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Transcriptional Repression in the Drosophila Embryo

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Transcriptional repression in the *Drosophila* embryo

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SUMMARY

Transcriptional repression is essential for the conversion of crude maternal gradients into sharp territories of tissue differentiation in the *Drosophila* embryo. Evidence will be presented suggesting that some of the embryonic repressors function through a short-range 'quenching' mechanism, whereby a repressor works over short distances (*ca.* 50 b.p.) to block neighbouring activators within a target enhancer. This type of repression can explain how different enhancers work autonomously within complex modular promoters. However, at least one of the repressors operating in the early embryo works through a long-range, or silencing, mechanism. The binding of a silencer to a given enhancer leads to the inactivation of all enhancers within a complex promoter. The analysis of chromatin boundary elements suggest that silencers and enhancers might work through distinct mechanisms. We speculate that silencers constrain the evolution of complex promoters.

1. INTRODUCTION

In this report we shall focus on transcriptional repression in the early *Drosophila* embryo. First, we shall briefly review evidence that repression is at least as important as activation in establishing localized patterns of gene expression during embryogenesis. Afterwards, specific mechanisms of repression will be discussed and evidence will be presented that the early embryo employs at least two distinct modes of repression.

Embryonic patterning is initiated by two maternal regulatory proteins, bicoid (bcd) and dorsal (dl) (St Johnston & Nusslein-Volhard 1992). The bcd protein is distributed in a broad concentration gradient, with peak levels present in anterior regions and progressively lower levels in posterior regions. The bcd gradient is responsible for the differentiation of head structures and is also important for initiating the segmentation cascade. The dl protein is distributed in a broad ventral-to-dorsal gradient, with peak levels present along the ventral surface. This dl gradient initiates the differentiation of several different embryonic tissues during early development. Both bcd and dl are transcription factors, and to determine how they control development we have analysed target genes that they directly regulate.

2. ANTEROPOSTERIOR PATTERNING

We have characterized the bcd target gene, *even-skipped* (*eve*). *eve* is expressed in a series of seven stripes along the anteroposterior axis of precellular embryos (Harding *et al.* 1986; Macdonald *et al.* 1986; Frasch *et al.* 1987). These *eve* stripes foreshadow the subdivision of the embryo into a repeating series of body segments. The *eve* promoter contains several non-overlapping enhancers that control the expression of individual stripes (Harding *et al.* 1989; Goto *et al.* 1989; summarized in figure 1). For example, *eve* stripe 2 is regulated by a 500 b.p. enhancer that is located about 1 kb upstream of the *eve* transcription start site (Small *et al.* 1992; figure 1*b*). This enhancer directs an authentic stripe of expression when taken out of the context of the complex *eve* promoter. Thus, a stripe2-lacZ fusion gene is expressed exactly within the limits of the endogenous *eve* stripe 2 pattern in transgenic embryos (Small *et al.* 1992).

The modular *eve* promoter contains a series of autonomous enhancers. The first 5 kb of the *eve* promoter is sufficient to direct the expression of stripes 2, 3 and 7 (figure 1). The stripe 2 and stripe 3 enhancers are each 500 b.p. in length and are separated by a 1.8 kb spacer sequence (Small *et al.* 1993). A major goal of our studies is to determine how these enhancers work independently of one another within the same promoter.

Repression plays an important role in the regulation of both stripes. The stripe 2 and stripe 3 enhancers are activated by broadly distributed regulatory factors in the early embryo. The stripe borders are formed through repression (Stanojevic *et al.* 1989, 1991; Small *et al.* 1991, 1992). For example, *eve* stripe 2 can be activated in nearly the entire anterior half of the embryo. The broad bcd gradient triggers a steeper pattern of *hunchback* (*hb*) expression, and then bcd and hb act in concert to activate the stripe 2 enhancer in anterior regions. An anterior repressor, giant (gt), defines the anterior stripe border, while another repressor, Kruppel (Kr), establishes the posterior border. All four regulatory proteins, bcd, hb, gt, and Kr, bind with high affinity to multiple sites in the minimal stripe 2 enhancer (Small *et al.* 1991). There is a total of 12 factor binding sites, including six activator sites and six repressor sites. There is tight linkage of the activator and repressor sites: four of the six activator sites directly overlap a gt or Kr repressor site. This arrangement of binding sites suggest that gt and Kr

