Short-range repression permits multiple enhancers to function autonomously within a complex promoter

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Transcriptional repressors play a key role in establishing localized patterns of gene expression in the early *Drosophila* embryo. Several different modes of repression have been implicated in previous studies, including competition and direct interference with the transcription complex. Here, we present evidence for "quenching," whereby activators and repressors co-occupy neighboring sites in a target promoter, but the repressor blocks the ability of the activator to contact the transcription complex. This study centers on a zinc finger repressor, *snail* (*sna*), which represses the expression of neuroectodermal regulatory genes in the presumptive mesoderm. We show that *sna* can mediate efficient repression when bound 50–100 bp from upstream activator sites. Repression does not depend on proximity of *sna*-binding sites to the transcription initiation site. *sna* is not a dedicated repressor but, instead, appears to block disparate activators. We discuss the importance of quenching as a means of permitting separate enhancers to function autonomously within a complex promoter.

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Transcriptional repression is essential for the establishment of localized patterns of gene expression in the *Drosophila* embryo. Different modes of repression have been proposed, including competition, direct repression, and quenching (for review, see Levine and Manley 1989; see Fig. 1). Competition has been invoked to account for spatially restricted expression of segmentation genes in the early embryo. For example, *even-skipped* (*eve*) stripe 2 expression is regulated by a 480-bp enhancer that contains six activator and six repressor sites (Small et al. 1992). Four of the six activator sites directly overlap four of the repressor sites. The core recognition sequence of the *bicoid* (*bcd*) activator shares a 6 out of 10 match with the binding sites of the *Krüppel* (*Kr*) repressor (Driever and Nüsslein-Volhard 1989; Stanojevic et al. 1989; Treisman and Desplan 1989). These observations prompted the proposal that *Kr* defines the posterior stripe 2 border by blocking the binding of *bcd* activators to overlapping sites (Small et al. 1991, 1992; Stanojevic et al. 1991). A similar mechanism has been suggested for the regulation of *Kr* (Hoch et al. 1992). Competition has also been implicated in cross-regulatory interactions among members of the steroid receptor superfamily (e.g., Liu et al. 1993).

Localized patterns of *zerknüllt* (*zen*), *decapentaplegic* (*dpp*), and *tolloid* (*tld*) expression appear to depend on a long-range mechanism of direct repression, perhaps akin

to "silencing" (Ip et al. 1991; Huang et al. 1993; Jiang et al. 1993; Kirov et al. 1993, 1994). All three genes are repressed in ventral regions of early embryos in response to the *dorsal* (*dl*) gradient. Repression of the *zen* pattern is mediated by a distal promoter element, the ventral repression (VR) element, that contains high affinity *dl*-binding sites and closely linked corepressor sites. The VR is able to repress the ventral expression of a number of different heterologous promoters, including *hunchback* (*hb*), *Kr*, and *eve* (Doyle et al. 1989; Ip et al. 1991; Jiang et al. 1993). Ventral repression is observed even when the *dl*-corepressor-binding sites in the VR map several kilobases away from the closest activators in the heterologous promoters (Hoch et al. 1990; Ip et al. 1991). These observations suggest that the *zen* VR might act directly on the transcription complex. Certain repressors appear to interfere with the transcription complex when bound to proximal regions of target promoters. Among these are the *Drosophila* homeo domain proteins *eve* and *engrailed* [*en*] Han and Manley 1993a,b], as well as the unliganded form of the mammalian thyroid hormone receptor (Fondell et al. 1993).

Here, we present evidence for repression through "quenching," whereby activators and repressors bind adjacent sites, but the repressors mask the ability of the activators to contact the transcription complex (see legend to Fig. 1). This study centers on the zinc finger re-

Figure 1. Modes of transcriptional repression. The diagrams summarize three different modes of repression. Competition involves the occlusion of activator sites by the binding of overlapping repressors. Direct repression involves interference with the formation or activity of the transcription complex (TC). Finally, according to a quenching mechanism, activator and repressor co-occupy neighboring sites, but the repressor masks the ability of the activator to contact the transcription complex.

