

Quantitative contributions of CtBP-dependent and -independent repression activities of Knirps

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Summary

The *Drosophila* Knirps protein is a short-range transcriptional repressor that locally inhibits activators by recruiting the CtBP co-repressor. Knirps also possesses CtBP-independent repression activity. The functional importance of multiple repression activities is not well understood, but the finding that Knirps does not repress some cis-regulatory elements in the absence of CtBP suggested that the co-factor may supply a unique function essential to repress certain types of activators. We assayed CtBP-dependent and -independent repression domains of Knirps in *Drosophila* embryos, and found that the CtBP-independent activity, when provided at higher than normal levels, can repress an *eve* regulatory element that normally requires CtBP. Dose response analysis revealed that the activity of Knirps containing both CtBP-dependent and

-independent repression activities is higher than that of the CtBP-independent domain alone. The requirement for CtBP at certain enhancers appears to reflect the need for overall higher levels of repression, rather than a requirement for an activity unique to CtBP. Thus, CtBP contributes quantitatively, rather than qualitatively, to overall repression function. The finding that both repression activities are simultaneously deployed suggests that the multiple repression activities do not function as cryptic 'backup' systems, but that each contributes quantitatively to total repressor output.

Key words: CtBP, Knirps, Even-skipped, Enhancer, Repression, Transcription, *Drosophila*

Introduction

Dynamic patterns of gene expression in the *Drosophila* embryo are orchestrated by the combined action of transcriptional activators and repressors acting on complex *cis* regulatory elements, or enhancers. In a number of well-studied cases in the *Drosophila* embryo, multiple enhancers act independently on a single promoter, in part due to the action of so-called short-range repressors, proteins whose inhibitory action is restricted to ranges of ~100 bp from the factor binding site (Gray and Levine, 1996a). In cell culture and transgenic embryo assays, short-range repressors can selectively inhibit individual enhancers, or entirely silence a gene if bound close to the basal promoter (Arnosti et al., 1996; Gray and Levine, 1996b; Ryu and Arnosti, 2003).

The apparent impotence of short-range repression actually provides a highly flexible mechanism for specific gene regulation, allowing genes to be 'tuned' to respond to subtle differences in repressor protein concentration and by small changes in the positions of factor binding sites (Hewitt et al., 1999). Changes in the spacing of short-range repressor binding sites correlate with functional alterations observed during enhancer evolution (Ludwig and Kreitman, 1998; Ludwig et al., 2000).

The CtBP co-repressor is required for full activity of short-range repressors such as Knirps, Krüppel, Giant and Snail that play important roles in patterning the blastoderm embryo (Nibu

et al., 1998a; Nibu et al., 1998b). This evolutionarily conserved co-factor also interacts with a number of vertebrate transcriptional regulators, including the adenovirus E1A protein, Net, Ikaros, Zeb and, indirectly, the Retinoblastoma tumor suppressor protein (reviewed by Chinnadurai, 2002). Transcription factors typically bind to CtBP via a short peptide motif similar to the PLDLS sequence originally identified in E1A (Schaeper et al., 1995). CtBP is homologous to α -hydroxyacid dehydrogenases, and contains a conserved NAD-binding domain as well as conserved residues in the putative active site (reviewed by Chinnadurai, 2002; Turner and Crossley, 2001). Although not identified in previous studies, recent reports found a weak dehydrogenase activity associated with CtBP (Kumar et al., 2002; Balasubramanian et al., 2003; Shi et al., 2003). CtBP has been found to bind directly to histone deacetylases (HDACs), suggesting that the co-repressor may effect repression by chromatin remodeling (reviewed by Turner and Crossley, 2001; Chinnadurai, 2002). A recent biochemical purification of CtBP identified additional proteins in a complex, including histone methyltransferases, the CoREST repressor and a protein homologous to polyamine oxidases (Shi et al., 2003). This additional complexity suggests that CtBP itself may use multiple activities to effect transcriptional repression. *Drosophila* factors functionally characterized as short-range repressors all interact with CtBP, although it has not been established that all factors that bind

the co-factor are necessarily short-range repressors. The long-range repressor protein Hairy, in particular, is thought to interact with CtBP, although this might be in an antagonistic mode (Poortinga et al., 1998; Zhang and Levine, 1999).

CtBP-mediated repression is crucial for full activity of short-range repressors; however, *Drosophila* short-range repressors also possess CtBP-independent repression activities (La Rosee-Borggreve et al., 1999; Keller et al., 2000; Strunk et al., 2001; Nibu et al., 2003). In the case of Knirps, a form of the protein that lacks the CtBP-binding motif exhibits weak activity when overexpressed (Nibu et al., 1998b). The CtBP independent activity has been mapped to an N-terminal repression domain that lacks a CtBP-binding motif and is able to repress in the absence of CtBP (Keller et al., 2000). Although many transcriptional repressors have been found to possess multiple activities, the functional relevance of such activities is not well understood. Previous studies suggest that multiple repression activities underlie both gene specific and activator specific effects. In the case of the Zeb repressor, a protein with CtBP-dependent and -independent activities, it was found that specific repression activities are directed at distinct classes of transcriptional activators (Postigo and Dean, 1999). In another case, distinct mechanisms are used at different promoters: the NRSF repressor mediates HDAC and DNA methylation-dependent repression of the *NaCh II* gene and a distinct form of repression of the *Scg10* gene (Lunyak et al., 2002).

Previous studies also hint that the possession of CtBP-dependent and -independent activities may confer important quantitative effects. For example, the *Krüppel* promoter is activated by Bicoid in both anterior and central regions of the blastoderm embryo, and is repressed by Giant in either CtBP-independent or CtBP-dependent manners, depending on the region of the embryo (Strunk et al., 2001). The higher levels of Bicoid activator in anterior regions of the embryo might necessitate additional repression activities beyond those afforded by CtBP-independent pathways, suggesting that CtBP might contribute quantitatively to overall repressor output. In cell culture studies, CtBP-dependent and CtBP-independent repression activities of Knirps possess similar functional attributes, including distance dependence, trichostatin A insensitivity and activator specificity, suggesting that their quantitative effects might be mediated through similar pathways (Ryu and Arnosti, 2003).

The Knirps protein is able to regulate at least one known target, the stripe 3 enhancer of the *even-skipped* (*eve*) gene in a CtBP-independent fashion, yet this CtBP-independent activity is not sufficient to supply the full biological function of Knirps (Keller et al., 2000; Nibu et al., 1998b). For example, transheterozygous *knirps* and *CtBP* embryos have disruptions in *eve* expression, suggesting that Knirps function is partially impaired, and a frameshift mutation in *knirps* encoding a protein lacking the CtBP binding motif is a strong hypomorph (Gerwin et al., 1994; Nibu et al., 1998a). Furthermore, a point mutation in the CtBP binding motif results in a protein that lacks the dominant phenotype of the wild-type protein when misexpressed in a pattern of *eve* stripe 2 (Nibu et al., 1998b). These results suggest that Knirps requires CtBP for effective regulation of at least some of its targets. Here, we examine the regulation of several enhancers targeted by Knirps to test the possibility that the CtBP-dependent and CtBP-independent

repression activities of Knirps might be deployed to achieve qualitatively or quantitatively distinct effects. Our results suggest that in the case of the *eve* gene, the two activities are both required to achieve quantitatively sufficient levels of repression.

