/NF1, Sp1 and Pit1 (Courey and Tjian, 1988; Ingraham et al., 1988; Mermod et al., 1989), but since this sequence alone fails to mediate gene activation, we refer to it as the co-activating domain (CAD).

Combined TA1 and CAD caused reporter gene activation even in the presence of TR1 (compare GBD/1–167, Fig. 1h, and GBD/75–167, Fig. 1j). Thus, these two domains together override the TR1-dependent transrepression activity contained within these fusion proteins (Licht et al., 1990). In contrast, when TA1 and CAD were directly fused with the previously identified C64 repressor domain of Krüppel (Fig. 1l; Sauer and Jäckle, 1993) (giving rise to the GBD/93–167 + C64 protein), CAT expression was not affected (Fig. 1m). Therefore, the opposite regulatory activities of the TA1/CAD and C64 domains are extinguished when fused. Thus, it appears necessary that, as in the full-size Krüppel protein, these domains are separated in order to exert opposite regulatory functions on transcription.

2.2. Krüppel-dependent activation and repression of gene expression in vivo

Having identified regions of Krüppel which act in a trans-regulatory manner in cell culture, we were interested to determine whether these regions of Krüppel indeed play a critical role for *Kr* target gene expression in vivo. We made use of a recently identified enhancer element of the pair-rule gene *hairy* (*h*), which mediates *Kr*-dependent gene expression in place of the *hairy* stripe7 expression domain in the blastoderm stage embryo (Fig. 2a; for details see La Rosée et al., 1997). The *hairy* stripe7 enhancer element, termed *h*7, decodes the activity of three activators, the maternal homeodomain proteins Caudal and Bicoid, and the zinc finger protein Krüppel (La Rosée, 1997). Previous genetic analyses have suggested that Caudal and Krüppel activities are necessary, and sufficient, to activate *h*7-mediated *lacZ* reporter gene (*h*7-*lacZ*) expression but Bicoid activity is additionally required to achieve wildtype expression levels (for details see La Rosée, 1997).

Absence of *Kr* activity does not only significantly reduce the level of *h*7-dependent reporter gene activation in the posterior region of the embryo, but also results in the appearance of a second and novel expression domain (Fig. 2a,b) in a position corresponding to the highest levels of Krüppel in wildtype blastoderm embryos (Gaul et al., 1987). Thus, *h*7 not only mediates gene expression in response to low levels of Krüppel in the posterior region of the blastoderm embryo, but at the same time, prevents reporter gene expression at high concentrations in the central region of the embryo.

To determine the ability of Krüppel to directly interact with the *h*7 element, we performed DNaseI footprinting experiments using bacterially produced Krüppel and subfragments of the *h*7 element. Fig. 2c,d indicates that the *h*7 element contains five in vitro Krüppel binding sites and opens the possibility that Krüppel may act through multiple binding sites within the *h*7 element.

2.3. The C-terminal region of Krüppel is essential for repression in vivo

To determine whether Krüppel can also cause *h*7-mediated repression in response to increased concentrations of the protein in the posterior region of the embryo, we ectopically expressed *Kr* at uniform levels throughout the early blastoderm embryo using a heat shock-inducible transgene containing the *Kr* cDNA (*hsp-Kr*).

No *h*7-*lacZ* expression was observed in response to transgene-dependent *hsp-Kr* expression by a 30 min heat-shock treatment starting at syncytial blastoderm stage (compare Fig. 3b,d). This shows that, upon Krüppel overexpression, Krüppel activity overrides the activators Bicoid, Caudal and Krüppel itself, resulting in suppression of *h*7-mediated gene expression. Since maternally derived activities of Caudal and Bicoid are not altered by zygotically expressed Krüppel, the ectopically increased levels of Krüppel must either repress or quench (Han et al., 1989) the function of the *h*7-bound activators.

