Short-range transcriptional repressors mediate both quenching and direct repression within complex loci in Drosophila

Susan Gray and Michael Levine
Department of Biology, Center for Molecular Genetics, University of California at San Diego, La Jolla, California 92093-0347 USA

The early Drosophila embryo provides a unique system for the analysis of transcriptional repression since a broad spectrum of repressors are distributed in spatially distinct patterns. Krüppel (Kr) and snail (sna), two zinc finger repressors, are essential for segmentation and for the establishment of the mesoderm/neuroectoderm boundary, respectively. Both repressors were examined in the context of synthetic gene complexes containing modular promoters and divergently transcribed reporter genes. These studies indicate that Kr and sna function as short-range repressors, which can mediate either quenching or direct repression of the transcription complex, depending on the location of repressor sites. When located within an upstream enhancer, the repressor locally quenches nearby activators and permits other enhancers to interact with the transcription complex (enhancer autonomy). In contrast, when bound to promoter-proximal regions the repressor functions in a dominant fashion and blocks multiple enhancers. Local quenching and dominant repression require close linkage (<100 bp) of the repressor with either upstream activators or the transcription complex. These studies establish short-range repression as a flexible form of gene regulation and suggest that the key distinction among repressors is their range of action.

[Key Words: Drosophila embryo, transcriptional repression; zinc finger repressors, Krüppel, snail; gene regulation]

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Previous studies have demonstrated the importance of transcriptional repression in establishing cell type-specific patterns of gene expression. The yeast a2 homeo domain protein specifies the a-cell phenotype by repressing a-specific genes [Keleher et al. 1992]. In the Drosophila embryo, stripes of segmentation gene expression are established by repressors that define the borders of individual stripes (for review, see Pankratz and Jackle 1990; Small and Levine 1991). Repression is also essential for establishing the boundary between the presumptive mesoderm and neuroectoderm [Alberga et al. 1991; Kosman et al. 1991; Leptin 1991]. In addition, repressors both define and maintain the expression limits of homeotic selector genes [Paro 1993; Busturia and Bienz 1993; Chan et al. 1994]. Numerous examples of transcriptional repression have been observed in mammalian systems. For example, repression establishes the rhombomere-specific pattern of Hoxb-1 expression within the hindbrain region of the mouse embryo [Studer et al. 1994]. Repression is also essential for B-cell specific expression of the immunoglobulin heavy-chain enhancer [Genetta et al. 1994] and the regulation of CD4 expression in different T-cell lineages [Sawada et al. 1994; Siu et al. 1994].

Several different modes of repression have been proposed, including competition, quenching, and direct repression of the transcription complex [for review, see Levine and Manley 1989; Johnson 1995]. The yeast a2 homeo domain protein represents the most thoroughly characterized eukaryotic repressor. Occupancy of a2 operator sites depends on cooperative DNA-binding interactions with a general transcriptional activator, Mcm1 [Vershon and Johnson 1993]. Once bound, a2 recruits the Tup1 repressor, which directly inhibits the transcription complex over distances of 200 bp to 1 kb [Keleher et al. 1992; Herschbach et al. 1994; Cooper et al. 1994]. It is unclear whether Tup1 also works in a local fashion to inhibit [quench] the neighboring Mcm1 activator.

In vitro transcription experiments and transient transfection assays suggest that a number of mammalian repressors, including nuclear receptor proteins such as the glucocorticoid and thyroid hormone receptors, might directly repress the transcription complex [Stromstedt et al. 1991; Drouin et al. 1993; Fondell et al. 1993]. However, in many instances repressor binding sites are contained within composite cis regulatory elements that map far (>200 bp) from the transcription start site [Pearce and Yamamoto 1993]. It is conceivable that such
Short-range repressors