Materials and methods

Plasmid construction

To generate transgenic flies that carry inducible, double-tagged Knirps genes, the P-element transformation vector pCaSpeR-hs (Pirrotta, 1988) was modified to incorporate an N-terminal hexahistidine tag and a C-terminal double FLAG tag in frame with a *KpnI-XbaI* insert (reading frame commencing with GGT). First, an oligonucleotide containing the ribosome binding site (Kozac) consensus sequence for *Drosophila* (Cavener and Ray, 1991) and an N-terminal sequence encoding MARGS(his)₆ was introduced into the unique *EcoRI* site of pCaSpeR-hs. This fragment was generated by annealing and extending the following primers: 5' CCG CGG AAT TCA CAA CCA AAA TGG CGA GAG GAT CGC ATC 3' and 5' GGC CGA ATT CGG TAC CGT GAT GGT GAT GGT GAT GCG ATC C 3', to generate an *EcoRI*-Kozac-MARGS(his)₆-*KpnI-EcoRI*-containing oligonucleotide, which was restricted with *EcoRI*, PAGE-purified and cloned into pCaSpeR-hs. Two FLAG epitope sequences were introduced in two successive steps using annealed oligonucleotides. To introduce the first FLAG epitope tag the vector containing the hexahistidine tag was cut with *KpnI* and *StuI* and ligated with two annealed oligonucleotides (5' CGA TCG ATC GTC TAG AGA TTA CAA GGA TGA CGA TGA CAA GGC GGC CGC TTA GTA ATT AGT TAG 3' and 5' CTA ACT AAT TAC TAA GCG GCC GCC TTT TCA TCG TCA TCC TTG TAA TCT CTA GAC GAT CGA TCG GTA C 3', FLAG-codons in bold) to generate a *KpnI*-(9bp)-*XbaI*-FLAG-(stop)s fragment. A second FLAG epitope was generated by annealing and cloning two *NotI*-compatible, FLAG-containing oligonucleotides (5' GGC CGC TGA TTA CAA GGA TGA CGA TGA CCA GGC 3' and 5' GGC CGC CTT GTC ATC GTC ATC CTT GTA ATC AGC 3') into the unique *NotI* site of the vector. The correct orientation of the second FLAG oligonucleotide was determined by PCR. The final vector, pCaSpeR-hs(H2xF), was sequenced to confirm the correct frame and orientation of the tags inserted. Different *knirps* fragments were subcloned as *KpnI-XbaI* inserts into pCaSpeR-hs(H2xF), generating hsKni1-429, which contains full-length Knirps, hsKni1-330, which contains the CtBP-independent repression domain of Knirps, hsKni1-105, which contains the Knirps DNA-binding domain (amino acids 1-74) and its nuclear localization signal (amino acids 75-95) (Gerwin et al., 1994), hsKni75-429 and hsKni75-330. All fragments were PCR amplified using as template pBS-N741, which contains a full-length *knirps* cDNA (kindly provided by Michael Levine). To amplify full-length Knirps (1-429), the primers used were DA-502 (5' CGC GCG GTA CCA TGA ACC AGA CAT GCA AAG TG 3') and DA-503 (5' CGG CCT CTA GAG ACA CAC ACG AAT ATT CCC CT 3'). To amplify Knirps75-330 the primers used were DA-504 (5' CGC GCG GTA CCG GAT CCC GCT ACG GAC GTC GC 3') and DA-505 (5' CGG CCT CTA GAT CCT TCT TGA GCG GAA ACG GTG G 3'). To amplify Knirps1-330, DA-502 and DA-505 were used. To amplify Knirps75-429, DA-504 and DA-503 were used. To amplify Knirps1-105 the primers used were DA-502 and DA-773 (5' CGG CCT CTA GAA GGC GCC TTG CCC GCC GCT GC 3').

Heat-shock experiments

To induce expression of recombinant Knirps proteins, 2- to 4-hour-old embryos collected on apple-juice plates at room temperature (22-23°C) were incubated for 5, 10, 20 or 30 minutes at 38°C in a 10 liter water bath to ensure rapid and even heating. After induction, embryos were allowed to recover in a water bath at room temperature for 30

minutes prior to fixation or sonication. Heat-shock inductions of Knirps1-330 and Knirps1-429 were also performed with no recovery. For the experiments described in Fig. 6, *hairy* expression pattern was monitored after 10 or 30 minutes of heat shock, *ftz* expression pattern was determined after 5, 10, 15, 20 or 30 minutes of heat shock, *runt* expression pattern was determined after 15 or 30 minutes of heat shock and *hb* expression pattern was determined after 30 minutes of heat shock. Thirty minutes of recovery after heat shock was applied for all the experiments described in Fig. 6.

Crude embryo lysate preparation

Approximately 50 mg of dechorionated embryos were resuspended in 1.2 ml of lysis buffer (25 mM HEPES pH 7.9, 150 mM NaCl, 1 mM DDT, 1 mM PMSF, 1 mM Na-metabisulfite, 1 mM benzamidine, 10 μ M pepstatin A) and disrupted by sonication using a Branson Sonifier 250 (two cycles of 12 pulses each, output 3, duty cycle 60%). After sonication, lysates were centrifuged for 15 minutes at 16,000 g using an Eppendorf centrifuge, and the protein concentration of the supernatant was determined using the Bradford assay, with BSA as the standard.

Western blot analysis

Immunoblotting was performed according to standard protocols (Harlow and Lane, 1999) using a tank transfer system (Mini Trans-Blot Cell, Biorad). Sequi-Blot™ PVDF membranes (BioRad) were used and antibody incubation was in TBST (20 mM Tris-HCl, pH 7.5, 120 mM NaCl, 0.1% Tween-20) supplemented with 5% (w/v) nonfat dry milk as blocking agent. The primary anti FLAG M2 monoclonal antibody (Sigma) was used at 1:10,000 dilution. The secondary ImmunoPure® Goat Anti-Mouse HRP-conjugated antibody (Pierce) was used at 1:20,000 dilution. Western blots were quantitated using a Fluor-S® MultiImager (Biorad) set on high sensitivity and with an exposure time of 50 minutes. The QuantityOne package (BioRad) was used to analyze the data. Four independent quantitations of four gels were performed, analyzing lysates from an experiment performed as described for Fig. 3 and Table 1.

P-element transformation, whole mount *in situ* hybridization

P-element transformation vectors were introduced into the *Drosophila* germline by injection of y^w embryos and *in situ* hybridization was performed using digoxigenin-UTP-labeled antisense RNA probes to *eve*, *h*, *kni*, *runt*, *ftz* and *lacZ* as described (Small et al., 1992).

lacZ reporters

The *eve* stripe 3/7 and 4/6 *lacZ* reporter genes used in Fig. 1 were described elsewhere (Small et al., 1996; Fujioka et al., 1999). Germline mutants of CtBP were generated as previously described (Keller et al., 2000) using CtBP⁰³⁴⁶³/TM3, Sb (Bloomington stock no. P1590). The *even-skipped* stripe 2/3 *lacZ* reporter used in Fig. 4 contains ~500 bp minimal elements separated by a 340 bp spacer sequence and 2 UAS sites (not used in this experiment) fused to the *eve* basal promoter (Keller et al., 2000). The *eve* stripe 3/7 reporter used in Fig. 4 (stock E9) was kindly provided by Steve Small and the *eve* stripe 4/6 *lacZ* reporter (stock B45C52-B) was kindly provided by Jim Jaynes (Fujioka et al., 1999).