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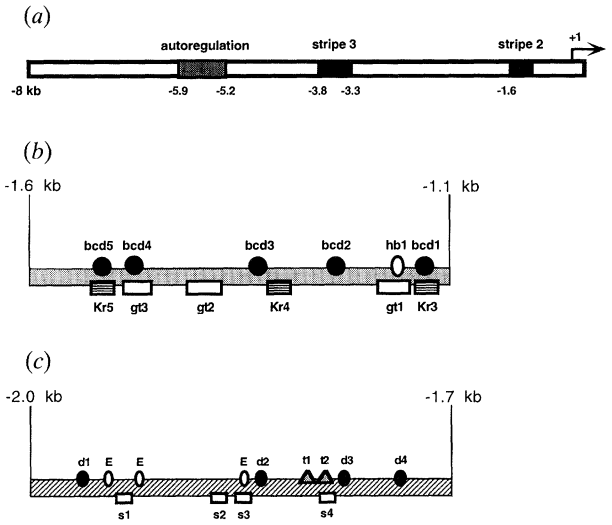

Figure 1. Summary of the *eve* promoter and minimal stripe enhancers. (*a*) Summary of the first 8 kb of the *eve* promoter region. The stripe 2 and stripe 3 enhancers are 500 b.p. apiece and are separated by a 1.8 kb spacer region. (*b*) Factor binding sites in the minimal stripe 2 enhancer. The horizontal bar represents the 500 b.p. sequence, which maps about 1 kb upstream of the *eve* transcription start site. There is a total of 12 factor binding sites, including six activator sites (five bcd and one hb) and six repressor sites (three gt and three Kr). (*c*) Factor binding sites in the *rho* lateral stripe enhancer (NEE). This 300 b.p. enhancer maps about 1.7 kb upstream of the *rho* transcription start site. It contains nine activator sites (four high affinity dl sites and five E boxes) and four sna repressor sites.

define the stripe borders through a simple competition mechanism of repression.

Recent studies suggest that competition might not represent an essential mechanism of *eve* regulation. There is a total of three gt repressor sites (summarized in figure 1*b*). Two of these sites, gt-1 and gt-3, directly overlap a bcd and hb activator site, respectively. The third site, gt-2, does not overlap an activator site, but instead it maps about 50 b.p. away from the closest bcd activator. Nonetheless, systematic mutations in each of these gt sites suggest that the gt-2 site is the most critical repressor site mediating the formation of the anterior stripe 2 border. Mutations in the gt-1 and gt-3 sites cause only a slight disruption in the stripe 2 pattern. In contrast, mutations in the gt-2 site result in an abnormal pattern of expression, including a severe anterior expansion of the anterior stripe border. These results argue against a competition mechanism of repression, and instead suggest that gt binds to the stripe 2 enhancer and then works over short distances to interfere with neighbouring bcd activators already bound to DNA. We refer to this mode of repression as 'quenching' (see below).

3. DORSOVENTRAL PATTERNING

Repression also plays an essential role in establishing localized patterns of gene expression along the dorsoventral axis of the early embryo. The dl gradient is responsible for initiating the differentiation of three basic embryonic tissues, the mesoderm, neuroectoderm and dorsal ectoderm (Jiang & Levine 1993). Peak levels of dl in ventral regions activate the expression of regulatory genes that are important for initiating the differentiation of the mesoderm (Thisse *et al.* 1991; Pan *et al.* 1991; Jiang *et al.* 1991). Lower levels of dl in lateral regions trigger the expression of neuroectodermal regulatory genes (Ip *et al.* 1992*a*). Finally dl also functions as a transcriptional repressor that restricts the expression of certain genes to dorsal regions where they are important for the differentiation of the dorsal ectoderm (Doyle *et al.* 1989; Jiang *et al.* 1993; Kirov *et al.* 1993, 1994; Huang *et al.* 1993). To determine how the dl regulatory gradient establishes these three territories of tissue differentiation, we have analysed target genes that are directly regulated by different concentrations of dl protein.