Efficient ventral repression of the rho neuroectoderm enhancer element [NEE] requires close proximity of sna and dl binding sites [Gray et al. 1994]. sna was also found to inhibit the heterologous bcd activator when bound within the eve stripe 2 enhancer or a synthetic enhancer containing bcd and dl activator sites. These findings prompted the proposal that short-range repression should allow enhancers to function autonomously within complex, modular promoters [Gray et al. 1994]. An example of a synthetic modular promoter is shown in Figure 1A. A nearly additive staining pattern [lateral and transverse stripes] is observed when a minimal 152-bp rho NEE is placed upstream of the 480-bp eve stripe 2 enhancer. sna bound to the rho NEE represses dl/bHLH activation in the presumptive mesoderm but does not interfere with ventral activation of the neighboring stripe 2 enhancer. The most closely linked sna and bcd sites are separated by ~175 bp, beyond the range of efficient sna repression [see diagram below Fig. 1A and summary in Fig. 1B].

Kr represses in a dominant fashion, within the limits of the eve stripe 2 enhancer [Fig. 1B]. However, there is a slight attenuation of the rho lateral stripes in central regions containing peak levels of Kr protein [arrows, Fig. 1A]. Subsequent studies [see Fig. 5] suggest that the Kr site at the 3' end of the stripe 2 enhancer, located just 45 bp from the transcription start site, mediates direct repression of the transcription complex. This repression is relatively weak, as the bcd activator and Kr repressor compete for overlapping binding sites [Small et al. 1991, 1992; see below].

Kr appears to mediate direct repression of the transcription complex

Kr can repress a defective 700-bp rho NEE that lacks all four sna repressor sites [NEE Δsna]. As shown previously [Ip et al. 1992b], removal of the sna site causes a loss of ventral repression, so that there is equal expression in both ventral and lateral regions (Fig. 2A). In these experiments, the NEE Δsna was placed between two divergently transcribed promoters, a leftward white reporter gene and a rightward lacZ gene. The expression of either gene can be assayed independently by in situ hybridization using digoxigenin-labeled white or lacZ antisense RNA probes [Tautz and Pfeifle 1989; Cai and Levine 1995].

Kr-binding sites positioned 50 bp upstream and 55 bp downstream of the nearest dl activator sites result in an almost complete loss of staining in central regions where
there are high concentrations of Kr protein (Fig. 2A). The simplest explanation for this observation is that Kr locally quenches or inhibits nearby dl activators. This is supported by the observation that white is also repressed by Kr (Fig. 2B), even though the nearest repressor site maps $>550$ bp from the white initiation site (see diagram below Fig. 2A). Previous tissue culture assays are also consistent with the notion that Kr functions via quenching (Zuo et al. 1991).

Further evidence for short-range quenching was obtained by moving the Kr sites away from the dl activators. When positioned $>150$ bp from dl, Kr no longer represses white (Fig. 2D). This result is consistent with the notion that Kr must map within $>100$ bp of upstream activators to inhibit expression, similar to the situation shown previously for sna (Gray et al. 1994).

However, we were surprised that this arrangement of Kr sites resulted in substantial repression of lacZ (Fig. 2C).

**Figure 1.** Enhancer autonomy in a modular promoter. The transgenic embryo is oriented with anterior to the left and dorsal up. It contains a fusion promoter with a minimal, 152-bp rho NEE placed upstream of the 480-bp eve stripe 2 enhancer (see summary diagram below the embryo). The expression pattern was visualized after hybridization with a digoxigenin-labeled lacZ antisense RNA probe. (A) Embryo at the midpoint of nuclear cleavage cycle 14. The staining pattern consists of rho lateral stripes and the transverse eve stripe. The stripe 2 enhancer contains five bcd activator sites and three Kr repressor sites, whereas the rho NEE contains two dl activator sites and three sna repressor sites. The most closely linked rho NEE repressor site and stripe 2 activator are separated by 175 bp. (B) Summary of short-range repression and enhancer autonomy. The staining pattern obtained with the fusion promoter is additive, as repressors bound to one enhancer do not affect the neighboring enhancer. According to this diagram, the sna repressor functions in a local fashion to inhibit, or quench, nearby activators in the rho NEE. Activators in the neighboring stripe 2 enhancer map beyond the range of sna repression ($>50$–100 bp). Similarly, stripe 2 repressors, such as Kr, do not interfere with the expression of the NEE.