Results

Repression by Knirps of *even-skipped* stripe 3/7 enhancer is independent of CtBP, while repression of stripe 4/6 enhancer is CtBP-dependent

The expression of the endogenous *eve* gene is strongly perturbed by a loss of CtBP, consistent with the important role of this co-repressor in the activity of gap repressors Giant,

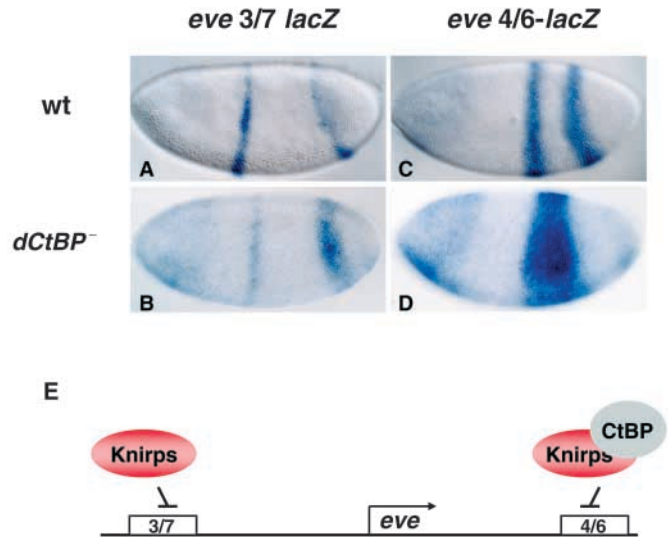


Fig. 1. CtBP is required for Knirps repression of *even-skipped* (*eve*) stripe 4/6 enhancer, but not stripe 3/7 enhancer. Expression patterns of *eve* stripe 3/7 (A,B) and *eve* stripe 4/6 *lacZ* reporter genes (C,D) in wild-type (A,C) and *CtBP* mutant (B,D) embryos, showing derepression only of the *eve* stripe 4/6 element in the *CtBP* mutant. (E) Schematic representation of *eve* regulatory regions, showing co-factor requirements for Knirps repression. Expression patterns were characterized in transgenic embryos by *in situ* hybridization. Embryos are oriented anterior towards the left, dorsal side upwards.

Krüppel, and Knirps (Nibu et al., 1998a; Nibu et al., 1998b; Strunk et al., 2001). To study the effectiveness of Knirps repression of individual *eve* regulatory elements, we assayed the expression of *eve-lacZ* reporter genes. Knirps is required for correct regulation of the *eve* stripe 3/7 and 4/6 enhancers, as demonstrated by the expression patterns of *lacZ* reporter genes in *kni* mutant embryos (Fujioka et al., 1999; Small et al., 1996). As previously observed (Keller et al., 2000) the posterior border of *eve* stripe 3 was not derepressed in a *CtBP* mutant, consistent with the CtBP-independent activity of Knirps on this enhancer (Fig. 1A,B). By contrast, Knirps repression of *eve* stripe 4/6 is compromised in a *CtBP* mutant background, indicating that the CtBP-independent repression activity of Knirps is insufficient to regulate this enhancer (Fig. 1C,D). Therefore, depending on which part of the *eve* gene is bound by the Knirps protein, its repression activity is either dependent or independent of the CtBP co-factor (Fig. 1E).

Ectopic expression of Knirps proteins in embryos

To determine whether the *eve* stripe 4/6 enhancer is intrinsically resistant to repression by the CtBP-independent activity of Knirps, or just less sensitive to this activity, we overexpressed full-length (1-429) FLAG epitope tagged Knirps or a truncated form of the protein that contains only the N-terminal, CtBP-independent repression activity (1-330) in embryos (Fig. 2A). As controls, proteins lacking the N-terminal DNA-binding domain were also overexpressed to test for specificity of repression. All proteins were expressed from a *hsp70* promoter construct introduced by germline transformation into *Drosophila*. *In situ* analysis showed a uniform distribution of *knirps* mRNA in embryos after heat

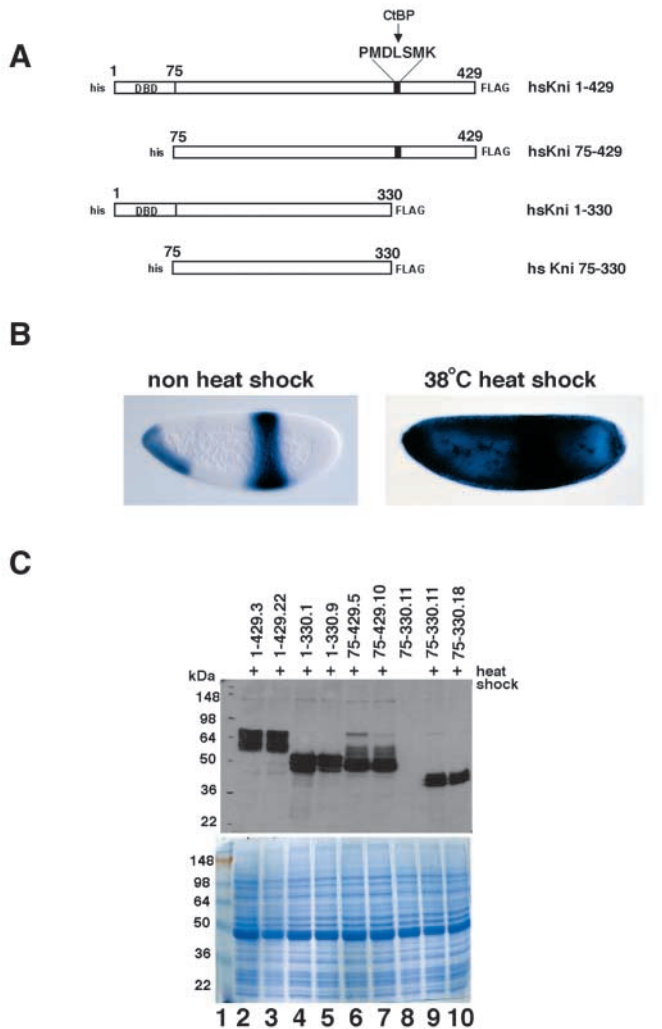


Fig. 2. Expression of full-length and CtBP-independent regions of the Knirps transcriptional repressor in transgenic *Drosophila*. (A) Structure of proteins expressed from *hsp70* promoter: 1-429, full-length Knirps protein; 75-429, non-DNA binding control protein; 1-330, CtBP-independent Knirps repression domain; 75-330, non-DNA binding control protein. (B) In situ analysis of expression of *knirps* mRNA produced from *hsp70-knirps* transgene before and after heatshock. (C) (Top) Proteins expressed from representative lines of the four constructs measured by western blot. M2 α -FLAG antibody was used to detect recombinant proteins. Lines shown in lanes 2 and 4 were used in subsequent experiments. Lane 8, non heat shock control. (Bottom) Coomassie blue stained gel illustrates equal loading.

shock, reaching levels comparable with the endogenous *knirps* gene (Fig. 2B). The different forms of the Knirps protein were expressed at similar levels after heat shock induction of the transgenes, with undetectable levels present before heat shock (Fig. 2C; data not shown). Heat-shock induction of full-length Knirps in the embryo was lethal (data not shown), as is expected for this regulatory factor whose expression usually exhibits tight temporal and spatial regulation.

