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The gap protein knirps mediates both quenching and direct repression in the *Drosophila* embryo

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Transcriptional repression is essential for establishing localized patterns of gene expression during *Drosophila* embryogenesis. Several mechanisms of repression have been proposed, including competition, quenching and direct repression of the transcription complex. Previous studies suggest that the knirps orphan receptor (kni) may repress transcription via competition, and exclude the binding of the bicoid (bcd) activator to an overlapping site in a target promoter. Here we present evidence that kni can quench, or locally inhibit, upstream activators within a heterologous enhancer in transgenic embryos. The range of kni repression is ~50–100 bp, so that neighboring enhancers in a modular promoter are free to interact with the transcription complex (enhancer autonomy). However, kni can also repress the transcription complex when bound in promoter-proximal regions. In this position, kni functions as a dominant repressor and blocks multiple enhancers in a modular promoter. Our studies suggest that short-range repression represents a flexible form of gene regulation, exhibiting enhancer- or promoter-specific effects depending on the location of repressor binding sites.

Keywords: *Drosophila* embryo/knirps/nuclear receptor/repression/transcription

Introduction

Complex patterns of gene expression in the early *Drosophila* embryo are regulated by spatially localized transcriptional repressors. The *Drosophila* knirps (kni) protein, a member of the nuclear receptor family of transcription factors, is expressed in abdominal regions of pre-cellular embryos and anterior regions of the presumptive germ band (Rothe *et al.*, 1989). It plays an essential role in the segmentation process, both by refining the expression patterns of gap genes and by establishing pair-rule stripes of gene expression (Nüsslein-Volhard *et al.*, 1987; Pankratz *et al.*, 1989; Small *et al.*, 1996). kni is a repressor of the *even-skipped* (*eve*) stripe 3 pattern; it binds to multiple sites in the stripe 3 enhancer element and functions as a repressor to establish the posterior border of expression (Small *et al.*, 1996). Recent studies suggest that the stripe 3 enhancer is activated by d-STAT, a *Drosophila* homolog of mammalian STAT transcription factors (Yan *et al.*, 1996). It is conceivable that kni represses this element via a competition mechanism, since one of the kni binding sites maps within 10 bp of a d-STAT activator site. Indeed, a competition mechanism for kni action was suggested initially by studies on the regulation of the gap gene *Krüppel* (*Kr*). The *Kr* promoter contains closely linked binding sites for the bcd activator and kni repressor, and it was proposed that kni might specify the posterior border of the *Kr* expression pattern by blocking the binding of bcd to an overlapping site (Hoch *et al.*, 1992).

In the current study, we seek to determine the mechanism of kni repression. This information may be of general relevance, because it might provide an understanding of the repression activity of other members of the nuclear receptor superfamily, including the thyroid hormone receptor, the retinoic acid receptor and the glucocorticoid receptor (Diamond *et al.*, 1990; Chen and Evans, 1995; Hörlein *et al.*, 1995; Kurokawa *et al.*, 1995). We have determined that kni can mediate two forms of transcriptional repression in the embryo: 'quenching', or local inhibition of adjacent activators, and direct repression of a basal promoter. When bound within an enhancer, kni can work over distances of 50–100 bp to quench activators in the *rhomboid* (*rho*) lateral stripe enhancer. kni can also directly repress basal promoter elements when bound to promoter-proximal sequences. This latter form of repression is dominant and results in the inhibition of multiple enhancers. A gal4–kni fusion protein lacking the kni DNA binding domain can also mediate repression in transgenic embryos, indicating that repression activity is independent of this zinc finger domain. We discuss these findings in the context of different models of transcriptional repression, and propose that the key distinction among repressors is whether they function over short or long distances.

Results

Enhancer autonomy

Previous studies have suggested that short-range repression permits enhancer autonomy within complex promoters. For example, the stripe 2 and stripe 3 enhancers in the *eve* gene function independently when separated by a short 'spacer' DNA. The removal of this spacer causes repressors on the stripe 2 enhancer to interfere with stripe 3 activity (Small *et al.*, 1993).

To address the issues of kni repression and enhancer autonomy, we analyzed the expression of a fusion gene that contains the 500 bp *eve* stripe 3 enhancer placed 5′ of the 300 bp *rho* lateral stripe enhancer (*rho* NEE) (Figure 1B). A fully additive pattern of expression is observed; the expression pattern directed by each enhancer is not influenced by the other. This result suggests that repressors bound to one of the enhancers do not affect activators in the neighboring enhancer. For example, the lateral expression directed by the *rho* NEE is undiminished in