To determine if both, or just the C64 transrepressor domains of Krüppel are required to provide *h*7-mediated repression in vivo, we made use of a previously identified

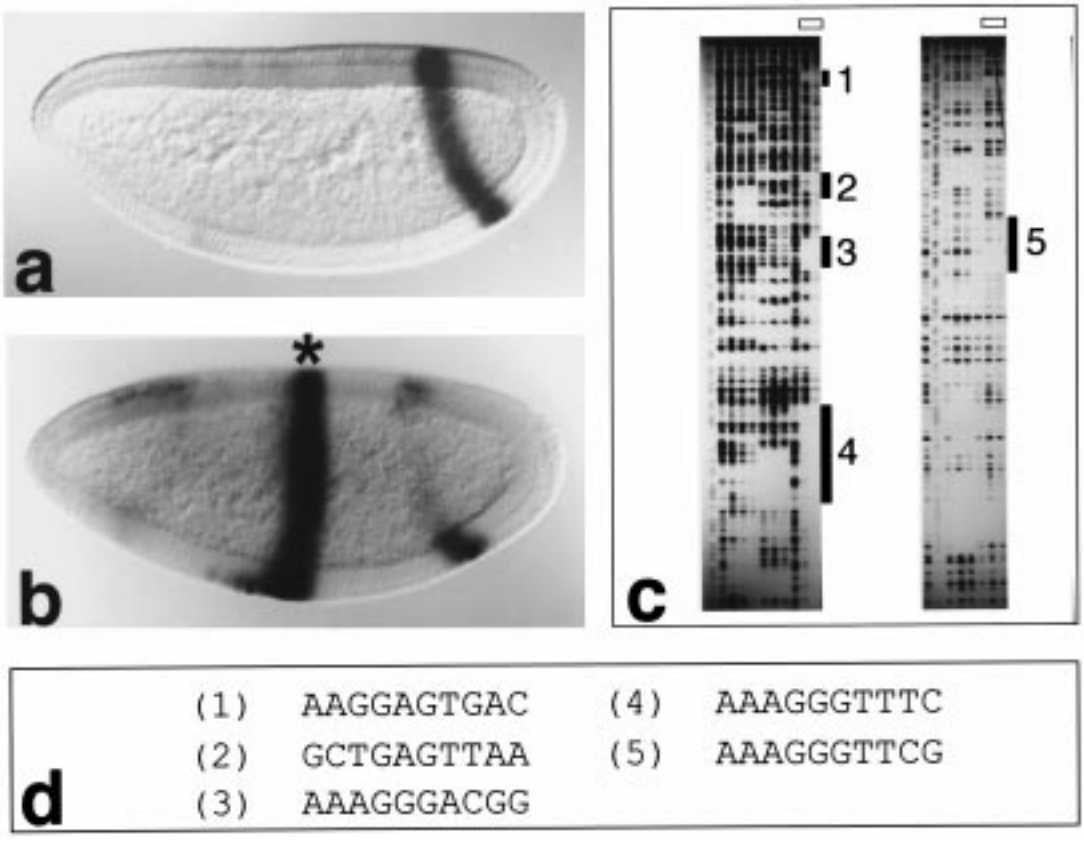

Fig. 2. *Kr*-dependent expression of *h*7-*lacZ* reporter gene expression and detection of Krüppel binding sites within the *h*7 stripe element. (a) *h*7-mediated *lacZ* expression in wildtype embryos at blastoderm stage showing a single stripe in position of *hairy* stripe 7 (for details see La Roseé et al., 1997). (b) *h*7-mediated *lacZ* expression in a homozygous *Kr* embryo. Note weak stripe 7 expression and the appearance of a strong central expression domain. Thus, absence of *Kr* activity affects the level of *h*7-mediated gene expression in a posterior position of the embryo (where Krüppel concentration is low in wildtype) and results in a new expression domain in the center (where the concentration of Krüppel is highest in wildtype; see Fig. 3a). Orientation of embryos: anterior is left, dorsal up. (c) DNase I in vitro footprints showing four Krüppel binding sites (1–4; core sequence in d) within DNA fragment AflII/ClaI and one site (5; core sequence in d) in BalI/AvaI DNA of the *h*7 element (for sequence and diagnostic restriction sites see La Roseé et al., 1997). Left panel: AflII/ClaI fragment. Maxam–Gilbert reaction (lane 1), footprinting reaction without protein (lanes 2,3), control proteins previously shown to bind (lanes 4–9;La Roseé et al., 1997), 1 and 5 μg of Krüppel (lanes 10,11; open box). Right panel: BalI/AvaI fragment. Footprinting reaction without protein (lane 1), Maxam-Gilbert reaction (lane 2), control proteins previously shown to bind (lanes 3–6; La Roseé et al., 1997), 1 and 5 μg of Krüppel (lanes 7,8; open box). (d) Core sequence of Krüppel binding sites 1–5.