pressor *snail* (*sna*), which is a key mesoderm determinant in the *Drosophila* embryo (Simpson 1983; Boulay et al. 1987). *sna*[−] mutants fail to gastrulate and lack mesoderm derivatives such as muscles and connective tissues (Simpson 1983; Alberga et al. 1991; Leptin 1991). *sna* defines the mesoderm/neuroectoderm boundary of the early embryo by repressing the expression of neuroectodermal regulatory genes in the presumptive mesoderm (Kosman et al. 1991; Leptin 1991). *sna* homologs have been implicated in the gastrulation of zebrafish (Hammerschmidt and Nüsslein-Volhard 1993; Thisse et al. 1993), frogs (Sargent and Bennett 1990), chicks (Nieto et al. 1994), and mice (Smith et al. 1992), so there is every expectation that its mechanism of action is evolutionarily conserved.

The *rhomboid* (*rho*) promoter is an early target of *sna* activity (Bier et al. 1990; Ip et al. 1992). It is expressed in two lateral stripes that coincide with the presumptive neuroectoderm of early embryos. *rho* stripes are regulated by the 300-bp neuroectoderm enhancer element (NEE), which contains *dl*- and HLH-binding sites that activate expression in both the lateral neuroectoderm and ventral mesoderm. Expression is excluded from the mesoderm by closely linked *sna* repressor sites. In *sna*[−] mutants, the *rho* expression pattern is completely derepressed and spans both the lateral neuroectoderm and ventral mesoderm (Kosman et al. 1991).

We present evidence that *sna*-binding sites need not overlap activator sites to repress *rho* expression in the ventral mesoderm. Repression is observed when *sna* sites map 50 or 120 bp from the closest *dl* activator sites, although better repression occurs when the *sna* sites are 50 bp from the activators. Efficient repression is not correlated with the proximity of the *sna* sites to the transcription initiation site. *sna* does not appear to be a “dedicated” repressor but, instead, can block the heterolo-

gous *bcd* activator. We discuss the importance of quenching as a means for permitting separate enhancers to function autonomously within a complex promoter.

Results

sna repressor sites need not overlap *dl*/HLH activator sites

The *rho* NEE contains four high affinity *dl*-binding sites, five E boxes [helix–loop–helix (HLH) recognition sequences], and four *sna* repressor sites (see legend to Fig. 2). Previous studies have shown that mutations in the *sna* sites result in the ventral derepression of the *rho* pattern, so that there is equally intense staining in both the lateral neuroectoderm and ventral mesoderm (see Fig. 2C, D; Ip et al. 1992). To investigate the mechanism by which *sna* represses *rho* we have introduced synthetic *sna*-binding sites at various positions in and near a defective *rho* NEE lacking all four native *sna* repressor sites. The s2 recognition sequence was used in these experiments because other *sna* sites, such as s3 and s4, also include an E-box activator site (Ip et al. 1992; Kasai et al. 1992). Modified NEEs were attached to a *lacZ* reporter gene and then introduced into embryos via P transformation. The resulting expression patterns were visualized by whole mount in situ hybridization using a *lacZ* antisense RNA probe labeled with a digoxigenin-tagged UTP analog (Tautz and Pfeifle 1989; Jiang et al. 1991).

The first set of experiments involved the use of a 700-bp DNA fragment that maps between −2.2 and −1.5 kb upstream of the *rho* transcription start site. This fragment encompasses the entire 300-bp NEE as well as ~200 bp of flanking sequence on either side of the NEE. The flanking sequences lack high affinity *dl*, basic HLH (bHLH), and *sna*-binding sites (Ip et al. 1992). However, we cannot exclude the possibility that these sequences include low affinity *sna* repressor sites, because gastrulating embryos carrying the defective 700-bp NEE show slightly reduced levels of staining in the invaginating mesoderm (Fig. 2D).