Differential effects of Knirps protein on the even-skipped gene

The effect of misexpression of Knirps proteins was monitored

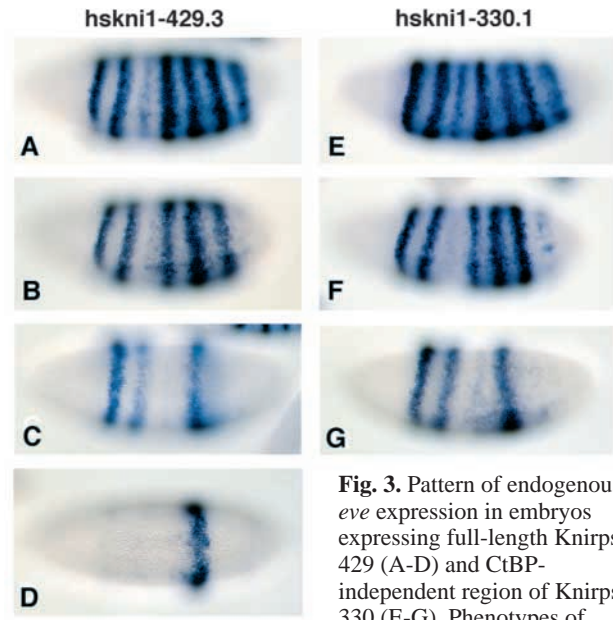


Fig. 3. Pattern of endogenous *eve* expression in embryos expressing full-length Knirps 1-429 (A-D) and CtBP-independent region of Knirps 1-330 (E-G). Phenotypes of

increasing severity are illustrated. Class I pattern (A,E) repression of stripe 3; Class II (B,F), repression of stripe 3 and 7; Class III (C,G) repression of stripe 3,4,6 and 7; Class IV, all stripes repressed except stripe 5. Endogenous *eve* patterns were visualized by in situ hybridization; embryos are oriented with anterior towards the left, dorsal side upwards. More severe phenotypes were produced by expression of full-length Knirps 1-429 than Knirps 1-330, as documented in Table 1.

by measuring endogenous *eve* expression by in situ hybridization. Heat shocks of variable duration were performed to test the effects of increasing levels of the Knirps protein (Fig. 3). Misexpression of the full-length Knirps protein, 1-429, resulted in repression of stripe 3 expression even after a short (5 minutes) heat shock pulse, with almost as frequent repression of stripe 7 (Fig. 3A,B; Table 1). Heat shocks of longer duration resulted in significant repression of stripe 4 and 6 (Fig. 3C). A 20 minutes heat shock also resulted in repression of stripe 1 and 2, leaving only stripe 5 expression (Fig. 3D). The selective repression of a subset of multiple enhancer elements is a striking demonstration of the way short-range repressors can repress individual regulatory elements without shutting down a gene entirely. No disruption of the *eve* pattern was noted in lines expressing Knirps proteins lacking amino acids 1-74, demonstrating that an intact DNA-binding domain is required for the effects observed (data not shown). The hierarchy of *eve* stripe 3-7 enhancer sensitivity to Knirps is consistent with the relative positions of these stripes within the Knirps protein gradient, whereby stripes 3 and 7 are sensitive to lower concentrations of Knirps than are 4 and 6 (Fujioka et al., 1999; Clyde et al., 2003).

Similar to the case with Knirps 1-429, stripes 3 and 7 were the first to be affected by misexpression of the Knirps 1-330 protein, which bears only the CtBP-independent repression activity. In this latter instance, however, the numbers of embryos showing repression was smaller (Fig. 3E,F; Table 1). Unexpectedly, overexpression of Knirps 1-330 also led to repression of *eve* stripes 4 and 6, indicating that this regulatory element is sensitive to the CtBP-independent activity of Knirps

Table 1. Percentage of transgenic embryos showing repression of *eve* stripes after heat shock

<i>eve</i> stripe	Heat shock duration					
	5 minutes		10 minutes		20 minutes	
	1-330	1-429	1-330	1-429	1-330	1-429
1	0	0	0	2.5	0	53
2	0	0	0	2.5	0	53
3	10	50	71	72	80	75
4	0	9	11	45	28	66
5	0	3	11	0	10	0
6	0	8	11	39	26	66
7	1.4	40	51	67	71	68
<i>n</i>	718	430	674	446	688	408

(Fig. 3G). Again, the relative number of affected embryos was smaller, indicative of a quantitative difference in repression between full-length Knirps and the CtBP-independent domain alone (Table 1). Unlike the case for Knirps 1-429, no embryos were observed that showed repression of stripes 1 and 2 by overexpression of Knirps 1-330. This result suggests that either these enhancers require still higher levels of Knirps 1-330 to be effectively repressed, or that there are qualitative as well as quantitative differences between the repressors. In a small percentage of cases, stripe 5 expression was also observed to be repressed in embryos misexpressing Knirps 1-330 and Knirps 1-429 (Table 1); however, a small percentage of nontransgenic controls also appear to show loss of stripe 5 expression (not shown), indicating that this phenotype may be a nonspecific heat shock effect.

Heat-shock experiments were also performed with no recovery time after induction to test whether Knirps might be repressing *eve* indirectly. We found that the order of repression of *eve* stripes was identical as in Fig. 3, although for each heat-shock regiment repression was not as complete (data not shown), possibly because the *eve* mRNA had less time to turn over. This result is consistent with a direct action of Knirps on *eve* enhancers.

The activity of Knirps 1-330, which contains the CtBP-independent domain of Knirps may reflect the previously identified CtBP-independent autonomous activity. Alternatively, some or all of the activity may be due to competition of the DNA-binding domain for activator binding sites. Therefore, we overexpressed the DNA-binding domain of Knirps (residues 1-105, containing the previously defined DNA binding domain and nuclear localization signal) (Gerwin et al., 1994) and determined its effect on *eve* expression pattern. As measured by quantitative western blotting, this protein was readily induced to levels almost as great as Knirps 1-330. Even at high expression levels, however, Knirps 1-105 was unable to perturb *eve* expression (data not shown), suggesting that Knirps represses *eve* by means other than direct competition for activator binding sites.

Differential effects of Knirps protein on minimal *eve*-skipped stripe enhancers

Next, we tested the effects of overexpression of full-length Knirps and the N-terminal, CtBP-independent domain of Knirps on minimal *eve* stripe 2-3, 3/7- and 4/6-*lacZ* reporters. We confirmed that the differential susceptibility to repression