The dl target gene *snail* (*sna*) is activated only by high concentrations of the dl gradient (Ip *et al.* 1992*b*). Consequently, *sna* expression is restricted to ventral regions corresponding to the presumptive mesoderm. Low levels of dl fail to activate *sna* in lateral regions that will form the neuroectoderm. *sna*-expressing cells invaginate through the ventral furrow to form the embryonic mesoderm. In *sna*$^{-}$-mutants, the furrow fails to form and mesodermal derivatives are absent (Simpson 1983). Another dl target gene, *rhomboid* (*rho*), is expressed in lateral 'stripes' that coincide with the presumptive neuroectoderm (Bier *et al.* 1990; Ip *et al.* 1992*a*). There are two *rho* stripes, one on either side of the ventral midline. In principle, both high and low levels of the dl gradient can activate *rho* in ventral and lateral regions, corresponding to the presumptive mesoderm and neuroectoderm. However, *rho* is excluded from the ventral mesoderm by sna (Ip *et al.* 1992*a*). As indicated above, *sna* is directly regulated by the dl gradient, but its expression is restricted to the ventral mesoderm. The sna protein functions as a sequence-specific repressor (see below), which keeps *rho* off in ventral regions and restricted to the lateral neuroectoderm.

The lateral stripes of *rho* expression are regulated by a 300 b.p. enhancer (the 'NEE'), which contains an arrangement of factor binding sites that is reminiscent of the *eve* stripe 2 enhancer (Ip *et al.* 1992*a*; figure 1*c*). There is a total of nine activator sites, including four high affinity dl binding sites. In principle, these activator sites can initiate *rho* expression in both ventral and lateral regions in response to the dl gradient. However, expression is excluded from ventral regions by four sna repressor sites. As for the stripe 2 enhancer, these sna sites are tightly linked to activator sites (three of the four sna sites overlap an activator site). Again, this organization of activator and repressor sites suggests a simple competition mechanism of repression, although we shall present evidence that sna need not function in this fashion.

Repression is essential for allowing the dl regulatory gradient to establish three territories of tissue differentiation. The characterization of a number of different dl target genes suggests that there are two essential threshold responses to the dl gradient (Jiang & Levine 1993). Type I promoters contain low affinity dl binding sites, and are activated only in ventral regions where there are high concentration of dl protein. Type

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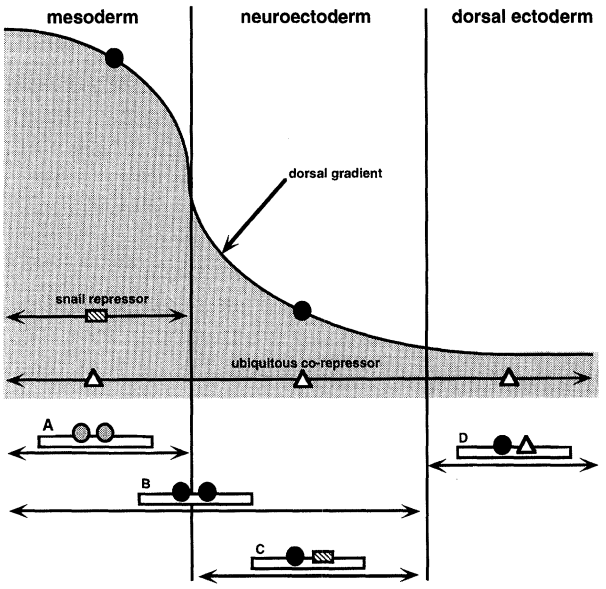