Synthetic *sna* s2 sites were placed ~150 bp upstream and ~120 bp downstream of the closest *dl* activators in the defective NEE (Fig. 2E,F). This modified NEE fusion gene, NEE-150, directs an abnormal pattern of expression in precellular embryos (Fig. 2E). Intense expression is detected in both the lateral neuroectoderm and ventral mesoderm, although there may be a slight attenuation in the levels of staining in the mesoderm. This pattern is quite similar to that observed for the unmodified NEE (cf. Fig. 2C). However, by the onset of gastrulation the NEE-150 fusion gene is slightly, but consistently, repressed in the mesoderm (Fig. 2F). This repression is more pronounced than the variably reduced ventral staining observed with the comparable NEE lacking synthetic *sna* sites (cf. Fig. 2D). It would appear that two *sna* sites, bracketing the defective NEE, can mediate slight repression when they map quite far (120–150 bp) from the closest *dl* activators. Repression may be more efficient in gastrulating embryos (Fig. 2, cf. F and E), as there

Figure 2. Uncoupling *sna* repressor sites and *dl*/bHLH activator sites. Embryos were collected from P transformants and stained to visualize the expression of the *lacZ* reporter gene. Embryos are oriented with anterior to the left. (A,C,E,G) Pcellular embryos; (B,D,F,H) gastrulating embryos. (A,B) Expression of a wild-type 600-bp NEE-*lacZ* fusion gene. Staining is excluded from the mesoderm and restricted to lateral stripes in the neuroectoderm. (C,D) Expression of a defective 700-bp NEE-*lacZ* fusion gene lacking all four *sna*-binding sites. Staining is derepressed in the ventral mesoderm. (E,F) Expression of a defective 700-bp NEE containing synthetic *sna* sites placed 150 bp upstream of the *dl* d1 and 120 bp downstream of the d4 site. There is a slight attenuation of the pattern in ventral regions early, which becomes more pronounced by the onset of gastrulation. (G,H) Expression of the defective NEE with synthetic *sna* sites placed 50 bp upstream of d1 and 50 bp downstream of d4. A nearly normal staining pattern is observed, with virtually complete repression in ventral regions. The diagrams (bottom) show the arrangement of factor-binding sites in the corresponding NEE. There are four *dl* activator sites (d1–d4), five E boxes (two of these bind the *twi* HLH protein), and four *sna* sites. Boxed X's represent mutagenized binding sites; the synthetic s2 *sna* sites are denoted by open rectangles.

is an increase in the levels of *sna* protein during cellularization (Alberga et al. 1991; Kosman et al. 1991; Lepetit 1991).

The NEE-50 fusion gene, containing one *sna* s2 site 50 bp upstream and another s2 site 50 bp downstream from the defective NEE directs an essentially normal pattern of expression (Fig. 2G,H). Lateral stripes are observed in precellular embryos, with only low levels of staining in the ventral mesoderm (Fig. 2, cf. G and A). Expression restricted to the neuroectoderm persists during cellularization and gastrulation (Fig. 2H). These results indicate that *sna*-binding sites need not overlap *dl*/bHLH activator sites to mediate efficient ventral repression.

To investigate the stoichiometry of *sna* activator interactions, single s2 sites were placed in or near the defective NEE. A solo site located 50 bp downstream of the NEE mediates efficient ventral repression (Fig. 3A), nearly comparable with that observed with the NEE-50

fusion gene containing two *sna* sites (Fig. 2G). A similar pattern is observed when the single site is located at its natural position within the NEE (Fig. 3B). Slightly less efficient repression is observed when the single site is placed 50 bp upstream of the NEE (data not shown). Thus, it would appear that a single *sna* protein can block multiple *dl* and bHLH activators.

sna can repress heterologous activators

To determine whether *sna* is a dedicated repressor that blocks only *dl* and/or bHLH activators, s2 sites were placed in both synthetic and natural promoter elements that are activated by the *bcd* homeo domain protein. *bcd* is distributed in a broad concentration gradient, with peak levels of protein in anterior regions and progressively lower levels in more posterior regions (Driever and Nüsslein-Volhard 1988). It is the primary activator of the

Figure 3. A single *sna* site can mediate repression. P-transformed embryos are presented as in Fig. 2. (A) Expression pattern of a precellular embryo containing the defective NEE with a single *sna* site placed 50 bp downstream of d4. There is nearly normal repression in ventral regions. (B) Expression pattern of a gastrulating embryo containing the defective NEE with a single *sna* site at the natural s2 position. An essentially normal *rho* pattern is observed.