mutant Krüppel allele, Kr^{v} (Gaul et al., 1989). Kr^{v} is a stop codon mutation that terminates Krüppel in position 351 and consequently, encodes Kr proteins that lack the C64 domain. In homozygous Kr^{v}-mutant embryos bearing the *h7-lacZ* transgene, a wildtype-like *h*7-*lacZ* stripe expression domain was observed in the posterior region of the embryos (Fig. 3e) indicating that the absence of the C-terminal region does not affect the activating function of Krüppel. Additionally, such embryos show a central *h*7-*lacZ* expression domain similar to that observed in *Kr* lack-of-function embryos. However, in comparison to *Kr* lack-of-function embryos, the expression level in this domain was significantly weaker (compare Figs. 2b and 3e), suggesting that the Kr^{v}-mutant protein is able to exert considerable repressor activity and that under wildtype conditions, the C64 domain is necessary to exert the full range of Krüppel's repressive activity. To test this proposal directly, we overexpressed a Krüppel protein which lacks the C64 domain from a heat shock-inducible transgene (hsp-$Kr^{\Delta C64}$). $Kr^{\Delta C64}$ protein did not repress *h7-lacZ* gene expression in the posterior region of the embryo (Fig. 3f) as had been observed in response to full-length ubiquitously expressed Krüppel (see above, Fig. 3d). Thus, the C-terminal region of Krüppel is required to override the activation of *h*7-mediated gene expression in the posterior region of the embryo by repression.

2.4. dCtBP is not essential to provide Krüppel-dependent repression mediated by the h7-element

The recent notion of a dCtBP-interacting motif in the C64 region (Nibu et al., 1998) made us ask whether dCtBP is necessary to mediate Krüppel-dependent repression. To determine if Krüppel-dependent repression of the *h7 element* is lost in the absence of dCtBP, we made use of the FRT/recombinase system (Golic, 1991; Chou and Perrimon, 1992; Chou et al., 1993) to generate dCtBP-deficient