Figure 2. Repressors convert the dl gradient into three territories of tissue differentiation. A diagrammatic representation of the dl protein gradient. This gradient is responsible for initiating the differentiation of the mesoderm, neuroectoderm and dorsal ectoderm. The characterization of various dl target genes suggests that there are two classes of target promoters, type I and type II. Type I promoters (including *twi* and *sna*) contain low affinity dl binding sites and are activated only in the presumptive mesoderm where there are peak levels of dl protein (A). In contrast, type II promoters contain high affinity dl binding sites and can be activated in both ventral and lateral regions, the mesoderm and neuroectoderm, in response to both high and low levels of the dl gradient (B). Transcriptional repression is essential for converting the dl gradient into three territories of tissue differentiation. The *sna* gene contains a type I promoter so that its expression is restricted to the presumptive mesoderm (A). The sna protein functions as a repressor, and type II promoters (or enhancers) that contain sna repressor sites are excluded from the mesoderm and restricted to the lateral neuroectoderm (C). The specification of the dorsal ectoderm requires that the dl gradient functions as both an activator and a repressor. Certain target genes can be activated throughout early embryos by ubiquitously distributed activators, but are excluded from ventral and lateral regions by dl. These target promoters contain high affinity dl binding sites, and neighbouring 'corepressor' sites that convert the dl activator into a long-range silencer (D).

II promoters contain high affinity dl sites and can be activated in both ventral and lateral regions, the presumptive mesoderm and neuroectoderm, in response to both high and low levels of dl. Thus, occupancy of binding sites is a critical determinant of the threshold response. Repression is responsible for converting these two thresholds into three territories (summarized in figure 2). First, as mentioned above, type II promoters (like the *rho* NEE) that contain sna repressor sites are restricted to the presumptive neuroectoderm. That is, the sna repressor plays an important role in restricting gene expression to the neuroectoderm. The third territory of tissue differentiation, the dorsal ectoderm, requires that the dl regulatory gradient also functions as a repressor (Doyle *et al.* 1989; Jiang *et al.* 1993; Kirov *et al.* 1993; Huang *et al.* 1993). There is a number of genes that can be activated throughout the embryo, in all three territories. However, the dl gradient represses their expression in ventral and lateral regions, so that expression is restricted to the dorsal ectoderm. These promoters contain 'corepressor' sites, so that dl can function as a repressor (see figure 2).

4. MODES OF REPRESSION

At least three distinct mechanisms of repression can be envisaged (reviewed by Levine & Manley 1989; see figure 3). First, activators and repressors can compete for the same site (or overlapping sites), so that the repressor works by blocking the interaction of an activator with the target promoter. As mentioned earlier, this type of mechanism is suggested by the arrangement of factor binding sites in the *eve* stripe 2 enhancer and the *rho* NEE. Second, direct repression involves the binding of a repressor to an upstream region in a target promoter or enhancer. The repressor does not block upstream activators, but instead it directly interferes with the assembly or function of the basal transcription complex. This type of mechanism appears to account for the way in which the alpha2 homeodomain protein regulates mating type in yeast (Keleher *et al.* 1992). Finally, according to a quenching scenario, activators and repressors bind to nearby sites in a target promoter or enhancer. The repressor does not interfere with the binding of the activator, but instead, it works over short distances to block the ability of the activator to contact the transcription complex. Although this a logical mechanism of repression, it has not been rigorously established in any eukaryotic organism. Studies on the sna repressor suggest that it might function via quenching (Gray *et al.* 1994).

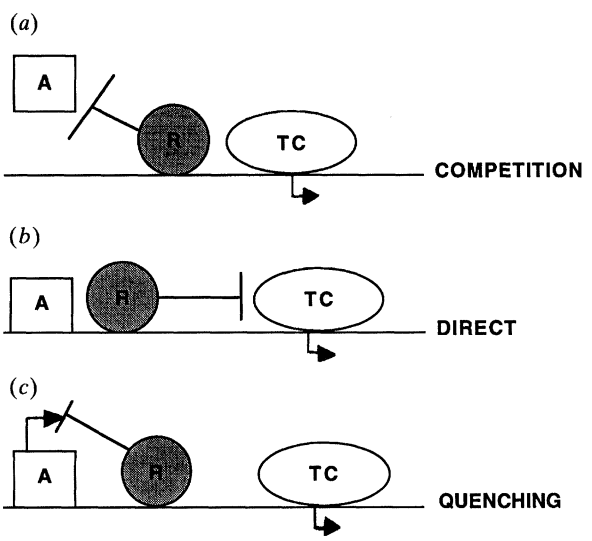

Figure 3. Modes of repression. (*a*) Repressors and activators compete for a common binding site (or an overlapping sequence), so that the repressor blocks the ability of the activator to bind a target promoter. (*b*) A repressor binds to an upstream site and then directly blocks the assembly or function of the transcription complex. (*c*) Repressors and activators bind to nearby sites and then the repressor somehow blocks the upstream activator from contacting the transcription complex.