$sna$ can repress the transcription complex

Our previous analysis of sna repression did not include a critical test for direct repression (Gray et al. 1994). sna sites located $>50$ bp from dl activators restore ventral repression to an otherwise defective NEE &sna (Fig. 3A). This arrangement of sna sites results in efficient repression of both the rightward lacZ gene (Fig. 3A) and the leftward white gene (Fig. 3B). Repression is probably caused by local quenching of dl activators, as moving the sna sites another 100 bp results in a near loss of sna-mediated repression (Gray et al. 1994). Moreover, sna represses white expression even though the closest sna site maps 550 bp from the white initiator (Fig. 3B).

To determine whether sna can directly repress the transcription complex, two sna-binding sites were placed $>50$ bp upstream of the lacZ transcription start site. This results in efficient repression of the lacZ pattern in ventral regions (Fig. 3C). In contrast, white is depressed completely in ventral regions (Fig. 3D), suggesting that sna does not interfere locally with dl but, instead, represses the lacZ promoter (the sna sites map 215 bp from the nearest dl activator site in the NEE &sna).

Further evidence that sna can mediate direct repression was obtained by placing the NEE &sna 3′ of lacZ (Fig. 4). The insertion of two sna sites $>50$ bp upstream of the lacZ transcription start restores an essentially normal pattern (Fig. 4A). Repression is almost certainly caused by direct repression of the transcription complex, as the nearest NEE &sna activators now map $>4.5$ kb from sna. Moving the sna sites another 75 bp upstream of lacZ abolishes repression (Fig. 4B). These results suggest that sna can repress the transcription complex only over short distances, similar to the range observed for sna-mediated quenching of upstream activators.

**Dominant repression of multiple enhancers**

As discussed earlier, short-range repressors bound to one enhancer do not interfere with neighboring enhancers in a modular promoter (e.g., Fig. 1A). The next series of experiments examined how the activity of multiple enhancers might be affected by the placement of sna and Kr repressor sites near the transcription start site.

Previous studies identified a 260-bp enhancer [PE] from the twist [twi] promoter region that directs expression in the ventral-most 12–14 cells in response to peak concentrations of the dl regulatory gradient (Jiang et al. 1991; Pan et al. 1991; Thisse et al. 1991). Two tandem
copies of this enhancer (2XPE) direct a somewhat more robust staining pattern than a single copy (Jiang and Levine 1993). A fusion promoter containing 2XPE placed upstream of the rho NEE directs an additive staining pattern, which consists of rho lateral stripes and a band of expression within the presumptive mesoderm (Fig. 5A; arrow indicates the gap between the expression patterns directed by the two enhancers). A single Kr-binding site placed between the two enhancers, >150 bp from activators in either the 2XPE or rho NEE, has no discernible effect on the pattern (Fig. 5A). In contrast, both enhancers are repressed when the same Kr site is placed ~75 bp from the transcription start site (Fig. 5B). The rho NEE is attenuated in central regions, whereas 2XPE expression is nearly abolished.

sna was also tested for its ability to repress multiple enhancers when placed near the transcription start site. Figure 6A shows the staining pattern obtained with a fusion promoter containing the 480-bp eve stripe 2 enhancer placed upstream of a 500-bp stripe 3 enhancer. The two enhancers are separated by a 160-bp neutral spacer sequence from the native eve promoter region (Small et al. 1993). An additive staining pattern is observed, although stripe 2 expression is slightly weaker than stripe 3 staining (Fig. 6A). A variable head stripe results from cryptic regulatory sequences within the P-transformation vector (Jiang et al. 1991; Small et al. 1992). In addition, stripe 7 expression is mediated by regulatory sequences located primarily in the stripe 3 enhancer (Small et al. 1993, 1996). A cluster of four sna-repressor sites has no effect on the ventral expression of either stripe 2 or stripe 3 when located in the spacer region. These map far (>150 bp) from the closest activators in either enhancer.