Figure 4. *sna* can repress activation by *bcd*. Lateral (A) and ventral (B) views of a precellular embryo containing a modified 200-bp NEE with *dl*-, *bcd*-, and *sna*-binding sites. A combinatorial *bcd*-*dl* staining pattern is observed, with the progressive diminishment of lateral stripes in posterior regions. Staining is excluded from ventral regions coinciding with the limits of the *sna* repressor pattern. Lateral (C) and ventral (D) views of a precellular embryo containing the modified NEE shown in A and B, except that the *dl*-binding sites were abolished by mutagenesis. Staining is restricted to anterior regions where there are peak levels of *bcd* but is excluded from ventral regions where there are high levels of the *sna* repressor. The diagrams below the embryos show the arrangement of factor-binding sites in the modified element, which includes three *bcd* sites, three *dl* sites, and three *sna* sites.

gap gene *hb* and the pair-rule gene *eve* (Driever et al. 1989; Struhl et al. 1989; Stanojevic et al. 1991; Small et al. 1992). The first test of potential *sna*-*bcd* interactions involved the use of a synthetic promoter that contains three high affinity *bcd* sites, three s2 sites, and three *dl* sites (Fig. 4, see summary diagram below A and B). This synthetic element is a highly modified form of a 200-bp minimal *rho* NEE. It retains three of the native *dl*-binding sites (d2, d3, and d4), but the three E boxes were eliminated; two of the sites were replaced with the *bcd1* (b1) recognition sequence, which is a crucial activator of the *eve* stripe 2 enhancer (Small et al. 1992). In addition, the s3 site, which normally includes an E box, was converted into an s2 site. Finally, synthetic s2 and b1 sites were placed between the d3 and d4 *dl* sites.

The synthetic element directs a staining pattern that is consistent with synergistic activation by *dl* and *bcd*, and repression by *sna* (Fig. 4A,B). Intense staining is observed in the anterior-most 20–30% of the embryo, where there are peak levels of *bcd* protein. Progressively lower levels of staining exist in more posterior regions, coinciding with reduced amounts of *bcd*. Interestingly, the lateral limits of the pattern are broadest in anterior regions, but there is a progressive narrowing commensurate with reduced levels of *bcd*. There is no staining in the posterior-most regions in response to *dl* alone. Staining is completely eliminated in the ventral mesoderm along the entire length of the embryo, suggesting that *sna* can repress activation by both *dl* and *bcd* (Fig. 4B).

It is conceivable that *dl*-*sna* interactions are required for the efficient occupancy of the s2 sites by the *sna* repressor. To determine whether *sna* can repress the *bcd* activator in the absence of *dl*, all three *dl* sites in the synthetic promoter element were mutagenized (Fig. 4, see summary diagram below C and D). This results in the loss of the lateral stripes in the presumptive thorax and abdomen, providing additional evidence that these

stripes depend on the combined action of *bcd* and *dl* (Fig. 4, cf. C and A). Staining is restricted to the anterior-most 20–30% of the embryo, where there are peak levels of *bcd*. Thus, activation of the modified element depends solely on *bcd*. Nonetheless, the staining pattern is excluded from the presumptive mesoderm in ventral regions (Fig. 4D), suggesting that *sna* is able to repress *bcd*-mediated activation in the absence of *dl*.

Another test of *sna*'s ability to repress heterologous activators involved inserting *s2* sites in the *eve* stripe 2 enhancer (Fig. 5, see summary diagram below B). Previous studies have shown that stripe 2 is activated by *bcd* and *hb*, whereas the borders of the stripe are defined by the *giant* (*gt*) and *Kr* repressors in anterior and posterior regions, respectively (Stanojevic et al. 1991; Small et al. 1992). The minimal 480-bp stripe 2 enhancer contains

Figure 5. *sna* represses a heterologous enhancer. (A) Precellular embryo expressing a *lacZ* fusion gene containing the 480-bp *eve* stripe 2 enhancer. Uniformly intense staining is observed in dorsal and ventral regions. (B) Precellular embryos expressing a modified stripe 2 enhancer containing three synthetic *sna* sites. Staining is excluded from ventral regions containing high levels of the *sna* repressor. The diagrams below the embryos show the wild-type and modified stripe 2 enhancers. There are six activator sites (five *bcd* and one *hb*), and six repressor sites (three *Kr* and three *gt*). The synthetic *sna* sites are indicated by open rectangles.