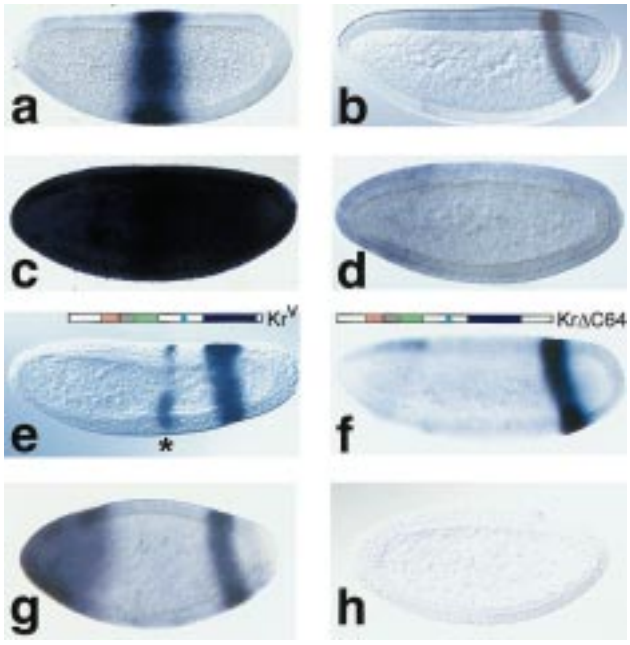

Fig. 3. Whole mount in situ hybridization on blastoderm embryos showing *h7-lacZ* reporter gene expression in response to: Krüppel (a–d), Krüppel mutants (e,f) and corepressor dCtBP (g,h). Orientation of embryos: anterior is left, dorsal up. (a) *Kr* expression and (b) *h*7-mediated *lacZ* expression in wildtype embryos. (c) Ubiquitous *Kr* expression in response to heat shock induced *Kr* transgene expression and (d) the absence of *h7-lacZ* reporter gene expression in such embryos, indicating that increased levels of *Kr* cause repression of *h*7-mediated *lacZ* expression throughout the embryo. (e) *h7-lacZ* reporter gene expression in homozygous Kr^{V} embryos. Note strong *h7-lacZ* expression in position of *hairy* stripe 7 and a new, but weak expression domain in the centre of the embryo. Thus, the C64 domain lacking in the Kr^{V} mutant protein (colour code as in Fig. 1a) is required for full suppression of *h*7-mediated gene expression in the centre, but not for coactivation in the posterior region. (f) Ubiquitous *hsp*-KrΔC64 (colour code as in Fig. 1a) transgene expression in otherwise wildtype embryos does not affect *h7-lacZ* expression in position of *hairy* stripe 7, indicating that the C64 domain is required for mediating repression. (g) dCtBP-deficient embryo (lack maternal dCtBP activity due to germ line clones; homozygous dCtBP genotype) lacking the *hs-Kr* transgene (as indicated by *hb-lacZ* expression in the anterior region; see Section 4) shows normal *h7-lacZ* expression in position of *hairy* stripe 7 after a 30 min heat shock treatment (serving as a control for h). Thus, in the absence of functional dCtBP, endogenous *Kr* activity in the embryo can repress *h7-lacZ* activation. (h) dCtBP-deficient embryo (lack maternal dCtBP activity due to germ line clones; homozygous dCtBP genotype) bearing the *hs-Kr* transgene (as indicated by the absence of *hb-lacZ* expression in the anterior region; see experimental procedures) lacks *h7-lacZ* expression in response to Krüppel induced by a 30 min heat shock treatment. Thus, dCtBP activity is not required for this aspect of Krüppel-dependent repression (see text).

embryos bearing the *h7-lacZ* and the *hsp-Kr* transgenes. Fig. 3g shows that dCtBP-deficient embryos express the *h7-lacZ* reporter gene in a wildtype-like pattern (compare Fig. 3b,g). However, upon ubiquitous ectopic expression of *Kr* in such embryos, *h7-lacZ* reporter gene expression is completely repressed (Fig. 3h) indicating that the absence of dCtBP has no effect on the repressive ability of Krüppel. While this result does not exclude the possibility that dCtBP contributes to some aspects of Krüppel-dependent repression, it clearly establishes that dCtBP is not required to mediate Krüppel-dependent repression in the context of the *h7* element.

3. Discussion

Krüppel contains three discrete transferable regions which possess specific regulatory properties. TA1 is necessary but not sufficient to activate transcription. Its presence in the protein can counteract TR1- but not C64-dependent repression, whereas the presence of the combined TA1/CAD domain mediates activation autonomously and overrides TR1-dependent repression. The combined TA1/CAD domain is also capable of counteracting C64-dependent repression upon direct fusion, but, it cannot override C64 repressor function by activation. The data presented here argue that TR1 and C64 are not equivalent in function but are individual parts of the greater protein that are likely to have discrete functions. In addition to the fact that C64 is the stronger of the two repressor domains in cell culture (Hanna-Rose et al., 1997), several pieces of in vivo evidence suggest that the two repressor domains of Krüppel are likely to mediate alternative modes of repression. When the C64-containing C-terminal region of Krüppel was removed by a mutation that prematurely terminates the open reading frame, the truncated Krüppel continues to function as a transcriptional co-activator of *h*7-mediated gene expression in the posterior region of the embryo, but its repressor activity in the central region of the embryo is strongly reduced. Furthermore, if full-length Krüppel is ectopically expressed throughout the embryo, it antagonizes *h*7-mediated gene activation by Bicoid and Caudal (La Rosée et al., 1997) in the posterior region of the embryo. This ability is not a feature of Krüppel proteins lacking the C64 domain and thus demonstrates that TR1 cannot functionally compensate for all aspects of C64-dependent repression in the embryo.

In mammalian tissue culture, the two transferable repression domains TR1 and C64 were shown to exert a specific regulatory profile suggesting that they may have distinct transcriptional targets and even different biological functions during *Drosophila* development (Hanna-Rose et al., 1997). The activator specificity of the two domains suggested that TR1 interacts with a factor that is more generally required for transcriptional activation (Hanna-Rose et al., 1997). Our results showing the prevalence of C64 over TR1 in mediating Krüppel repressor function in the embryo are consistent with this proposal.