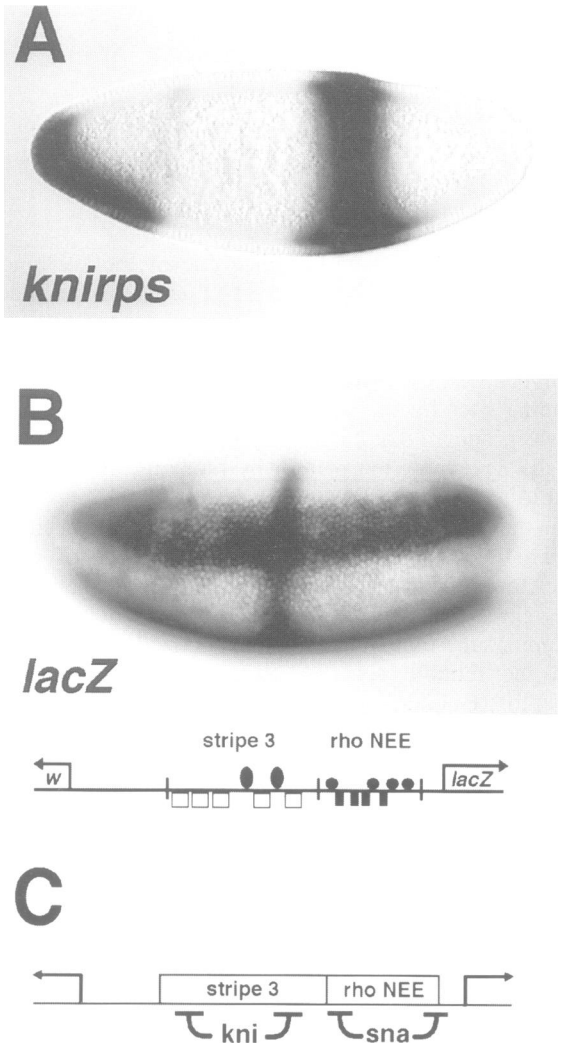

Fig. 1. The kni repressor permits enhancer autonomy. Cellularizing transgenic embryos are oriented with anterior to the left and dorsal up. Embryos were stained after hybridization with a digoxigenin-labeled *lacZ* or *knirps* antisense RNA probe. (**A**) Expression pattern of the endogenous *kni* gene. (**B**) The 500 bp *eve* stripe 3 enhancer (transverse stripe) was placed upstream of a 330 bp *rho* NEE (lateral stripes). The enhancers were placed between two divergently transcribed promoters, the *white* gene to the left and *lacZ* to the right (see diagrams below embryos). An additive pattern of expression of *lacZ* is observed, suggesting that kni repressor bound within the stripe 3 enhancer does not interfere with *rho* NEE expression. Similar results were observed for *white* expression (data not shown). In the promoter diagram shown below the embryo, dorsal activator sites are indicated by black circles, d-STAT activators by black ovals, kni repressor sites by white squares and sna repressor sites by black rectangles. (**C**) Local repression by kni and sna proteins permits enhancer autonomy. Heavy black lines indicate limited range of repression activity by kni and sna within each enhancer.

the portion of the embryo posterior to stripe 3, where the kni protein is expressed at high levels (Figure 1A and B). The nearest kni binding site in the stripe 3 enhancer is 240 bp from the 5′-most dorsal (dl) activator protein bound within the *rho* NEE, which suggests that kni is unable to act over this range to mediate repression. Similarly, snail (sna) repressor bound to the *rho* NEE in ventral regions of the embryo does not abolish the ventral expression of stripe 3. The nearest sna binding site in the *rho* NEE is 260 bp from the 3′-most d-STAT activator site in the stripe 3 enhancer, which is beyond the range of sna repression (50–100 bp) as defined in previous experiments (Gray *et al.*, 1994). Thus, both kni and sna proteins confer enhancer autonomy by acting in a local fashion (Figure 1C).

kni can locally quench heterologous activators

To define the distance requirements for kni repression and to determine if kni is capable of repressing heterologous activators, we introduced kni binding sites into a well-characterized enhancer which is not normally subject to kni repression. A *rho* NEE that lacks sna repressor sites (*rho* NEE Δ sna) directs equally intense staining in ventral and lateral regions (e.g. Figure 2D). The *rho* NEE Δ sna was positioned between two divergently transcribed promoters, *white* and *lacZ*, which can be assayed independently by *in situ* hybridization (see diagrams below the panels in Figure 2). The embryo shown in Figure 2A was hybridized with an antisense *lacZ* RNA probe, in order to monitor the expression of the 'rightward' *lacZ* gene. The insertion of two kni binding sites causes the *rho* NEE Δ sna to be repressed in the presumptive abdomen (arrow, Figure 2A), where kni protein is present. One kni binding site is located 50 bp 5′ of the distal dl activator site, while the other is located 50 bp 3′ of the proximal dl site. Figure 2B shows the expression of the leftward *white* gene; there is a similar reduction of the staining pattern in abdominal regions. These experiments suggest that both promoters are repressed by kni.

kni is expressed in a virtually identical pattern to a related gene, *knirps-related* (*knrl*) (Oro *et al.*, 1988). Evidence that kni mediates the observed repression was obtained by assaying the transgene shown in Figure 2A and B in a *kni*$^{-}$ mutant background (Figure 2D). There is a complete loss of the abdominal gap, and the staining pattern is now identical to the one generated by the *rho* NEE Δ sna lacking kni binding sites.