six *bcd* and *hb* activator sites, as well as six *gt* and *Kr* repressor sites (Fig. 5, see summary diagram below A). The stripe 2 enhancer is active in dorsal and ventral regions of early embryos (Fig. 5A). However, the introduction of three *s2*-binding sites within the stripe 2 enhancer results in the repression of the pattern in the ventral mesoderm (Fig. 5B). The domain of repression coincides with the limits of the *sna* expression pattern, suggesting that *sna* is able to repress the modified stripe 2 enhancer. It is unlikely that repression involves competitive binding to DNA, as the most closely linked *s2* repressor site and *bcd* activator site (Fig. 5, see summary diagram below B) map ~40 bp from each other (see Discussion).

Short-range repression permits enhancer autonomy

The preceding results are consistent with the notion that *sna* acts locally, within the limits of the enhancer, to mask the ability of activators to contact the transcription complex. An important implication of this mechanism is that a repressor bound to one enhancer should not interfere with another enhancer located elsewhere in the same promoter. To test this idea, composite promoters were examined that contain the *eve* stripe 2 enhancer attached to the *rho* NEE. These experiments involved the use of the 480-bp stripe 2 enhancer (Small et al. 1992) and either a 200- or 152-bp minimal NEE.

The first set of experiments involved placing the 152-bp NEE upstream of the stripe 2 enhancer. This NEE contains two *dl* sites, three E boxes, and three *sna* sites, including the *s2* sequence. The composite promoter directs an additive expression pattern that includes both *eve* stripe 2 and *rho* lateral stripes (Fig. 6A,B). There is no detectable diminishment in the ventral expression of stripe 2, suggesting that *sna* bound to the NEE does not interfere with stripe 2 activators (Fig. 6, A and B; cf. with Fig. 5B). The most closely linked *sna* repressor site in the NEE (*s4*) and activator site in the stripe 2 enhancer (*bcd* 5; *b5*) map ~75 bp away from each other, just beyond the range of efficient *sna*-mediated repression as judged by earlier experiments (e.g., Fig. 2). Although *sna* appears to exert little or no effect on stripe 2 expression, it would appear that the binding of *Kr* to the stripe 2 enhancer attenuates the expression of the *rho* lateral stripes (Fig. 6A,B). There is reduced expression in central regions, just posterior to the limit of stripe 2. This coincides with the *Kr* expression domain (Rosenberg et al. 1985; see below). Very similar staining patterns, including attenuated expression of the *rho* stripes, were observed when the 152-bp NEE was placed in either a direct or inverted orientation upstream of the stripe 2 enhancer (Fig. 6, cf. A and B). The two orientations result in similar spacings of activators and repressors in the two enhancers.

Kr may repress over longer distances than *sna*, as the *Kr* and *sna* sites map a similar distance from the closest activator site in the neighboring enhancer (Fig. 6, see summary diagrams below A and B). Perhaps *Kr* exerts a stronger effect on the composite NEE/stripe 2 pattern, as the *Kr*-binding sites map downstream of the NEE, close

Figure 7. *Kr* represses a heterologous enhancer. (A) Precellular embryo expressing a defective 700-bp NEE lacking all four *sna* sites (same as those shown in Fig. 2, C and D). A derepressed staining pattern is observed, with nearly uniform expression in lateral and ventral regions. (B) Precellular embryo expressing the same NEE as in A, except that synthetic *Kr*-binding sites were placed 50 bp upstream of d1 and 50 bp downstream of d4. Staining is repressed in central regions coinciding with the limits of the *Kr* pattern. The diagrams below the embryos show the arrangement of factor-binding sites in the wild-type and modified NEE.

of the pattern in both lateral and ventral regions, as the *Kr* repressor is equally distributed along the dorsoventral axis. This result indicates that *Kr* can efficiently repress heterologous activators in the context of a distinct enhancer. The simplest interpretation of this result is that *Kr*, like *sna*, can mask the ability of closely linked activators to contact the transcription complex (see Discussion).