Stripe 2 is repressed in ventral regions when several sna sites are inserted within the stripe 2 enhancer (Fig. 6B). These sna sites map ~30–40 bp from critical bcd sites within the modified enhancer. Stripe 3 is unaffected, as the stripe 3 enhancer is located beyond the range of sna repression. The insertion of sna repressor sites just upstream of the lacZ initiation site results in the ventral repression of both stripes (Fig. 6C). The stripe 2 pattern is somewhat more efficiently repressed than stripe 3; the stripe 2 enhancer may be inherently weaker than the stripe 3 enhancer (see Cai and Levine 1995). Nonetheless, these results suggest that sna can function as a dominant repressor to block multiple enhancers when bound near the transcription complex.
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Figure 3. The sna repressor can silence the transcription complex. Transgenic embryos are oriented as described previously. They contain the 700-bp NEE Asna enhancer between divergently transcribed white and lacZ reporter genes. (A) lacZ staining pattern in a precellular embryo with synthetic sna sites placed ~50 bp from dl activator sites (see diagram beneath the embryo). Efficient repression is observed in ventral regions where there are high concentrations of sna protein. (B) Gastrulating embryo expressing the same fusion gene as A except that white expression is monitored. Repression is observed in the presumptive mesoderm. (C) lacZ staining pattern in a precellular embryo obtained with two sna sites placed ~50 bp upstream of the lacZ transcription start site. These mediate efficient repression in the presumptive mesoderm. (D) Gastrulating embryo expressing the same fusion gene as C except that white expression is monitored. The sna sites located near lacZ fail to repress white expression. Consequently, the staining pattern is derepressed in ventral regions.

The sna repression domain can be uncoupled from DNA binding

Previous studies have established that transcriptional activators possess distinct DNA-binding domains and activation domains (e.g., Chi et al. 1995). Repressors have not been characterized to the same extent, although recent tissue culture assays have identified discrete repression domains (Han and Manley 1993a, b; Gerwin et al. 1994; Licht et al. 1994). To determine whether the non-DNA-binding domain of sna is sufficient for repression in vivo, we analyzed the activities of a sna–Gal4 fusion protein in transgenic embryos.

A 200-bp rho NEE was modified extensively to make it responsive to the fusion protein (see Materials and methods). The modified NEE contains three dl, three twi, and three Gal4 upstream activating sequence (UAS) binding sites (see diagram below Fig. 7A) and directs strong expression in ventral and ventrolateral regions, encompassing ~20–22 cells (Fig. 7A).

The sna–Gal4 fusion protein consists of the non-DNA-binding domain of sna fused to the DNA-binding domain of the yeast activator Gal4. sna is composed of 390-amino acid residues; the carboxy-terminal sequences located between amino acids 245 and 390 contain five zinc fingers that mediate DNA binding (Boulay et al. 1987; Ip et al. 1992b; Mauhin et al. 1993). sna coding sequences for amino acids 1–244 were placed upstream of the Gal4 amino acid 2–93 coding region. The fusion gene was placed under the control of a chimeric promoter containing the 2XPE twi enhancer attached to a 1.6-kb sequence from the sna promoter region. As shown previously, this twi–sna chimeric promoter directs expression in the ventral-most 14–16 cells (Ip et al. 1994). The modified NEE was introduced into transgenic embryos expressing the sna–Gal4 fusion gene (Fig. 7B). The fusion protein mediates efficient repression in the ventral-most 14–16 cells, thereby converting the wide band of ventrolateral staining into two narrow stripes (cf. Fig. 7B and 7A).