In vitro experiments have previously shown that C64 provides a homodimerization surface that permits Krüppel homodimer formation at high protein concentrations. The homodimer acts exclusively as a transcriptional repressor, whereas the Krüppel monomer has been shown to function as a transcriptional activator both in vitro and in *Drosophila* tissue culture assays (Sauer and Jäckle, 1993). Based on the in vitro results, it has been proposed that Krüppel acts as a transcriptional repressor in the central region of the blasto-

derm embryo and may function as an activator of target genes outside the central region where the concentration of Krüppel gradually decreases (Sauer and Jäckle, 1993). The *h7*-mediated expression pattern in Kr^{V} mutant embryos is consistent with this proposal by showing that the lack of the C-terminus, and hence the dimerization domain, does not affect Krüppel's ability to co-activate *h*7-mediated gene expression in a position of low Krüppel concentration in the embryo, but rather, strongly reduces its repressor function at high concentrations.

Gray and Levine (1996) proposed two models to explain Krüppel-mediated repression. One model suggests that Krüppel possesses two separate activities, one interfering with enhancer-bound activators by quenching, and the other directly inhibiting transcription by interacting with components of the basal transcription machinery. The second model proposes that Krüppel recruits a repressor complex that only functions locally. Some aspects of the results presented here fit with the first model, others with the second. For example, TR1 could be the repressor domain that acts through quenching. In this case, TR1 would interfere with Bicoid-dependent activation mediated by *h*7 in the central region of the embryo, but not with Caudal-dependent activation which is predominant in the posterior region of the embryo. This assignment would be consistent with the finding that repression of *h*7-mediated gene expression is strongly reduced in the central region of the Kr^{V} mutant embryo but no effect is observed in the posterior region of the embryo when Krüppel lacking the C-terminal region is expressed throughout the embryo. Alternatively or additionally, C64 could act either by blocking activation via inhibiting basal transcription, or it may interfere with, and thereby extinguish, both Caudal and Bicoid activities directly. Direct inhibition of the basal transcription machinery would be consistent with in vitro data showing that C64 prevents transcription by interacting with the general transcription factor TFIIEβ (Sauer et al., 1995). This proposal would, however, only be consistent with the recent finding that the C-terminal repression region of Krüppel inhibits certain activators (Hanna-Rose et al., 1997) if the subset of affected activators would target TFIIEβ to exert their function. The second model which explains transcriptional repression via a repressive complex formation is consistent with the observation that the C-terminal domain enables Krüppel to form heterodimer complexes with other transcription factors such as Knirps (Sauer and Jäckle, 1995). A further possibility is that the C-terminal domain could serve to recruit more general co-repressors such as Groucho (Hartley et al., 1988; Delidakis et al., 1991) or CtBP (Poortinga et al., 1998) to template DNA.

A CtBP-binding motif has indeed been noted in the C-terminal repressor region of Krüppel (Nibu et al., 1998). This co-repressor was originally identified as an attenuator of adenovirus E1A-dependent transcriptional activation in human cells (Boyd et al., 1993; Schaeper et al., 1995). The *Drosophila* homologue, dCtBP, has been shown to interact with the gap gene product Knirps in vitro and gene-dosage interaction studies with dCtBP and *knirps* mutants have suggested that Knirps–dCtBP interactions are also able to occur in vivo (Nibu et al., 1998). The recruitment of dCtBP by short-range repressors, such as Knirps and Krüppel, may theoretically be able to alter the chromatin structure, its status of acetylation or the presence of transcriptional activators bound to a nearby site within the enhancer. Nevertheless, the weakest known *knirps* mutant, $knirps^{14F}$, which lacks the dCtBP–interaction motif (see Gerwin et al., 1994), develops an almost normal abdominal segment pattern with the exception that the abdominal segment 4 is consistently missing. This suggests that dCtBP may possibly be important for some specific but not all aspects of Knirps-dependent repressor function. The results shown here indicate that dCtBP is neither required for Krüppel-dependent repression of *h*7-mediated activation in the central region of the embryo, nor for Knirps-dependent repression of the expression domain in the posterior region of the embryo (La Rosée et al., 1997; Fig. 3g). Furthermore, dCtBP is also not required for repression of this expression domain in response to ubiquitously expressed Krüppel (Fig. 3h). These observations suggest that dCtBP could represent one of several co-repressors for gap gene factors as has been described for the transcriptional repressor encoded by the pair-rule gene *hairy* (Poortinga et al., 1998).