Discussion

Evidence is presented that *sna* functions through a short-range mechanism of transcriptional repression. We favor the view that the repression involves quenching, whereby *sna* blocks the activities, but not binding, of nearby *dl* and HLH transcriptional activators. There are numerous examples of short-range interactions among transcriptional activators, such as protein–protein contacts that facilitate cooperative occupancy of DNA-bind-

ing sites (e.g., Jiang and Levine 1993). In principle, quenching could involve similar interactions between repressor and activator, although *sna* appears to function in a nonstoichiometric manner, in that one *sna* repressor site can efficiently block numerous activators. *sna* does not appear to be a dedicated repressor but, instead, may be able to block disparate activators. Quenching is a more flexible form of repression than competition because it does not require that activators and repressors recognize related DNA-binding sites. Short-range repression provides a basis for understanding how different enhancers can function autonomously within a complex promoter.

Modes of repression

Several lines of evidence suggest that *sna* does not repress *rho* by directly interfering with the transcription complex (direct repression; see Fig. 1). As *sna* sites are moved farther from a defective NEE but closer to TATA, there is a progressive loss in *sna* repression activity (Fig. 2). The NEE-150 promoter is only poorly repressed by *sna*, even though one of the synthetic *sna* sites maps just 78 bp from TATA. In contrast, efficient repression is observed for the NEE-50 promoter, although the proximal *sna* site now maps 178 bp from TATA. Analysis of stripe 2–NEE fusion promoters supports the view that proximity to TATA is not a critical determinant of repression efficiency. For example, *sna* sites in the NEE do not necessarily repress stripe 2 expression when placed near TATA. Attenuated expression is observed only when activators in the distal stripe 2 enhancer map within 100 bp of *sna* repressor sites in the NEE (see Fig. 6).

The native configuration of activator and repressor sites in the *rho* NEE suggested that *sna* might function through a competition mechanism of repression (see footnote to Fig. 1). One of the *sna* sites (s4) directly overlaps an E box (t2), and gel mobility retardation assays indicate that there is mutually exclusive binding of the *twist* (*twi*) HLH activator and *sna* repressor to this sequence (Ip et al. 1992; R. Park and M. Levine, unpubl.). Two of the remaining *sna* sites map within 14 bp of either an E box or *dl* site. Despite this tight linkage, *sna* does not appear to function through competition.

Efficient repression of the *rho* pattern is observed when *sna* s2-binding sites are placed 50 bp from the closest activator sites in a defective NEE (see Fig. 2). Weak, but consistent, repression is also observed when the s2 sites map as far as 120 bp from the closest activator. Given these large distances, it seems unlikely that *sna* functions via competition. Gel mobility retardation assays indicate that mixtures of a truncated *dl* protein (*dl*-378) that is over half the size of the full-length protein and a glutathione *S*-transferase (GST)–*sna* fusion protein that is about the same size as full-length *sna* can co-occupy neighboring *dl*- and *sna*-binding sites separated by just 6 bp (J. Jiang and M. Levine, unpubl.). Similarly, the demonstration that *Kr* can repress the *rho* NEE over a distance of 50 bp (Fig. 7) suggests that competition

might not be important for the regulation of the *eve* stripe 2 enhancer.

Mechanism of quenching

It is conceivable that *sna* represses transcription by making local protein–protein contacts with closely linked activators. These interactions might be similar to the weak protein associations that permit certain activators to bind DNA in a cooperative fashion (e.g., Jiang and Levine 1993). According to this view a discrete domain within the *sna* protein might contact a neighboring activator so that its activation domain is unable to promote transcription. Although the nature of the *sna*–*dl* or *sna*–HLH interaction is still unclear, the results presented in this study indicate that *sna* blocks repression in a nonstoichiometric manner. There are several potential explanations for the ability of a single *sna* repressor to block many activators. Perhaps *sna* recruits a “repression complex” after it is bound to DNA, and this complex is able to interfere with multiple activators. This scenario would be similar to the recruitment of the *tup1/ssn6* repression complex by the $\alpha 2$ homeo domain protein in yeast (Keleher et al. 1992). Alternatively, it is possible that multiple activators coordinate communication with the transcription machinery by recruiting an “adaptor” protein. A single repressor might be capable of disrupting a higher order conformation arising from interactions among activators and thereby block transcription. Ultimately, it will be interesting to determine whether *sna* shares any common features with another zinc finger repressor in *Drosophila*, *Suppressor of Hairy Wing*, which mediates directional repression over long distances (Geyer and Corces 1992).