The repression mediated by the sna–Gal4 fusion protein is probably caused by short-range inhibition of nearby dl activators, rather than direct repression of the transcription complex. The proximal-most Gal4 site is located >100 bp from the lacZ transcription start site. A competition mechanism of repression is also unlikely, as Gal4 fusion proteins that activate gene expression do not repress the modified NEE. For example, the modified NEE is activated in anterior regions of transgenic em-
Short-range repressors

Multiple enhancers over distances of several kilobases regardless of location within a gene complex (Huang et al. 1993; Jiang et al. 1993; Kirov et al. 1993; Lehming et al. 1994). We propose that the most crucial distinction among repressors is their range of action.

Short-range vs. long-range repression

Previous studies have identified several apparently distinct modes of repression, including competition, quenching, and direct repression of the transcription complex (for review, see Levine and Manley 1989; Johnson 1995). Evidence for competition stems from the organization of enhancers and promoter elements active in the early embryo. For example, four of the six repressors containing an anteroposterior gradient of a bcd–Gal4 fusion protein (Simpson-Brose et al. 1994; data not shown).

Discussion

We have shown that short-range repression represents a flexible form of gene regulation. Binding of repressor to remote locations in the promoter region results in local inhibition, or quenching, of upstream activators. Other enhancers contained in the promoter are not influenced by the repressor and can interact freely with the transcription complex. In contrast, the binding of repressor to promoter–proximal regions results in the dominant repression of the promoter, so that multiple enhancers are blocked. This flexibility contrasts with long-range repressors (summarized in Fig. 8), which can block multiple enhancers over distances of several kilobases regardless of location within a gene complex [Huang et al. 1993; Jiang et al. 1993; Kirov et al. 1993; Lehming et al. 1994]. We propose that the most crucial distinction among repressors is their range of action.

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Figure 4. sna can silence a rho NEE located far from the transcription start site. Precellular, transgenic embryos are oriented as described previously. Both embryos contain a 700-bp rho NEE Δsna downstream of the lacZ gene. (A) lacZ staining pattern obtained when two sna sites are placed ~50 bp from the transcription start site [see diagram beneath the embryo]. Efficient repression is observed in the presumptive mesoderm. [B] Same as A except that the sna sites were moved another 75 bp from the lacZ start site. Little or no repression is observed, so that the staining pattern is derepressed completely in ventral regions. Different transgenic lines containing the fusion gene in A exhibit somewhat variable staining patterns. The staining pattern in A represents efficient ventral repression; other lines show slightly derepressed patterns [data not shown].

Figure 5. The Kr repressor can silence multiple enhancers. Precellular transgenic embryos are oriented as in Figs. 1–4. They express a synthetic modular promoter containing the 2XPE mesoderm-specific enhancer placed upstream of the rho NEE. (A) lacZ staining pattern obtained with the fusion gene containing a single Kr-binding site located near the 5’ end of the 700-bp rho NEE [see diagram beneath the embryo]. Neither enhancer is repressed, in that uniform staining is observed along the anteroposterior axis in both the presumptive mesoderm and neuroectoderm. The arrow shows the gap between the 2XPE and NEE expression domains. (B) Same as A except that the Kr-binding site was placed 75 bp upstream of the lacZ transcription start site. Both enhancers are repressed; mesoderm staining is nearly abolished in central regions containing high concentrations of Kr (2XPE enhancer), whereas the NEE lateral stripes are attenuated in the same domain.
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Figure 6. The sna repressor can both quench and silence a modular eve promoter. Precellular transgenic embryos contain a modular promoter consisting of the 480-bp stripe 2 enhancer placed upstream of the 500-bp stripe 3 enhancer. A 160-bp spacer sequence separates the two enhancers. (A) lacZ staining pattern obtained with the fusion promoter containing four sna repressor sites inserted in the spacer sequence (see diagram beneath the embryo). These sna sites map far from activators in both enhancers (>150 bp). Consequently, neither enhancer is repressed, and both stripes are expressed uniformly in dorsal and ventral regions. Note that stripe 7 expression depends on regulatory sequences located in both enhancers, primarily the stripe 3 enhancer. In addition, the anterior head stripe (asterisk) is attributable to cryptic regulatory sequences in the P-transformation vector. (B) Same as A except that three sna repressor sites were inserted within the stripe 2 enhancer. This results in the ventral repression of stripe 2 (arrow). The neighboring stripe 3 enhancer is unaffected. (C) Same as A and B except that two sna sites were placed 55 bp from the lacZ transcription start site. Both stripes 2 and 3 are repressed in ventral regions (arrows), suggesting that sna functions as a dominant silencer when bound near the initiation site.