There is a substantial loss of repression activity when the two kni sites are moved further from the nearest dl activators (Figure 2C; compare with A). In this experiment, one of the kni sites maps 150 bp 5′ of the distal dl activator site, while the other kni site maps 120 bp from the proximal dl site (see diagram below Figure 2C). kni activity is barely detectable in this configuration, with only a slight reduction in the staining pattern in the presumptive abdomen. A similar pattern is observed with the *white* promoter (not shown). These results suggest that kni represses the *rho* NEE through a short-range quenching mechanism. The effective range of action appears comparable with that observed for sna (~50–75 bp). However, in both experiments (50/50 bp spacing and 150/120 bp spacing), kni repressor sites map far from the *white* and *lacZ* transcription start sites (no closer than 440 and 140 bp, respectively). The next series of experiments examine the possibility that kni can also mediate direct repression of a basal promoter when bound near the initiation site.

kni can function as a dominant repressor

The *rho* NEE Δ sna activates both the *white* and *lacZ* reporter genes when placed 3′ of the *lacZ* transcription unit. Insertion of tandem kni binding sites 55 bp 5′ of the *lacZ* transcription start site represses the staining pattern

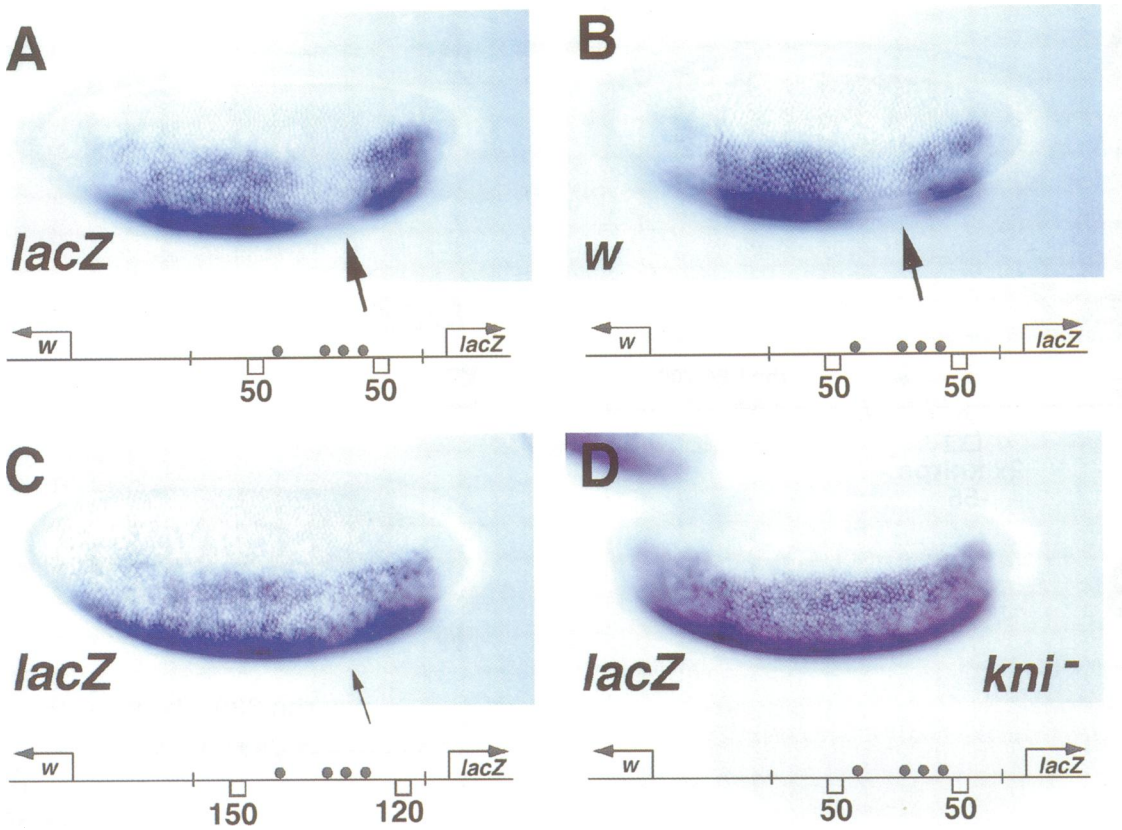

Fig. 2. kni can quench a heterologous enhancer over short distances. Transgenic embryos are oriented as in Figure 1. The leftward *white* (*w*) gene and rightward *lacZ* gene were visualized independently with separate digoxigenin-labeled antisense RNA probes. (**A**) *lacZ* staining pattern directed by a modified 700 bp *rho* NEE containing two synthetic kni binding sites located ~50 bp from the nearest dl activator sites (see diagrams below the embryos). Strong repression (arrow) is observed in posterior regions containing kni protein. In the diagrams, the black circles represent dl activator sites in the NEE, while the synthetic kni sites are depicted by white squares. The four native sna repressor sites were eliminated. (**B**) Same as (A), except that staining was done with the *white* probe. Again, repression is observed in the presumptive abdomen (arrow). This embryo is just slightly older than the one in (A), and repression is observed both in abdominal and anterior regions. This latter repression is probably due to a second area of kni expression that appears during the late phases of cellularization. (**C**) *lacZ* staining of a modified *rho* NEE containing kni binding sites located 150 bp 5′ and 120 bp 3′ of the nearest dl activator sites. This increased spacing nearly abolishes kni-mediated repression, although there may be a slight attenuation of the pattern (arrow). A similar pattern was observed for *white* expression (data not shown). (**D**) Same as (A) and (B), except that the fusion gene was crossed into a *kni*$^-$ embryo. There is no longer any detectable repression in the presumptive abdomen.

in the presumptive abdomen (Figure 3A). This repression is comparable with that observed when the kni sites are positioned near the upstream dorsal activator sites within the *rho* NEE (see Figure 2A). As in the case of quenching, direct repression of the basal transcription complex appears to occur over short distances. Repression is lost when the kni binding sites are positioned 130 bp 5′ of the *lacZ* transcription start site (Figure 3B). These results suggest a similar range for the inhibition of upstream activators and the transcription complex (~50–100 bp).