sna does not appear to be a dedicated repressor that specifically blocks *dl* and/or HLH activators. Several of the experiments presented in this study demonstrate that *sna* is able to block activation by *bcd*, even though none of the embryonic promoters characterized to date appear to be jointly regulated by these two regulatory factors. It is possible that *sna* can quench disparate activators that employ distinct mechanisms for contacting the transcription complex. Alternatively, it is possible that *dl* and *bcd* possess structurally related activation domains, even though they do not share any obvious sequence homologies. Future experiments will address this issue of specificity by analyzing chimeric *bcd* or *dl* proteins containing each of the well-characterized activation domains, including acidic, glutamine-rich, and proline-rich activators (for review, see Mitchell and Tjian 1989).

Long-range vs. short-range repression

Repression by quenching can account for the autonomous expression of different enhancers within a complex promoter. For example, the *eve* promoter contains separate enhancers for the regulation of individual stripes (Goto et al. 1989; Harding et al. 1989). The *eve* stripe 3 enhancer is expressed in central regions of the

embryo where there are high levels of the *Kr* repressor. It evades repression by *Kr*, as it lacks high affinity *Kr*-binding sites (Stanojevic et al. 1989). Moreover, *Kr* bound to the stripe 2 enhancer fails to interfere with the expression of the stripe 3 enhancer because the two enhancers are separated by a 1.7-kb spacer sequence (Small et al. 1993). According to a quenching mechanism, *Kr* acts only locally within the limits of the stripe 2 enhancer to block the *bcd* and *hb* activators. Previous studies have shown that *Kr* can attenuate stripe 3 expression when the spacer sequence is removed and the two enhancers are directly coupled (Small et al. 1993).

Fusion promoters containing both the NEE and stripe 2 enhancer direct an additive pattern of expression consisting of *rho* lateral stripes and *eve* stripe 2. This suggests that *sna*, like *Kr*, also acts over short distances, within the limits of the NEE. Cross-enhancer interactions are observed only when repressors in one enhancer map near activators in the other. For example, there is reduced expression of stripe 2 when the upstream stripe 2 enhancer is placed in a direct orientation relative to the proximal NEE. In contrast, stronger expression is observed when the orientation of the stripe 2 enhancer is inverted (Fig. 6). The former fusion promoter contains a *sna* site within 80 bp of a *bcd* activator site in the stripe 2 enhancer while the latter separates the two classes of binding sites by >190 bp.

The passive, short-range effects of the *sna* and *Kr* repressors contrast with the results obtained with fusion promoters containing the *zen* VR silencer element. The VR is repressed in response to *dl*, and when attached to heterologous promoters, such as the *eve* stripe 2 enhancer, it eliminates their ventral expression (Ip et al. 1991; Jiang et al. 1993). Thus, long-range repressors such as those that bind the *zen* VR exert a dominant effect on the activities of other regulatory elements in a complex promoter, whereas the *sna* and *Kr* repressors act only over short distances and therefore do not interfere with activators present even in neighboring enhancers.

Materials and methods

Plasmid constructions

rho promoter elements were derived from a *HindIII*–*SspI* genomic DNA fragment spanning from –2.2 kb to +90 bp relative to the putative *rho* transcription start site (Bier et al. 1990) in a pGEM 7Zf+ vector (Ip et al. 1992). The 700-bp *rho* NEE fragments were obtained by digestion with *Bam*HI (located in the polylinker of the vector) at the 5' end and *Bsu*36I at the 3' end. The *Bsu*36I end was blunted with Klenow, and *Bam*HI linkers were added to insert the element into the *Bam*HI site of the P-transformation vector, C4PLZ, which contains the P-element promoter and transposase amino-terminal sequence fused in-frame with the *lacZ* gene (Wharton et al. 1993). In the NEE-150 construct (see Fig. 2), the blunted *Bsu*36I end was unchanged, and the element was inserted into the *Bam*HI site and blunted *Sph*I site of the C4PLZ vector. A 600-bp NEE (Fig. 2A,B) was obtained by digestion with *Bam*HI and *Ava*II, and blunted into the *Stu*I site of the pWHL P-transformation vector, which contains a minimal *hsp70* promoter (Driever et al. 1989). A 200-bp NEE (Fig. 6C,D) was obtained by digestion of the 600-bp pWHL