Figure 7. sna contains a discrete repression domain. Ventrolateral views of transgenic embryos expressing a modified rho NEE that lacks sna repressor sites and contains Gal4 recognition sequences (UAS; see summary diagrams beneath the embryos). (A) lacZ staining pattern obtained with the modified NEE in a normal embryo. A sharp staining pattern is observed in the ventral 20–22 cells, which encompasses the presumptive mesoderm and mesectoderm. (B) Same as A except that the modified NEE was crossed into an embryo that expresses a sna–Gal4 fusion protein in the ventral-most 14–16 cells. The NEE is repressed in regions containing the fusion protein.

The yeast α2/Tup1 complex appears to function via direct repression of the transcription complex (Herschbach et al. 1994). Repression is observed over distances of several hundred base pairs, and genetic studies suggest that the Tup1 repressor interacts with one or more SRBs (suppressor of RNA polymerase B) in the RNA polymerase II (Pol II) holoenzyme complex (Herschbach et al. 1994, Barberis et al. 1995, Liao et al. 1995). It is unclear...
whether the α2–Tupl complex can also quench the neighboring Mcm1 activator or whether Tup1 must be linked closely to activators to contact the transcription complex.

Direct repression has been implicated in both Drosophila and mammalian tissue culture assays. eve has been shown to silence transcription, possibly through direct interactions with the TATA-binding protein (TBP) [Han and Manley 1993a, Um et al. 1995]. A similar mechanism might apply to engrailed [Han and Manley 1993b], although there is also evidence that it can quench upstream activators when bound at a distance from the transcription start site [Jaynes and O'Farrell 1991]. In vitro transcription assays suggest that the unliganded form of the thyroid hormone receptor might block transcription, over short distances, by directly contacting TBP or TFIIIB [Fondell et al. 1993]. Finally, other members of the nuclear receptor family, such as glucocorticoid receptor (GR), can work as repressors when bound >200 bp from the transcription start site [Pearce and Yaffe 1993] and in promoter–proximal regions [Drouin et al. 1993]. Thus, previous studies on repressors raise a number of basic issues. Do they quench, directly repress the transcription complex, and work over short or long distances?

Our analyses of transcriptional repression in the early embryo suggest that there may be two basic forms of repression: short-range and long-range. We have analyzed the activities of six different repressors that are localized in precellular embryos: sna, Kr, kni, giant (gt), dl, and hairy [Gray et al. 1994; Paroush et al. 1994; Arnosti et al. 1996; Arnosti et al., in prep.]. Four of the six embryos, sna, Kr, kni, and gt, appear to function over short distances, <100 bp, to either quench neighboring activators within a target enhancer or directly repress the transcription complex (this study; Arnosti et al., in prep.). In contrast, both dl [and associated corepressors] and hairy appear to work over long distances (>1 kb) to silence the transcription complex [Ip et al. 1991; Jiang et al. 1993; Kirov et al. 1993; Lehming et al. 1994]. For example, dl/corepressors can repress the ventral expression of the eve stripe 2 enhancer over a distance of ~5 kb [H. Cai and M. Levine, unpubl.]. The hairy repressor can function in a dominant fashion and block multiple enhancers when bound ~1 kb upstream of the transcription start site [S. Barolo and M. Levine, unpubl.].