To determine whether direct repression (Figure 3) results in the dominant inhibition of multiple enhancers, we combined enhancers which direct transcription in different regions of the embryo. A 260 bp enhancer from the *twist* (*twi*) promoter region (PE) directs expression in the ventral-most 12–14 cells. A 330 bp NEE, containing four native sna repressor sites, was placed upstream of two tandem copies of the *twi* PE (2×PE). An additive pattern of expression is observed, consisting of lateral stripes in the neuroectoderm and a swath of staining in the ventral mesoderm (Figure 4B). Two kni binding sites 55 bp 5′ of the *lacZ* transcription start site cause repression of both enhancers in the presumptive abdomen (Figure 4A). This result suggests that kni can function as a dominant repressor, and block multiple enhancers in a modular promoter.

Although kni blocks both enhancers from activating the rightward *lacZ* gene (Figure 4A), the expression of the leftward *white* gene is unaffected (Figure 4B). In the latter case, both enhancers are uniformly active along the length of the embryo, including abdominal regions. Thus, the expression of the two genes is uncoupled when kni repressor sites are located near one of the basal promoters.

The kni repression domain is separable from the DNA binding domain

To determine whether the DNA binding domain of kni is necessary for repression, we analyzed the activities of a gal4–kni fusion protein in transgenic embryos. The kni protein is composed of 429 amino acid residues; the first 74 amino acids contain the two zinc fingers which mediate DNA binding (Nauber, 1988). The coding sequence encompassing codons 75–429 was fused to the first 93 codons of the yeast gal4 DNA binding domain. This gal4–

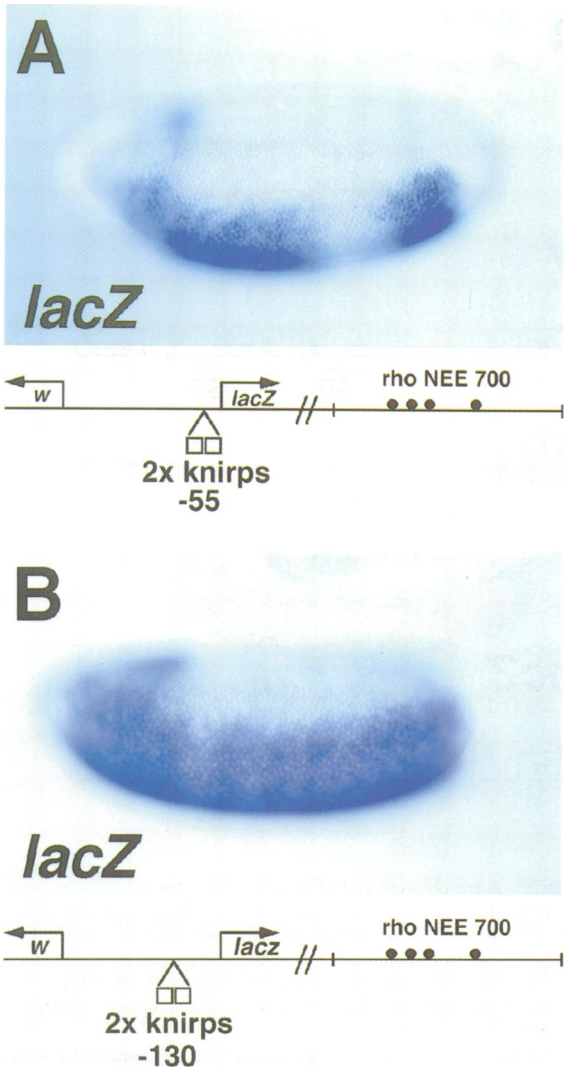

Fig. 3. kni can directly repress the basal promoter. Transgenic embryos are oriented as in previous figures. A modified 700 bp *rho* NEE which lacks sna repressor sites was placed 4.5 kb 3′ of the *lacZ* promoter. (**A**) *lacZ* staining of a pre-cellular embryo containing two kni repressor sites located 55 bp upstream of the transcription start site (see diagram below embryo). Efficient repression is observed in the presumptive abdomen. In the diagrams, dl activator sites in the *rho* NEE are indicated by black circles, while kni sites are depicted by white squares. (**B**) Same as (A), except that the kni sites were placed 130 bp 5′ of the start site. No repression of the pattern is observed.