fusion gene at the synthetic *NotI* (–1892 bp) and *BamHI* (–1694 bp) sites. This 200-bp fragment was inserted into the *NotI* and *BamHI* sites of C4PLZ. The 152-bp NEE (Fig. 6A,B) was obtained by digestion at the *NotI* site (–1892 bp) and synthetic *SphI* site (–1740 bp). This fragment was inserted into the C4PLZ polylinker between *NotI* and *SphI*, and then excised from this vector to make the 152-bp NEE–stripe 2 fusion promoter (see below).

The *eve* stripe 2 enhancer is 480 bp in length and maps between –1.55 kb and –1.07 kb upstream of the *eve* transcription start site. This was attached to a basal *eve*–*lacZ* fusion gene inserted into the CaSpeR injection vector (Small et al. 1992). The 152-bp NEE–stripe 2 fusion promoter was prepared by removing the 152-bp NEE from C4PLZ by digestion with *HindIII* (which cuts in the 3' polylinker). *EcoRI* linkers were attached to this fragment after blunting with Klenow. It was subsequently inserted into the *EcoRI* site located 5' of the 480-bp stripe 2 enhancer in the CaSpeR vector. The 200-bp stripe 2–NEE fusion promoter was prepared by inserting the 480-bp stripe 2 enhancer into the *EcoRI* site of the C4PLZ vector containing the 200-bp NEE (see above).

P transformation and whole-mount in situ hybridization

P transposons containing various *lacZ* reporter genes were introduced into the *Drosophila* germ line using standard methods (Spradling and Rubin 1982). Multiple independent transformed lines were examined for each construct. *lacZ* reporter gene expression was visualized by whole-mount in situ hybridization using a digoxigenin–UTP-labeled *lacZ* antisense RNA probe (Tautz and Pfeifle 1989; Jiang et al. 1991).

Site-directed mutagenesis

The pGEM7Zf+ plasmid containing the *HindIII*–*SspI* fragment from the *rho* 5'-flanking region, and the pBluescript SK+ plasmid containing the 480-bp *BstEII*–*FspI* fragment spanning the *eve* stripe 2 enhancer, were used to transform the CJ236 strain of *Escherichia coli* to prepare uracil-containing single-stranded DNA templates. Mutagenesis was done with the Muta-Gene kit (Bio-Rad, NY) by annealing the templates with various mutagenic oligonucleotides, as suggested by the manufacturer. The in vitro-synthesized double-stranded DNA was transformed into the DH5 α strain of *E. coli*. The identities of the mutagenized templates were confirmed by nucleotide sequence analysis. Synthetic *sna* s2 sites (CAGCAAGGTG) were created at the following positions in the *rho* promoter (all numbers refer to distance from the putative transcription start site): –2133 to –2124 bp; –2021 to –2012 bp; –1839 to –1830 bp; –1748 to –1739 bp; –1658 to –1649 bp; –1595 to –1586 bp. Synthetic s2 sites were created at the following positions within the *eve* stripe 2 enhancer: –1395 to –1386 bp; –1244 to –1235 bp; –1162 to –1153 bp. Synthetic *Kr1* sites (AAAACGGGT–TAAGC) were created at the following positions in the *rho* promoter region: –2023 to –2010 bp; –1652 to –1639 bp. Synthetic *bcd1*-binding sites (GGATTAGGG) were created at the following positions within the *rho* promoter: –1794 to –1786 bp; –1780 to –1772 bp; –1732 to –1724 bp. Mutations that disrupt native *sna* and *dl*-binding sites in the NEE are described by Ip et al. (1992).

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Short-range repression permits multiple enhancers to function autonomously within a complex promoter.

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