Mechanisms of short-range repression

Several different mechanisms of repression can be envisioned. The simplest view is that the short-range repressor directly inhibits upstream activators through protein–protein interactions. Perhaps sna and Kr repression domains block activator–promoter interactions. This direct mechanism of quenching may be compromised by the demonstration that both sna and Kr can silence the transcription complex when bound to promoter–proximal regions. However, it is conceivable that the repressors interact with upstream activators as they loop to the transcription complex. Alternatively, the repressors may recognize common motifs present in both upstream activators and the transcription complex [for review, see Goodrich and Tjian 1994].

Another potential mechanism involves the recruitment of one or more corepressors. As discussed above, the yeast α2 repressor recruits Tup1, which in turn inhibits the transcription complex [Hersbach et al. 1994]. Moreover, hairy recruits groucho, which is related to Tup1 by virtue of WD40 repeats [Paroush et al. 1994]. Perhaps hairy represses transcription through a groucho–promoter interaction similar to the situation described for α2–Tup1. Recent studies suggest that the Tup1 repression complex is quite large, >1 million daltons, and contains multiple Tup1 and Smn6 subunits [A.D. Johnson, pers. comm.]. Short-range repressors, such as Kr and sna, might recruit smaller repression complexes that function only over short distances.

Tissue culture assays and in vitro binding experiments suggest that Kr may repress transcription by interacting with the TFIIΕ-β subunit of the transcription complex.
Our results do not rule out such a mechanism; however, we note that the earlier study also suggests that Kr activates transcription through interactions with TFIIB. A model was presented whereby Kr monomers activate transcription, whereas dimers mediate repression. However, we have shown that a single Kr-binding site causes both the rho NEE and twi 2XPE enhancers to be repressed continuously in response to the Kr protein gradient in central regions of the embryo (e.g., Fig. 2). Activation is not observed at the margins of the pattern where there are diminishing levels of Kr protein.

Short-range repression in complex loci

The flexibility of short-range repression is particularly evident for complex genetic loci (summarized in Fig. 8). In the present study a complex locus was created by placing a modular promoter between two divergently transcribed reporter genes, white and lacZ. When either snail or Kr binds to a given enhancer (enhancer 1; see Fig. 8A), this enhancer is barred from interacting with both white and lacZ. However, the neighboring enhancer 2 is free to interact with both genes. In this case, local repression permits enhancers to function autonomously. In contrast, when snail or Kr repressor sites are placed near the lacZ transcription start site, both enhancer 1 and enhancer 2 are blocked (Fig. 8B). Despite this dominant repression of lacZ, both enhancers are unimpeded by interacting with the white gene. Thus, in this case, the short-range repressor permits promoter autonomy within a gene complex. This flexibility, enhancer autonomy or promoter autonomy, contrasts with the binding of long-range repressors within the complex, whereby both enhancers would be blocked on both promoters (Fig. 8C).

This form of repression might help explain localized patterns of homeotic gene expression within the Bithorax complex (BX-C). Repressors encoded by the gap genes hunchback [hb], Kr, and knirps [knii], have been shown to play a critical role in establishing the anterior boundaries of BX-C gene expression (Zhang et al. 1991; Qian et al. 1991; Busturia and Bienz 1993). For example, Kr binds within the iab3 enhancer, which is located downstream of the abdominal-A (abd-A) gene (Shimell et al. 1994). Kr is required for keeping abd-A off, at least initially, in parasegment [PS] 6. It does not influence the expression of the neighboring Ultrabithorax [Ubx] gene, which is expressed in PS6, possibly because of the short-range nature of Kr repression.