Our results shown here describe a previously missing piece of information surrounding Krüppel function, namely that Krüppel possesses both activator and repressor function in vivo. The switch between activator and repressor functions is dependent on the concentration of Krüppel protein and is mediated by the C-terminus. The precise mechanism by which this mode of switching is regulated and potential cofactors of Krüppel are still unknown and need to be addressed by future studies.

4. Experimental procedures

4.1. Drosophila strains and mutant embryos

Drosophila strains were kept under standard conditions. Mutant alleles are described previously (Lindsley and Zimm, 1992): Kr^{2}, Kr^{V}. Transgenic embryos carrying P-elements were collected from stocks balanced with CyO or TM3. The balancer chromosomes carried a *lacZ* reporter gene containing the *fushi tarazu* or the *hunchback* promoter which allow homozygous mutant embryos to be unambiguously identified on the basis of the lack of *hunchback* or *fushi tarazu* staining patterns. Production of germline mosaics for maternal dCtBP mutant embryos was done using the autosomal FLP-DFS technique (Perrimon et al., 1996; Poortinga et al., 1998).

4.2. In situ hybridization of embryos

The patterns of *Krüppel* or reporter gene (*lacZ*) expres-

sion were examined by in situ hybridization to whole mounted embryos using antisense RNA probes according to Klingler and Gergen (1993).

4.3. Construction of fusion genes and CAT reporter, transfections and transformation studies

GAL4/Krüppel fusion genes and the Krüppel binding site-depending chloramphenicol aminotransferase (CAT) reporter gene were generated as described previously (Sauer and Jäckle, 1991). Co-transfection experiments were done as described (Sauer and Jäckle, 1993). The expression of the effector genes and the subcellular localization of the protein products were monitored by anti-*Krüppel* antibody stainings, showing that they accumulate in the nuclei of the transfected cells (data not shown).

The *h*7-*lacZ* fusion gene was generated as described previously (La Rosée, 1997). The $Kr^{\Delta C64}$ transgene contains the inducible hsp70 promoter fused to *Kr* cDNA that was truncated by Hind II in the position of amino acid 401, inserted into the vector HS-pCaSpeR (Thummel and Pirrotta, 1992). For details see (Häder, 1998; Sauer, 1993). Recombinant plasmids were introduced into the fly genome by P-element-mediated germline transformation (Rubin and Spradling, 1982). Several independent transformant lines were established for each construct and expression of the transgenes were analysed by in situ hybridization using an RNA probe.

4.4. Footprinting experiments

The bacterial expression vector pRSET*Kr* encodes amino acids 29–466 of Krüppel (Hartmann, 1996). Expression of the protein was induced by 1 mM IPTG and continued for 2 h at 37°C. Krüppel extract was purified by affinity chromatography on a nickel trinitrilo acetic acid matrix and used for DNase I footprinting experiments as described (Kadonaga et al., 1987) except that the DNA fragments were end-filled by the Klenow fragment of DNA polymerase (Sambrook et al., 1989). For this we generated two *h*7 element subfragments of the 1.5 kb *Eco*RI/*Eco*RI fragment (Pankratz et al., 1990): the 455 bp *Cla*I/*Afl*II and the 555 bp *Bal*I/*Ava*I fragments.

4.5. Heat shock experiments

Embryos were collected for 1 h at 25°C from stocks that are heterozygous for an insert carrying the *Kr* cDNA or the $Kr^{\Delta C64}$ cDNA under the control of the heat-inducible hsp70 promoter on the second and homozygous for the *h*7 transgene on the third chromosome. The second chromosome was balanced with Cyo as described in Section 4.1. The embryos were aged for another hour at 25°C, transferred in a PCR tube filled with 37°C water and incubated 15–30 min at 37°C in a PCR-machine. The embryos were allowed to recover for 15 min at 25°C and then fixed for in situ hybridization as described in Section 4.2. To examine the effect of ectopic *Kr* expression in maternal *dCtBP* mutant embryos the embryos were collected from a cross of females bearing dCtBP-free eggs (see Section 4.1) and males carrying the heterozygous inducible *Kr* cDNA and homozygous *h*7 transgene.

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