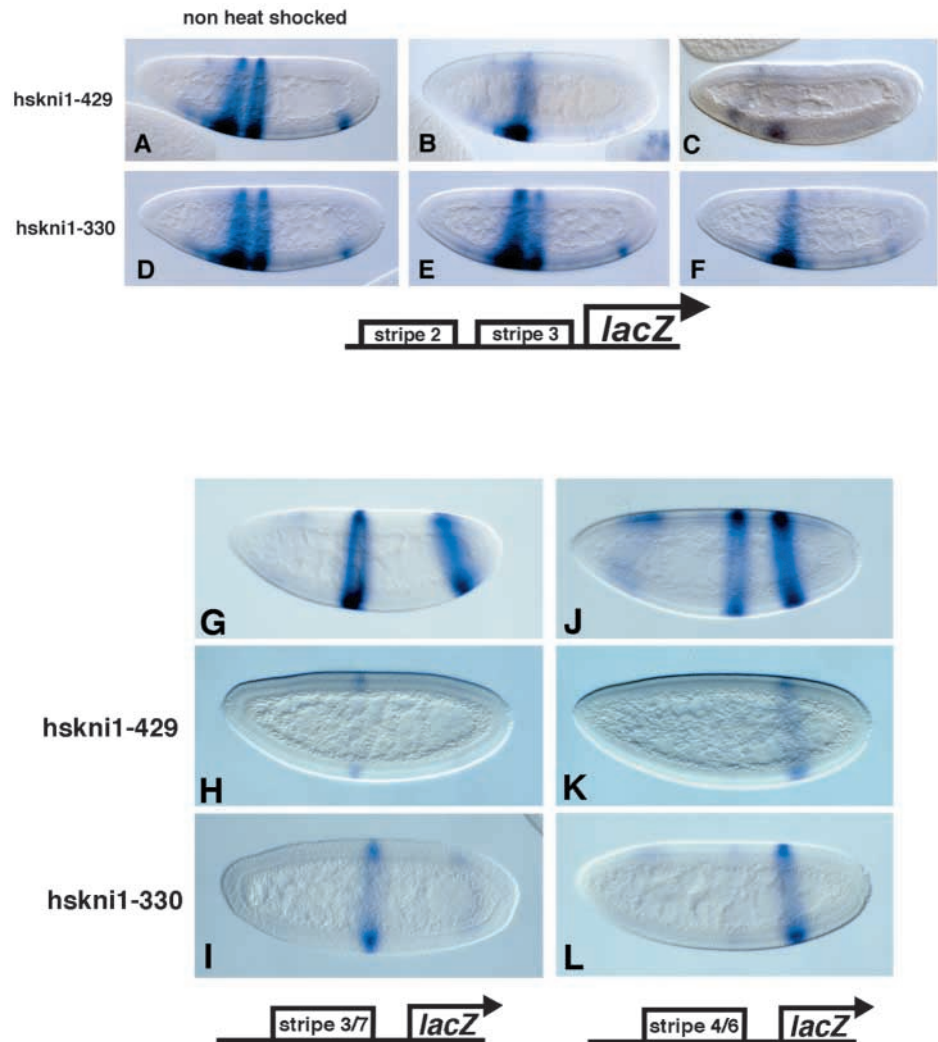
of *eve* stripe 3 compared to stripe 2 (Fig. 3) was directly associated with the previously defined regulatory regions using a *lacZ* reporter gene coupled to the minimal 500 bp stripe 2 and 3 enhancers. Both full-length Knirps 1-429 and Knirps 1-330 preferentially repressed stripe 3 over stripe 2 (Fig. 4B,F). The Knirps 1-429 protein was able to entirely repress stripe 3 in almost all embryos, and stripe 2 in a majority of embryos (Fig. 4A-C). By contrast, as noted with the endogenous *eve* gene, the CtBP-independent 1-330 repression domain was less potent than 1-429, resulting in more embryos with only partially repressed *eve* stripe 3, and fewer embryos in which stripe 2 was repressed (Fig. 4D-F). Embryos in which both stripes 2 and 3 were repressed were distinguishable from nontransgenic embryos by residual stripe 2 expression in ventral regions and an anterior stripe driven by vector sequences (Fig. 4C). The minimal stripe 2 enhancer is probably more sensitive to repression by Knirps 1-330 than the endogenous stripe 2 enhancer because it does not contain all sequences involved in stripe 2 regulation (M. Ludwig and M. Kreitman, personal communication). A previous study found that the minimal stripe 2 element was only slightly affected by Knirps overexpression (Kosman and Small, 1997), but here we are probably achieving higher levels of expression.

To further verify the relative activity of full-length Knirps versus the CtBP-independent activity of Knirps, we tested the effects of overexpression of Knirps proteins in embryos carrying *eve* stripe3/7- and 4/6-*lacZ* reporters (Fig. 4G-L). As observed on the endogenous *eve* gene, full-length Knirps was a more potent repressor than the CtBP-independent domain, causing complete repression of *eve* stripe 4 and 7 and almost complete repression of *eve* stripe 3 and 6 (Fig. 4H,K compare with 4G,J). A large decrease in the number of stained embryos also indicates that many *lacZ* reporter genes were completely repressed. Knirps 1-330 caused a similar repression pattern, but longer heat shocks were required to achieve comparable repression of the more sensitive *eve* stripe 4 and 7. After 30 minutes of heat shock, repression of *eve* stripe 3 and 6 was not as complete as that achieved by Knirps 1-429 after 15 minutes of heat shock (compare Fig. 4I with 4H, and compare 4L with 4K). Importantly, when expressed at high level, the CtBP-independent repression activity of Knirps was able to completely repress *eve* stripe 4, and partially repress stripe 6, confirming the results observed with the endogenous *eve* gene.

Higher specific activity of Knirps protein containing multiple repression activities

The lower activity of Knirps 1-330 protein relative to the full-length Knirps protein might be due to a greater potency of the protein containing two distinct repression activities, or it might merely reflect lower protein expression levels. To directly compare levels of ectopically expressed Knirps proteins, lysates from transgenic embryos were subject to western blot analysis, using the same heat shock regime as that used for the in situ analysis above (Fig. 5A). Equivalent amounts of total protein from whole embryo lysates were separated on SDS gels, transferred to membranes and probed with an antibody specific for the C-terminal FLAG epitope. Quantitation of the signals from the blots indicate that the weaker Knirps 1-330 repressor was actually expressed at approximately twofold higher levels than Knirps 1-429 at each time point tested (Fig. 5B). Therefore, the greater potency of the full-length Knirps is

Fig. 4. Differential repression of minimal *eve* stripe enhancers by Knirps1-429 and 1-330, demonstrates differential sensitivities to Knirps activity. Repression of *eve* stripe 2/3-*lacZ* reporter gene demonstrates differential sensitivities of *eve* stripe 2 versus stripe 3 enhancers to Knirps expression. Patterns of *lacZ* expression in embryos prior to heat shock (A,D) and after heat shock (B,C,E,F). After a 10-minute heat shock, the majority of embryos expressing Knirps 1-429 showed repression of *eve* stripe 3 (B). After a 30-minute heat shock, the majority of embryos showed repression of both stripe 2 and 3 (C). A significant percentage of embryos showed only partial repression of stripe 3 upon overexpression of Knirps 1-330 (E). The majority of embryos demonstrated a loss of stripe 3, but not stripe 2 after 30 minutes of heat shock (F). (G,J) Unperturbed reporter gene expression. Effects of overexpression of Knirps1-429 and 1-330 in embryos carrying the *eve* stripe 3/7 *lacZ* reporter (G-I) or *eve* stripe 4/6 *lacZ* reporter (J-L). Full-length Knirps was a more potent repressor, but Knirps 1-330 was capable of repressing the minimal *eve* stripe 4/6 element (see text for details). The patterns shown in H and K are representative of embryos heat shocked for 15 minutes, whereas the patterns shown in I and L are typical of embryos heat shocked for 30 minutes. Embryos are oriented with anterior towards the left, dorsal side upwards.



not just a function of greater expression or stability of this protein, but presumably reflects the greater activity of the combined repression domains.