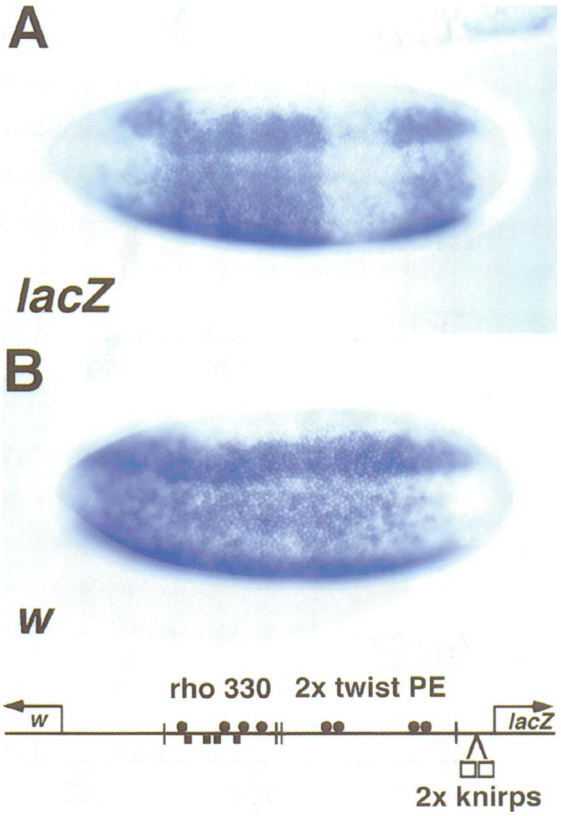

Fig. 4. kni can function as a dominant repressor to block multiple enhancers. Transgenic embryos are oriented to show ventrolateral views. Fusion genes contain the 330 bp *rho* NEE placed upstream of the 520 bp 2×PE mesoderm enhancer from the *twi* promoter region. kni repressor sites were placed 55 bp 5′ of the *lacZ* transcription start site (see diagram below embryos; dl activator sites are indicated by black circles, the black squares depict sna repressor sites in the *rho* NEE, while the white squares show the kni repressor sites). (**A**) *lacZ* staining pattern. Both enhancers are repressed in the presumptive abdomen. The *rho* NEE lateral stripes show a gap in the pattern where there are high levels of kni protein. In addition, the 2×PE mesoderm enhancer is also repressed in this region. Repression is also evident in response to the anterior domain of the kni pattern. (**B**) Same as (A), except that expression of the leftward *white* gene is being monitored. Neither enhancer is repressed by kni. Continuous staining is observed along the anteroposterior axis in both the presumptive mesoderm (2×PE pattern) and lateral neuroectoderm (*rho* NEE).

kni fusion protein was expressed in the ventral half of early embryos using a promoter element (2×PEe-Et; Jiang and Levine, 1993) derived from the *twi* gene.

A reporter gene including the *eve* stripe 2 enhancer was used to monitor the activities of the gal4–kni fusion protein. Two gal4 binding sites (UAS) were placed between the stripe 2 enhancer and the TATA sequence of the *lacZ* fusion gene. This gene is expressed uniformly in both dorsal and ventral regions (Figure 5A). However, when it is expressed in embryos containing the gal4–kni fusion protein, a very different pattern of expression is observed (Figure 5B). The fusion protein mediates efficient repression in ventral regions, thereby restricting the stripe to dorsal and lateral regions. Other gal4 chimeric proteins such as gal4–Sp1 and gal4–bcd do not repress this gene, indicating that the repression activity is mediated by amino acid residues 75–429 of the kni protein (data not shown). The UAS sites map close to the critical b1 bicoid activator site in the stripe 2 enhancer as well as the TATA box, so it is possible that the gal4–kni fusion protein mediates repression both by local quenching of upstream activators and by direct inhibition of the transcription complex.

Discussion

While previous studies have suggested that kni may regulate gene expression through a competition mechanism (Hoch *et al.*, 1992; Langeland *et al.*, 1994), we have presented evidence that kni can function as a short-range repressor, acting over distances of 50–100 bp to inhibit upstream activators or the basal promoter. We demonstrate that this type of repression allows for multiple modes of transcriptional regulation.

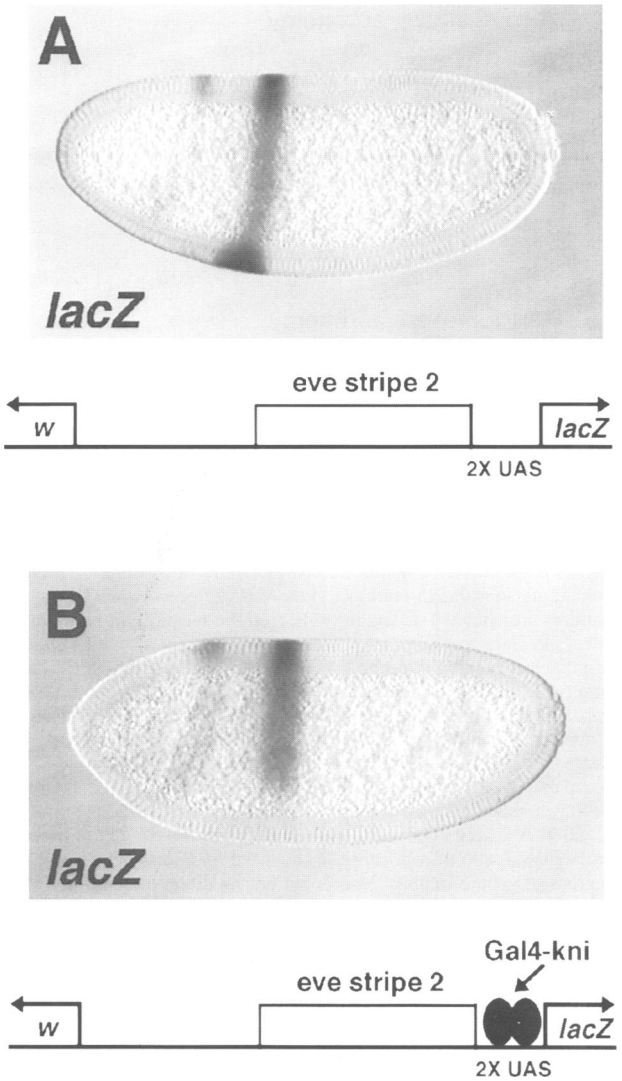

Fig. 5. kni repression domain is separable from the DNA binding domain. Lateral views of transgenic embryos are shown in parasagittal sections. Females carrying the *eve* stripe 2-UAS-*lacZ* reporter gene were mated with wild-type males or males carrying a transgene for zygotic expression of gal4 (1–93)–kni (75–429) protein in ventral regions of the embryo, under control of the 2×PEe-Et *twist* element. The reporter construct is diagrammed underneath the embryos. (**A**) Expression pattern of *eve* stripe 2-UAS-*lacZ* in a wild-type background. Strong ventral expression is observed in all embryos. (**B**) Expression of the *eve* stripe 2-UAS-*lacZ* gene in an embryo containing the gal 4–kni chimeric repressor protein in ventral regions. *lacZ* expression is repressed in ventral regions.