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5. SNA REPRESSION

The initial studies on sna involved the use of a defective *rho* NEE containing point mutations in all four sna repressor sites. The loss of these sites results in an abnormal pattern of expression, whereby the NEE directs equally intense expression in both lateral and ventral regions (Ip *et al.* 1992*a*). Synthetic sna binding sites were then introduced at variable distances from the dl activator sites contained within the defective NEE. The goal was to identify the maximum distance that synthetic sna sites could be placed from dl activator sites and still mediate repression in the ventral mesoderm. Synthetic sna sites placed 150 b.p. away from the closest dl activator sites are ineffective and fail to repress ventral expression. However, efficient repression is observed when the same sna sites are placed about 50 b.p. away from the dl activator sites. A modified NEE, lacking the native sna sites but containing the synthetic sites positioned at these distances, directs an essentially normal pattern of expression (lateral stripes) in transgenic embryos (Gray *et al.* 1994). These and other observations argue against a competition mechanism since efficient repression is observed with a spacing of 50 b.p. between activator and repressor. *In vitro* binding assays suggest that dl and sna can cooccupy sites that are separated by just 6 b.p. A direct mechanism of repression does not appear to be involved since the efficiency of repression depends on the linkage of the synthetic sna sites with upstream dl activator sites, and not proximity to TATA. For example, poor repression is observed when the sna sites map *ca.* 80 b.p. from TATA but over 150 b.p. from dl activator sites in the NEE. This leaves the possibility of repression by quenching. sna appears to bind to the *rho* NEE and then work over short distances to block nearby dl activators.

sna is not a 'dedicated' repressor, but instead it is able to block the action of heterologous activators, such as bcd. The first evidence for this view came from the analysis of a synthetic promoter containing several dl and bcd activator sites, as well as several sna repressor sites. This promoter directs a combinatorial pattern of expression in transgenic embryos, based on the known distribution patterns of the regulatory factors. Staining is broad and intense in anterior regions where there are high concentrations of both the dl and the bcd activators. The pattern progressively diminishes in posterior regions owing to limiting amounts of the bcd activator. Ventral repression extends along the entire length of the embryo, in both anterior and posterior regions, which suggests that sna can block both dl and the heterologous bcd activator (Gray *et al.* 1994).

More definitive evidence that sna can block bcd was obtained by analysing the expression of a modified *eve* stripe 2 enhancer containing synthetic sna repressor sites. Three sna sites were placed about 50 b.p. away from each of three different bcd activator sites in the stripe 2 enhancer. Normally, the enhancer directs an equally intense stripe in both dorsal and ventral regions. However, the sna sites repress the modified enhancer in ventral regions, the presumptive mesoderm, where there are high concentrations of sna protein. These results suggest that sna binds to a target enhancer, and then functions over short distances to block nearby activators. At present, the exact mechanistic details are obscure. It is possible that sna directly 'touches' the activation domain of neighbouring activators. Alternatively, sna repression might involve accessory proteins, as has been observed for the yeast alpha2 and *Drosophila* hairy proteins (Keleher *et al.* 1992; Paroush *et al.* 1994). These repressors recruit related non-DNA binding proteins, tup-1 and groucho, respectively.

6. ENHANCER AUTONOMY

Regardless of the detailed mechanism, the main point we wish to emphasize is that sna-mediated repression is short-range, and occurs only within the limits of a target enhancer. In principle, short-range repression can explain how different enhancers work independently of one another within a complex promoter. For example, the binding of the Kr repressor to the *eve* stripe 2 enhancer would not interfere with the expression of the stripe 3 enhancer if it works only over short distances, within the confines of the stripe 2 enhancer (Small *et al.* 1993).