Materials and methods

Plasmid constructions

The synthetic fusion promoter containing the 152-bp rho NEE and 480-bp eve stripe 2 enhancer (Fig. 1A) has been described previously (Gray et al. 1994). The 700-bp rho NEE (Ip et al. 1992b; Gray et al. 1994) was inserted into the lacZ 3' polylinker of the C4PLZ transformation vector (Wharton et al. 1993). Sna sites were created in the 5' polylinker sequence by inserting a synthetic 35-bp sequence, containing two sna sites (CAGCAAGGTG) separated by a 10-bp spacer, into either the SphI or the KpnI site. The spacer sequence separates the sna s2 and s3 sites in the native rho promoter (Ip et al. 1992b). The 2XPE twi enhancer was inserted between the EcoRI and KpnI sites of the C4PLZ vector; the 700-bp rho NEE (containing a synthetic Kr site) was cloned into the unique BamHI site. The eve stripe 2/stripe 3 fusion promoters (Fig. 6) were described previously (Small et al. 1993). Four sna sites were placed in the spacer between stripes 2 and 3 (Fig. 6A) by inserting two tandem copies of the 35-bp sequence, described above, into a synthetic SphI site located 150 bp 3' of the proximal bed site in the stripe 2 enhancer. Two sna sites were placed 55 bp 5' of the lacZ transcription start site (Fig. 6C) by inserting one copy of the 35-bp sequence into a synthetic SphI site located at the 3' end of the 500-bp stripe 3 enhancer. The sna sites in the stripe 2 enhancer (Fig. 6B) were created by site-directed mutagenesis (see below).

To construct the sna–Gal4 fusion sequence, an Xhol–XbaI fragment from the 2XPE twi–lacZ fusion gene (Jiang and Levine 1993) containing twi promoter sequences from −180 bp to +160 bp and the lacZ coding region was replaced by a truncated 1.6-kb snail promoter (Ip et al. 1992a), driving the snail cDNA through codon 244 fused to the 5' end of the coding region for Gal4 amino acids 2–93. A synthetic NotI site creates three alanine codons between snail amino acids 244 and Gal4 amino acid 2.

The UAS reporter (Fig. 7; modified from the 200-bp rho NEE described in Gray et al. 1994) was synthesized from a series of oligonucleotides and subcloned into the unique NEE site of the C4PLZ vector. Binding site identities were confirmed by sequence analysis. The d1, 2, 3, and 4 sites and the twi t1 E box were unchanged. The twi t2 E box/sna s4 site and the E box/sna s3 site were changed to the twi t1 binding sequence (CATATG). The sna s2 site was replaced by a 15-bp dimeric Gal4-binding site (GGAGGACAGTCCTCC). Additional Gal4-binding sites were created at the 5' end of the modified NEE, and between the d3 and d4 activator sites. Gal4-binding sites are located relative to the putative rho transcription start as follows: −1735 to −1749 bp, −1842 to −1856 bp, and −1865 to −1879 bp.

P element-mediated germ line transformation and whole mount in situ hybridization

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

Site-directed mutagenesis

The pBluescript SK + plasmid and CJ236 strain of Escherichia coli were used to prepare uracil-containing single-stranded DNA templates. Mutagenesis was performed by annealing the templates with various mutagenic oligonucleotides. The in vitro-synthesized double-stranded DNA was transformed into the DH5α strain of E. coli. The identities of the mutagenized templates were confirmed by sequence analysis.

Synthetic Kr sites [AAAACGGGTTAAGC] were created at the following positions in the rho NEE (all numbers refer to distance from the putative rho transcription start site): −2010 to −2023 bp, −1639 to −1652 bp, −2129 to −2142 bp, and −1533 to −1546 bp. Synthetic sna sites (CAGCAAGGTG)
were created in the rho NEE at −1202 to −2021 bp and −1649 to −1658 bp. Synthetic sna sites were created in the eve stripe 2 enhancer at −1153 to −1162 bp, −1235 to −1244 bp, and −1386 to −1395 bp relative to the eve transcription start site. Defective NEEs lacking native sna-binding sites were described by Ip et al. [1992b].

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S Gray and M Levine

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