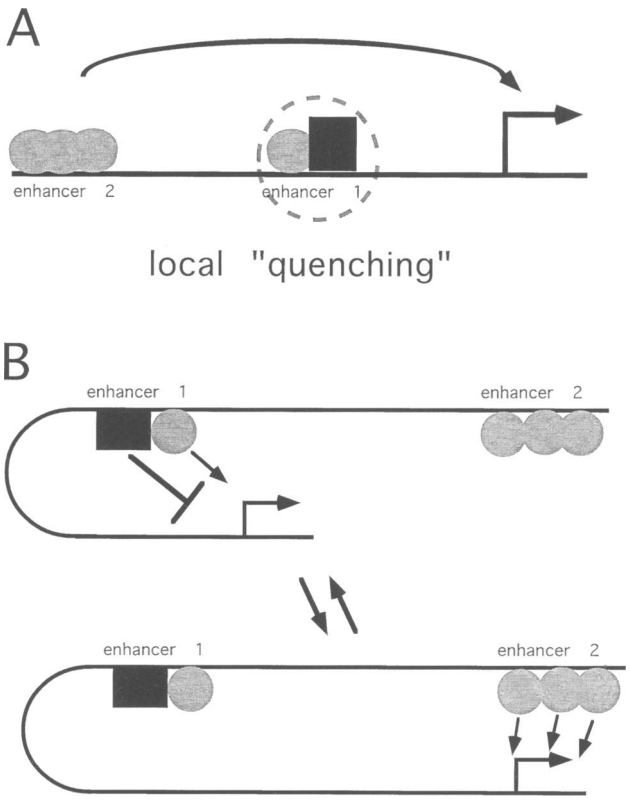

Fig. 6. Models for short-range repression. Activators are indicated by gray circles, repressors by black rectangles. (**A**) Local 'quenching' by short-range repressors (symbolized by a broken circle) would involve repressor interactions with nearby activator proteins, either by direct protein–protein contacts or via putative co-repressors. With this type of repression, positive and negative regulatory information is integrated by the enhancer itself. (**B**) 'Hitchhiking' by short-range repressors proposes that repressors do not interact with neighboring activators, but instead act directly on the basal transcription machinery. The repressor in enhancer 1 is able to contact its target by virtue of close linkage to activator proteins, which interact with other components of the basal machinery. The instability of the interaction between the repressed enhancer 1 and the basal machinery allows enhancer 2 access to the promoter. In this situation, integration of positive and negative transcriptional information from the enhancer is accomplished by the basal transcriptional machinery.

Mechanisms of repression

Studies on the sna repressor prompted the proposal that quenching might involve direct protein–protein interactions between repressors and upstream activators (Gray *et al.*, 1994). This type of mechanism has been proposed for the retinoblastoma (Rb) protein, where repressor–activator specificity is observed (Weintraub *et al.*, 1995). Rb can function in transient assays over distances of >1 kb to block specific activators bound to promoter-proximal elements (Weintraub *et al.*, 1995). This type of mechanism does not appear to apply to the kni repressor, since it is able to block a number of unrelated activators and functions only over short distances. In this study, we have shown that kni can quench dorsal and basic helix–loop–helix (bHLH) activators in the *rho* NEE (Figure 2). kni also appears to quench d-STAT activators in the *eve* stripe 3 enhancer. Moreover, a gal4–kni fusion protein can repress the *eve* stripe 2 enhancer, which is activated by the bicoid and hunchback proteins (Figure 5).

This lack of specificity suggests that kni may not quench upstream activators through direct protein–protein interactions. It is conceivable that kni, and other short-range repressors such as Kr and sna, recruit 'co-repressors', which cause local changes in chromatin structure (e.g. positioning a nucleosome) or in some other way interfere with access to the DNA by activators or basal transcription factors (Figure 6A).

An alternative view is that kni and other short-range repressors work solely through the basal transcription complex, and do not interact with neighboring activators. Perhaps the tight linkage requirement with upstream activators reflects an inherent inability of short-range repressors to contact the transcription complex over long distances. Instead, these repressors might 'hitchhike' with neighboring activators, looping to contact the basal promoter, and then inhibit components of the transcription

complex (Figure 6B). This situation can be simulated by the binding of kni to promoter-proximal regions, as seen in Figure 3A. Note that in this version of the 'hitchhiking' model, interactions of the repressed enhancer with the basal promoter element are transient, allowing other non-repressed enhancers to stimulate the promoter (Figure 6B). Similar transient enhancer–promoter interactions have been inferred from studies of the human globin gene cluster (Wijgerde *et al.*, 1995).