Potency of Knirps 1-429 versus Knirps 1-330 in regulation of *hunchback*, *runt*, *hairy* and *fushi tarazu*

To compare the activities of full-length Knirps 1-429 with the Knirps CtBP-independent repression domain on other endogenous target genes, we examined the effects of overexpressing Knirps 1-429 or Knirps 1-330 on *hunchback*, *runt*, *hairy* and *fushi tarazu*. Previous studies demonstrated that the *hunchback* parasegment 4 stripe is very sensitive to low levels of Knirps (Kosman and Small, 1997), and we found that both the full-length Knirps protein as well as the CtBP-independent Knirps repressor strongly downregulated this stripe (Fig. 6A-C). Consistent with genetic information about *knirps*-mediated regulation of *runt* (Klingler and Gergen, 1993; Kosman and Small, 1997), misexpression of Knirps 1-429 had a drastic effect on *runt* expression, leading to repression of up to six of the *runt* stripes (Fig. 6F). Stripe 1 was repressed less frequently than stripes 2-4 and 6, consistent with an earlier report that indicated it was not affected by levels of Knirps sufficient to inhibit stripe 2-3 (Kosman and Small, 1997). Knirps 1-330 was much less effective in perturbing *runt*

expression, except for weakened *runt* stripe 3 expression (Fig. 6E).

The stripe elements 3, 4, 6 and 7 of *hairy* have been found to be affected by misexpression of Knirps protein or mutations in the *knirps* gene (Pankratz et al., 1990; Langeland et al., 1994; Kosman and Small, 1997) and binding sites for Knirps protein have been mapped on the *hairy* stripe 6 and 7 enhancer elements (Langeland et al., 1994; Hader et al., 1998). Expression of Knirps 1-429 caused a strong repression of *hairy* stripe 3, 4, and 7 expression, while expression of Knirps 1-330 had no such inhibitory effect (Fig. 6G-I). The *ftz* pair-rule gene is also under control of gap gene regulators, as well as primary pair-rule genes (Carroll and Scott, 1986; Yu and Pick, 1995). In Knirps 1-429-overexpressing embryos, the central stripes are fused, but overexpression of Knirps 1-330 had a much milder effect, with partial weakening of *ftz* stripes 2 and 3 (Fig. 6J-K). As discussed below, the effects of Knirps misexpression on *ftz* might well represent secondary effects mediated through upstream regulators, in particular *eve* and *hairy*. These effects on *eve* and other endogenous pair-rule genes support the observation that the CtBP-independent repression domain of Knirps is capable of mediating repression on the most sensitive target genes, but is quantitatively less potent than the full-length protein.

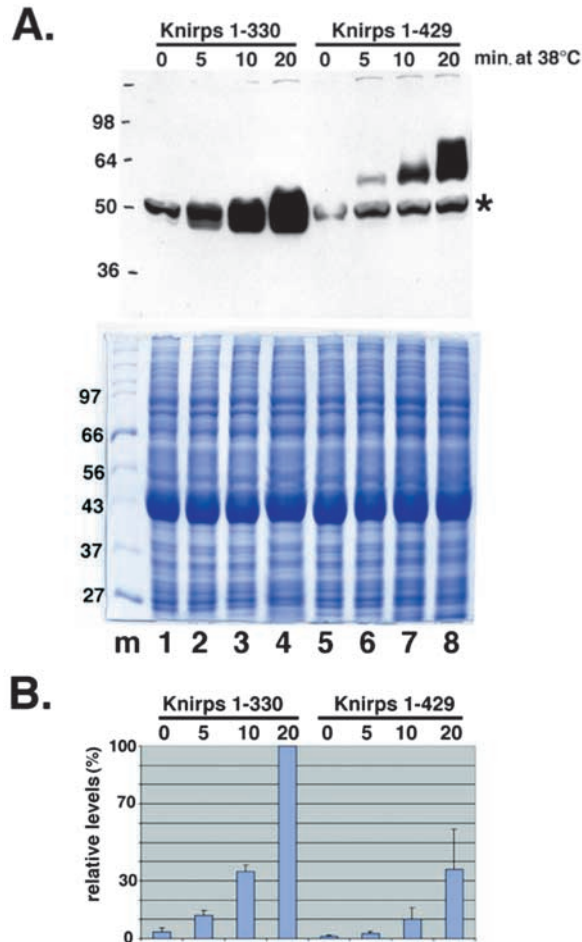


Fig. 5. Quantitation of proteins expressed from *hsp70-knirps* transgenes demonstrates that full-length Knirps 1-429 is less abundant than Knirps 1-330. (A) Western blot analysis of embryos subjected to the same heat-shock regimen (0, 5, 10 and 20 minutes) used for analysis shown in Fig. 3 and Table 1. Asterisk marks nonspecific cross-reacting protein that was also present in lysates from non-transgenic *Drosophila* (presence of nonspecific band appeared to vary with batch of antibody; data not shown). Because this nonspecific band co-migrates with the 1-330 protein, the signal from the nonspecific protein (averaged from lanes 5-8) was subtracted from each of the values in lanes 1-4 to determine levels of 1-330 protein. Below, Coomassie stained gel showing equal loading. (B) Quantitation of western blots demonstrates an approximately twofold higher level of Knirps 1-330 protein at each time point than Knirps 1-429, demonstrating that the higher activity of the 1-429 is not due to higher levels of this protein. Standard deviations are shown in B for four separate gels and quantitations of the heat-shock experiment shown in A.

Discussion

Multiple repression activities – quantitative contributions to reaching repression thresholds

Just as transcriptional activators are known to possess multiple activities to stimulate transcription, a growing number of transcriptional repressors have been found to have multiple activities that are dependent on distinct co-factors. In *Drosophila*, the Brinker repressor can interact with both the CtBP and Groucho co-repressors to mediate repression of Dpp-

regulated genes (Hasson et al., 2001; Zhang et al., 2001). For this repressor, distinct co-factors are required at different promoters. The *tolloid* gene is repressed by Brinker in the blastoderm embryo in a Groucho-dependent manner, while either CtBP or Groucho are sufficient to mediate *brk* autoinhibition. Interestingly, neither co-factor appears to be required for repression of *omb* and *sal*, suggesting a third pathway for repression, possibly direct competition (Hasson et al., 2001; Rushlow et al., 2001). Similarly, the Even-skipped, Runt and Engrailed proteins repress through Groucho-dependent and -independent pathways, again showing gene-specificity. In none of these cases is it known whether the requirement for specific repression activities at endogenous enhancers reflects qualitatively distinct mechanisms, or alternatively, distinct quantitative requirements for repression levels (Kobayashi et al., 2001; Fujioka et al., 2002; Aronson et al., 1997). Analysis of the Groucho-dependent and -independent activities of Eve protein on *lacZ* reporters, suggest that in combination these two domains do provide quantitatively superior level of repression (Fujioka et al., 2002).

Previous studies of Krüppel, Giant and Knirps have indicated that CtBP dependence or independence of their repression activities varies according to the specific cis regulatory element involved, suggesting that there are particular enhancer architectures that necessitate CtBP activity. The clearest example of enhancer specific requirements for CtBP is shown in the case of *eve* enhancers. In nuclei situated between *eve* stripes 4 and 6, the stripe 4/6 and 3/7 enhancers are both repressed by Knirps in the same nuclei, yet this repression is independent of CtBP on the 3/7 element and dependent on CtBP on the 4/6 element (Fig. 1). By expressing increasing levels of the CtBP-independent form of Knirps, the requirement for CtBP is obviated (Fig. 3). These results suggest that distinct requirements for the CtBP co-factor at different genes or cis regulatory elements can be based on the quantitative levels of repression activity. Indeed, the combination of the CtBP-dependent and CtBP-independent activities make a particularly powerful repressor, as judged by comparison of repression activities of Knirps 1-429 versus Knirps 1-330 on *eve* (Fig. 3 and Table 1) and other pair-rule genes (Fig. 6). These results suggest that both repression domains can be simultaneously engaged on a given cis regulatory element, rather than a particular repression activity being selectively engaged at particular enhancers. Consistent with this picture, when they are assayed separately as Gal4 fusion proteins in embryos, both CtBP-dependent and CtBP-independent repression domains of Knirps have equal, modestly effective repression activities. By contrast, a Gal4 protein containing both domains is much more effective at repressing a strongly activated promoter (Sutrias-Grau and Arnosti, 2004).