Recent studies suggest that repressor proteins such as the thyroid hormone receptor and the *Drosophila* Kr and eve proteins can interact with components of the basal machinery (Baniahmad *et al.*, 1993; Sauer *et al.*, 1995; Tong *et al.*, 1995; Um *et al.*, 1995). Biochemical and genetic evidence also suggests that the *Saccharomyces cerevisiae* TUP1 repressor directly contacts the RNA polymerase holoenzyme (Herschbach *et al.*, 1994; Johnson, 1995). However, in these studies, it was not clear whether the repressors also quench neighboring activators.

Short-range repression is a flexible form of gene regulation

Our studies indicate that kni functions as a short-range repressor, acting over ranges of 50–100 bp to repress the activity of nearby activators or basal promoter elements. Recent studies suggest that several other *Drosophila* repressors function in this manner, including giant, Kr and sna (Arnosti *et al.*, 1996; Gray *et al.*, 1994; Gray and Levine, 1996). Short-range repression represents a flexible form of gene regulation. For example, kni is capable of generating very different patterns of gene expression depending solely on the location of its binding sites (see Figures 2 and 3). This situation contrasts with long-range silencing, whereby a repressor functions in a dominant fashion to inhibit the transcription complex over long distances (1 kb and more), without regard to exact binding site location (Ip *et al.*, 1991; Jiang *et al.*, 1993; Studer *et al.*, 1994; Schoenherr and Anderson, 1995). The selective activity of short-range repressors may be especially important in the regulation of gene complexes. For example, the *Abdominal-B* gene of the bithorax complex is regulated in posterior regions of early embryos by the *iab-5* enhancer, which may be repressed directly by kni (Busteria and Bienz, 1993; Casares and Sánchez-Herrero, 1995). It would appear that the neighboring *abdominal-A* (*abd-A*) gene is unaffected by kni because the repressor works in a local fashion.

Promoter- and enhancer-autonomous gene regulation

Short-range repression can mediate two distinct types of transcriptional regulation, depending on whether the repressor acts on distal enhancers or the basal promoter element (see Figure 7 legend). Enhancer autonomy within a complex promoter, such as that seen with the *eve* gene, is possible when the repressor is bound close to activator sites, but far from a basal promoter element. Such enhancer-autonomous effects can allow one repressor to regulate multiple promoters, as seen in Figure 2A and B.

In contrast, promoter autonomy within gene complexes is the outcome when a short-range repressor is located close to a basal element. The expression of two closely spaced basal promoters can be uncoupled, as illustrated in Figure 4, when one promoter is independently regulated by a short-range repressor. This type of regulation may explain the apparent enhancer–promoter specificity of the *gooseberry* (*gsb*) locus, which contains two tissue-specific enhancers flanked by two divergently transcribed promoters (Li and Noll, 1994). The *gsb* epidermal enhancer activates only one of the two genes, and this specificity can be reversed by exchanging basal promoter regions. Such specificity might be achieved by the binding of a short-range repressor near the initiation site of the inactive promoter (Figure 7B). It is conceivable that other instances of enhancer-promoter 'compatibility' involve short-range repressors bound to promoter-proximal sequences (Foster *et al.*, 1985; Garcia *et al.*, 1986; Wefald *et al.*, 1990).

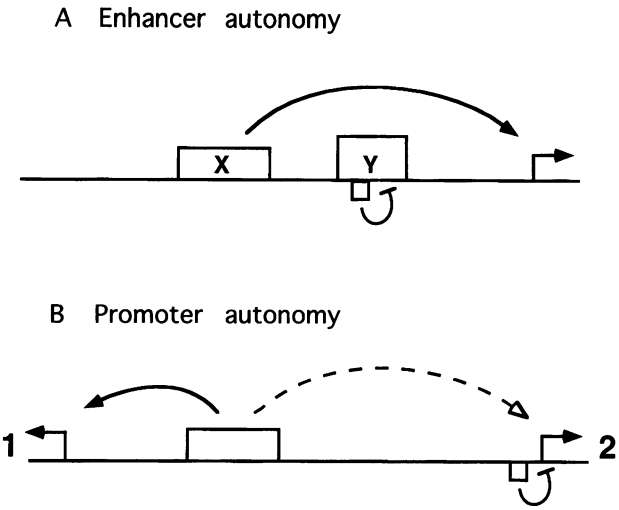

Fig. 7. Flexibility of short-range repression: enhancer-autonomous versus promoter-autonomous effects. Summary of modular promoters containing short-range repressor sites. Very different expression patterns are obtained depending solely on the location of the repressor sites. (**A**) Enhancer autonomy. The diagram depicts a modular promoter containing two non-overlapping enhancers. This situation is observed for the *eve* promoter. A short-range repressor bound within enhancer 'Y' represses nearby activators and blocks that enhancer. However, activators in the neighboring 'X' enhancer are beyond the range of the repressor, so X is free to interact with the transcription complex. (**B**) Promoter autonomy in a gene complex. The diagram depicts a gene complex containing two divergently transcribed genes, 1 and 2. A shared enhancer is located between the two genes (unfilled rectangle). A short-range repressor bound to promoter-proximal regions of gene 2 inhibits gene 2, but has no effect on gene 1.

Materials and methods

P-element transformation and whole mount in situ hybridization of embryos

P-element transformation vectors containing *lacZ* reporter genes and gal4–kni fusion genes were introduced into the *Drosophila* germline by injection of yw^{67} embryos as described in Small *et al.* (1992). *In situ* hybridizations were performed as described (Small *et al.*, 1992), using digoxigenin-UTP-labeled antisense RNA probes to *lacZ*, *white* or *knirps*. Multiple transgenic lines were generated for each construct, and at least three independent lines were tested. To analyze repression in a kni^{-} mutant, independent lines carrying the transgene shown in Figure 2A were crossed into a *kni IIV95* background and offspring carrying the kni 50-5′/kni 50-3′ *rho* NEE Δ sna transgene were crossed *inter se*. Embryos were analyzed as described above.

Construction of lacZ reporter genes

To make the *eve* stripe 3–*rho* NEE fusion gene shown in Figure 1B, a 500 bp *eve* stripe 3 enhancer (Small *et al.*, 1993) was inserted into the *Xba*I site of pBluescript II SK (Stratagene), and a 330 bp *rhomboid*

enhancer (–1.97 to –1.64, *Sty*I–*Xho*I, *rho* NEE) was cloned into the *Eco*RI site. The enhancers were excised on a *Not*I–*Eco*RV fragment and ligated into the *Eco*RI site of *eve* –42 pCaSpeR (Small *et al.*, 1992). In Figure 2A, B and D, the *rho* 700 NEE Δ sna used in Gray *et al.* (1994) was mutagenized as described in Small *et al.* (1992) to create kni binding sites [CTGATCTAGTTT (Hoch *et al.*, 1992)] 50 bp 5′ and 3′ of the dl1 and dl4 sites respectively, and the enhancer was inserted in C4PLZ (Wharton and Crews, 1993) at the *Bam*HI site, maintaining the original 5′ to 3′ orientation. The gene shown in Figure 2C contains kni sites created by mutagenesis 150 bp 5′ and 120 bp 3′ of the dl1 and dl4 sites, respectively. The gene shown in Figure 3A contains the *rho* 700 NEE Δ sna enhancer as described in Gray *et al.* (1994) cloned 4.5 kb 3′ of the *lacZ* transcription start site in the *Bgl*II site of C4PLZ, oriented with the dl4 site closest to the *lacZ* basal promoter. An oligonucleotide bearing two kni binding sites (bold) 5′ **CTGATCTAGTTT**GTACTA-GACAT**CTGATCTAGTTT**CATG 3′ was inserted into the *Sph*I site of C4PLZ, with the 3′ end of the proximal binding site at –55 bp. The gene shown in Figure 3B was prepared in a similar fashion, with two kni binding sites inserted in the *Kpn*I site of C4PLZ; the 3′ end of the proximal binding site is located at –130 bp. The gene shown in Figure 4 was made by modifying the gene shown in Figure 3A by digesting with *Xho*I, followed by religation to remove the downstream *rho* NEE 700 Δ sna enhancer. A 520 bp dimerized sequence from the *twist* promoter, 2×PE (Jiang and Levine, 1993), was inserted into the *Not*I site 5′ of *lacZ* in C4PLZ, and a 330 bp *rho* NEE was inserted into the *Eco*RI site of C4PLZ. Both enhancers retain the original 5′–3′ orientation. The *eve* stripe 2-UAS-*lacZ* reporter gene shown in Figure 5 was constructed as described in Arnosti *et al.* (1996) by ligating a wild-type stripe 2 MSE (Small *et al.*, 1992) to an oligonucleotide containing two tandem UAS sequences and fusing this to the *eve* –42 promoter in pCaSpeR.

Construction of gal4–kni chimeric repressor

The *gal4–kni* gene was expressed using the pTwiggy vector, which was created from pCaSpeR AUG β-gal by inserting the 520 bp *twi* promoter element 2×PEe-Et and *twist* basal promoter from –180 to +160 (Jiang and Levine, 1993) into the *Eco*RI–*Xho*I sites 5′ of *lacZ*, excising the *Bam*HI–*Xba*I *lacZ* fragment, and inserting a *Hin*dIII–*Xba*I fragment (whose *Hin*dIII site had been blunted and converted to *Bam*HI by ligation of a linker) from pSCTEVgal93-LF0-stop (Seipel *et al.*, 1992) containing a 5′-untranslated region (UTR) and the first 93 codons of the *gal4* gene. Codons 1–93 of gal4 are fused to a glycine and a threonine codon which provide a *Kpn*I site, and 3′ of this is an *Xba*I site followed by stop codons. A 1.3 kbp *Aat*II–*Bam*HI fragment containing codons 80–429 and 240 bp 3′ UTR of the *kni* gene was released from pN741 (in pCarnegie 20; G.Struhl, unpublished) and subcloned into the *Kpn*I–*Bam*HI site of pBS-SK(+) using a bridging oligo to recreate codons 75–80, and inserting an additional three alanine codons after the *Kpn*I site. A *Kpn*I–*Xba*I fragment from this subclone was inserted into the *Kpn*I–*Xba*I sites of pTwiggy, yielding a fusion gene coding for the protein gal4(1–93)-Gly-Thr-Ala-Ala-Ala-kni(75–429). We also assayed the activity of a gal4–kni fusion protein containing knirps amino acids 189–254, a domain which had been reported previously to mediate transcriptional repression in transient transfection assays (Gerwin *et al.*, 1994). This chimeric protein was not active in our assay, however; perhaps the protein has a low level of activity seen only when overexpressed at high levels in transient assays.

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