A model that explains the quantitative contribution of the CtBP co-repressor to Knirps repression activity is shown in Fig. 7. At the top, two lines depict the levels of repression activity generated by increasing Knirps concentrations, the top line illustrating the levels of repression achieved by the Knirps protein complexed with CtBP. Thresholds of repression required by the *eve* stripe 3/7 and 4/6 enhancers are depicted by horizontal lines. Below, relative levels of Knirps are shown with respect to position (egg length) in the embryo. At a

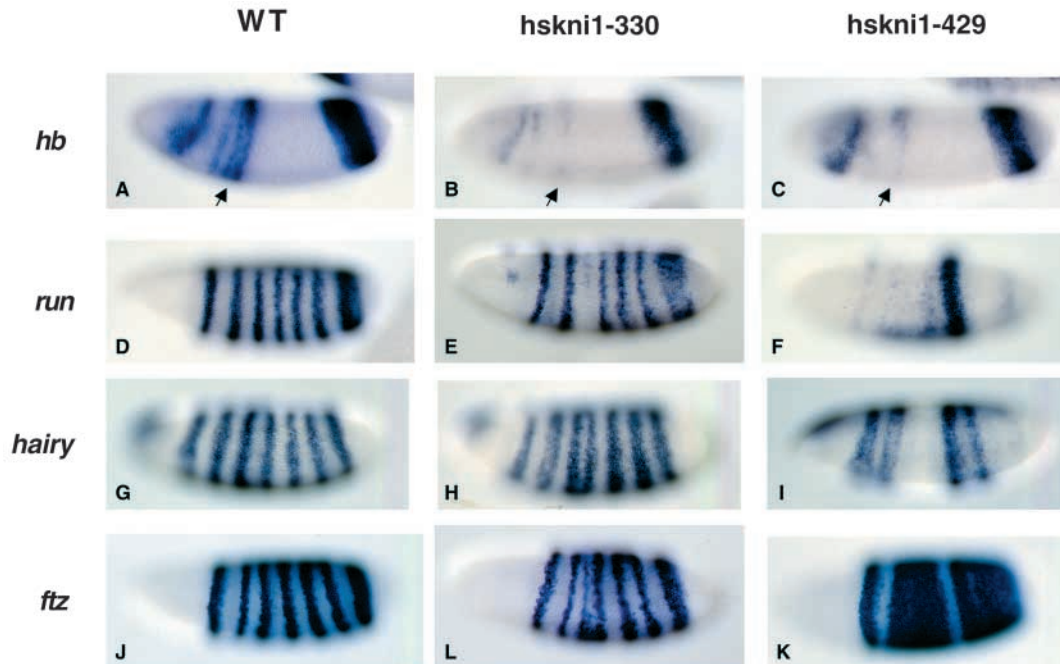


Fig. 6. Effect of expression of Knrtps proteins on *hunchback* (*hb*), *runt*, *hairy* and *fushi tarazu* (*ftz*) demonstrates differential activity of Knrtps 1-429 versus Knrtps 1-330 on all but the most sensitive target genes. *hb*, a sensitive target of Knrtps (A-C), showed similar repression by both Knrtps 1-330 and Knrtps 1-429 of the parasegment four zygotic expression pattern (arrow) after 30 minutes of heat shock. On other genes, Knrtps 1-330 was much less potent than Knrtps 1-429. Knrtps 1-330 repressed only stripe 3 of *runt* (E), while Knrtps 1-429 repressed all except *runt* stripe 5 (F) after 15 minutes of heat shock (similar patterns were observed at 30 minutes). Similarly, 1-330 had a modest effect only on *hairy* stripes 3/4 (H) and *ftz* stripe 3 (L), while Knrtps 1-429 completely repressed *hairy* 3,4 7 (I) and extensively disrupted *ftz* expression (K) after 5 and 10 minutes of heat shock respectively (similar patterns were noted at 15 and 30 minutes). Transcripts of endogenous Knrtps target genes were visualized by in situ hybridization. All embryos are oriented with anterior towards the left, dorsal upwards.

relatively low level of Knrtps protein activity, the *eve* 3/7 enhancer is repressed, and this level of repression activity is achieved at similar levels of Knrtps, regardless of whether or not CtBP contributes to repression. Thus, in the absence of CtBP, the positions at which the stripe 3/7 boundaries form shift very little. The much higher level of repression required by the stripe 4/6 element is achieved only near the peak of Knrtps protein levels. If CtBP is not complexed with Knrtps, the intercept shifts sharply to the right, to a level of Knrtps not normally present in the embryo. The sufficient level of repression in the absence of CtBP activity or protein is only

achieved under conditions where Knrtps is overexpressed, as in Fig. 3.

Setting thresholds

The threshold model explains how the contributions of separate repression activities act in a quantitative fashion to meet given thresholds, but what is the basis for distinct repression thresholds? There are at least two variables involved in dictating a threshold, namely, regulatory protein levels and the nature (number, affinity, and placement) of the relevant binding sites within a regulatory element. Varying intranuclear activator levels can influence repression thresholds, as suggested by regulation of the *Krüppel* gene: Giant requires CtBP for repression of this gene only in nuclei containing peak levels of the Bicoid activator (Strunk et al., 2001). Varying

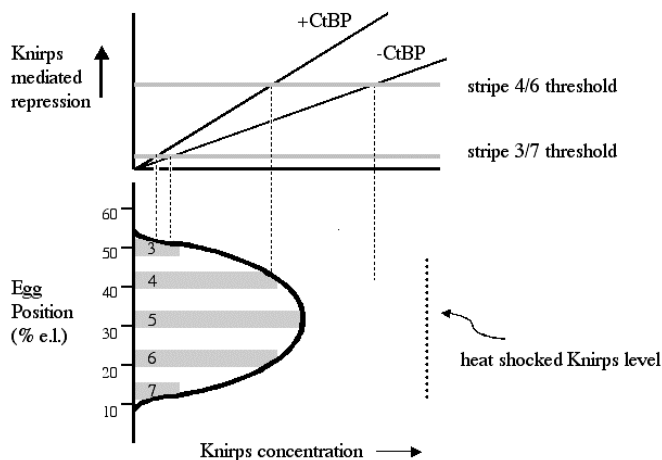


Fig. 7. Quantitative model for contribution of CtBP activity to repression by Knrtps. Protein levels of Knrtps (horizontal axis) are plotted against differential levels of repressor activity (vertical axis at top). With CtBP, Knrtps repression levels increase more sharply with increasing protein levels, allowing the activity to cross critical thresholds at lower protein levels. The position of the Knrtps protein levels in the embryo (lower part of figure indicated by % egg length) then dictates where appropriate stripe boundaries will form (vertical broken lines). This model predicts that, owing to the inherently high threshold of the *eve* stripe 4/6 enhancer, loss of CtBP activity will move the intercept off of the range of physiological Knrtps concentrations, while having little effect on the stripe 3/7 position.

intranuclear repressor levels will dictate how easily those thresholds are met with or without multiple repression activities. Gap genes, including *knirps*, generate protein gradients that have properties of morphogens, i.e. they trigger differential responses at different threshold levels (Kosman and Small, 1997). The stripe 4/6 and 3/7 modular enhancers of the *even-skipped* gene are designed to respond to different levels of Knirps protein, allowing the embryo to establish multiple stripe boundaries with a single protein gradient. The short-range activity of Knirps allows the two enhancers to act independently, so that activators bound to the stripe 4/6 enhancer activate the gene in nuclei where the levels of Knirps are already sufficiently high to inhibit the stripe 3/7 enhancer.

Binding site affinity and number have been clearly established to influence threshold responses in the case of transcriptional activators, such as Bicoid and Dorsal (Jiang and Levine, 1993; Szymanski and Levine, 1995; Struhl et al., 1989). A similar effect is likely to be true for repressors. Sequence analysis of the *eve* gene indicates that there are more high-affinity Knirps binding sites within the *eve* stripe 3/7 element than in the 4/6 enhancer, consistent with relative sensitivities of these elements that we determined experimentally (Fig. 3) (Papatsenko et al., 2002; Berman et al., 2002). Removal of some of the Knirps binding sites in the *eve* stripe 3/7 enhancer reduces the sensitivity of this element to the Knirps gradient (Clyde et al., 2003). However, the number of predicted high-affinity binding sites alone is not sufficient information to predict relative sensitivity to Knirps. If it were, one would expect the *eve* stripe 2 enhancer, with three predicted Knirps sites, to be more sensitive to Knirps than *eve* stripe 4/6, with only a single site, yet the reverse is true (Berman et al., 2002) (Fig. 3). This lack of correlation might be partly attributable to errors in prediction of binding sites; however, additional factors, such as affinity of binding sites and relative placement with respect to other proteins, are likely to make the decisive difference in determining enhancer sensitivity to Knirps. In the case of the Giant repressor, small shifts in the placement of the binding site allows detection of less than two-fold differences in repressor concentrations, a 'gene tuning' mechanism that seems to have been invoked during internal evolution of the *eve* stripe 2 enhancer (Ludwig et al., 2000; Hewitt et al., 1999). The stoichiometry of activators to repressors has also been suggested to be a crucial factor in determining repression levels, and direct tests indicate that Giant and Knirps respond sensitively to differences in activator binding site number and affinity on defined regulatory elements (Hader et al., 1998; Kulkarni and Arnosti, 2003).

eve stripe 1 lies just posteriorly to the weak anterior domain of *knirps* expression, suggesting a possible role of Knirps in regulating that element, but it is not clear whether the relative sensitivity of other *eve* stripe enhancers normally active outside of the main posterior domain of Knirps expression is of physiological significance. The *eve* stripe 2 pattern lies outside of the normal area of Knirps expression, and is only repressed at highest levels of Knirps (Table 1), suggesting that repression might be through cryptic Knirps sites in the element (Berman et al., 2002). The robust activity of the *eve* stripe 5 enhancer even under conditions of high levels of Knirps misexpression underlines that this regulatory element has been designed to function in nuclei containing peak levels of Knirps protein (Fig. 3). Similarly, *run*t stripe 5 also resists peak levels of

ectopic Knirps (Fig. 6). Both of these regulatory elements have few or no predicted Knirps-binding sites (Berman et al., 2002). These elements would provide a useful platform to test the number and placement of novel Knirps binding sites required to bring the element under the control of this repressor.

Knirps regulation of *hb*, *run*t, *h* and *ftz*

The effects of Knirps misexpression on other endogenous pair rule genes reinforce the lessons learned from *eve*, regarding the relative potency of the Knirps repression domains and the sensitivity of different enhancers. Both the CtBP-independent region of Knirps as well as the intact protein were capable of repressing the *hunchback* parasegment 4 stripe, a highly sensitive target of Knirps (Kosman and Small, 1997). However, *hairy*, *run*t and *ftz*, which have been previously noted to have a higher threshold to Knirps repression, were noticeably less affected by Knirps 1-330 compared with Knirps 1-429 (Fig. 6). Thus, it is likely that CtBP activity contributes quantitatively to repression of other Knirps target genes in addition to *eve*.

Repression of central *run*t stripes is consistent with previous findings of direct repression by Knirps and the greater sensitivity of stripes 2-4 relative to stripe 1 (Kosman and Small, 1997). We observed a greater effect of ectopic expression of Knirps on *hairy* than noted in previous experiments, probably on account of higher levels of expression. Knirps expressed under the control of an *eve* stripe 2 enhancer was previously found to have little effect on anterior *hairy* expression, except for a delay in stripe 3/4 separation (Kosman and Small, 1997). Heat shock expression of full-length Knirps 1-429, by contrast, resulted in strong repression of *hairy* stripes 3, 4 and 7 (Fig. 6I). The *hairy* stripe 3, 4 and 7 enhancers are predicted to contain Knirps-binding sites, in contrast to the unexpressed stripe 1 and 5 enhancers (Langeland et al., 1994; La Rosee et al., 1997; Berman et al., 2002). The weaker Knirps 1-330 protein had an effect similar to that of full-length Knirps expressed from an *eve* stripe 2 expression construct, i.e. a delay of stripe 3/4 separation (Fig. 6H). Interestingly, *knirps* is important for activation of *hairy* stripe 6, and the protein can bind to the stripe 6 enhancer directly in vitro (Riddihough and Ish-Horowicz, 1991; Langeland et al., 1994). We see no evidence of activation upon overexpression, however, suggesting that such activation might be indirect.

The derepression of *ftz* we observe between stripes 2-4 and 6-7 is likely to be due to indirect effects of repression of *hairy* and *eve* expression; both of these genes are thought to repress *ftz* directly (Jiménez et al., 1996; Manoukian and Krause, 1992). By contrast, previous work involving lower levels of anteriorly expressed Knirps observed only weakened *ftz* stripes 2 and 3, rather than stripe fusion. This lower level of Knirps had a much less profound effect on upstream regulators *hairy* and *eve*, suggesting that Knirps might be a direct gap gene input to this pair-rule gene, as suggested by earlier studies (Yu and Pick, 1995; Kosman and Small, 1997).

Repression mechanisms

Our study suggests that the multiple repression activities of Knirps can be simultaneously mobilized to provide quantitatively correct levels of repression activity, and that the design of *cis* regulatory elements can elicit CtBP dependence. CtBP-independent activity can in some cases be directly attributed to direct competition with activator for DNA binding

(Hoch et al., 1992; Nibu et al., 2003); however, the CtBP-independent activity of Knirps can repress activators on elements where sites are not overlapping (Keller et al., 2000; Ryu and Arnosti, 2003), and overexpression of the DNA-binding domain of Knirps (Knirps1-105) is insufficient to mediate repression of endogenous *eve* enhancers (data not shown). Cell culture and transgenic embryo assays indicate that both CtBP-dependent and independent repression activities of Knirps have very similar characteristics with respect to activator specificity, distance dependence and overall potency, thus the targets and molecular mechanisms might well be similar in each case (Ryu and Arnosti, 2003; Sutrias-Grau and Arnosti, 2004). Key to a deeper understanding of the molecular circuitry controlled by short-range repressors such as Knirps will be biochemical knowledge of the mechanisms of repression employed on these developmentally regulated enhancers.

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