An example of enhancer autonomy is observed with a synthetic promoter containing the *rho* NEE attached to the *eve* stripe 2 enhancer. This promoter directs a fully additive pattern of expression in transgenic embryos, which includes a normal *eve* stripe and *rho* lateral stripes (figure 4). Thus, a repressor bound to one enhancer does not interfere with the expression of the neighbouring enhancer. For example, sna bound to the *rho* NEE does not interfere with the bcd activators

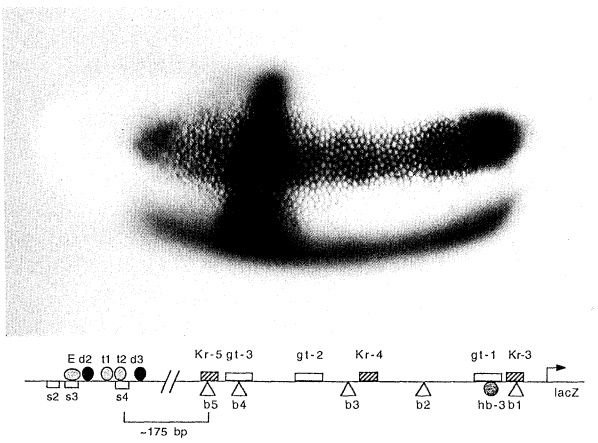

Figure 4. Enhancer autonomy and additive expression patterns. Lateral view of a transgenic embryo that contains a fusion promoter with a minimal *rho* NEE and the *eve* stripe 2 enhancer (see diagram below the embryo). The embryo is oriented with anterior to the left; it is undergoing cellularization (*ca.* 3 h after fertilization). The two enhancers function is a completely additive fashion, to yield a composite pattern (an *eve* stripe + the *rho* lateral stripes). This result suggests that repressors bound to one enhancer do not interact with activators bound to the neighbouring enhancer. For example, sna bound to the NEE does not interfere with bcd activators in the stripe 2 enhancer since they are separated by 175 b.p., which is beyond the range of efficient sna repression.

in the neighbouring stripe 2 enhancer, since these map too far away. As indicated above, sna works best when located no more than 50 b.p. from a neighbouring activator. In the case of the composite NEE-stripe 2 promoter, sna sites in the NEE map 175 b.p. from the closest bcd sites in the stripe 2 enhancer (see figure 4). This distance is beyond the range of effective sna repression. In summary, short-range repression can account for the evolution of complex modular promoters through the serial addition of separate autonomous enhancers.

7. SILENCING

Preliminary studies suggest that other embryonic repressors might also function through a similar short-range mechanism. These include the segmentation repressors gt, Kr and knirps. However, at least one repressor does not work in this fashion, dl. dl functions as a long-range silencer. To contrast the consequences of short-range versus long-range repression, consider a complex promoter containing two separate enhancers (figure 5). When a short-range repressor, such as sna, binds to one of the enhancers, it inactivates only that enhancer. The neighbouring enhancer is free to interact with the promoter. According to this view, the integration of regulatory information occurs at the level of the enhancer; in principle, the promoter is 'unaware' of the repressor. In contrast, a long-range repressor, or silencer, might function through the direct inactivation of the transcription complex. Consequently, the silencer causes the dominant inactivation of all enhancers within the promoter. It would appear that the dl repressor functions through this type of dominant long-range silencing mechanism.

We have characterized the dl target gene *zen* (Doyle *et al.* 1989; Ip *et al.* 1991; Jiang *et al.* 1992, 1993). *zen* expression is restricted to dorsal regions of early embryos where it is important for the differentiation of a specialized region of the dorsal ectoderm, the amnioserosa. In principle, *zen* can be activated throughout the embryo, in both dorsal and ventral regions, but it is repressed in ventral regions by the dl gradient. In *dl* mutants, *zen* is derepressed in ventral regions. The *zen* promoter region contains a silencer element that is located about 1 kb upstream of the transcription start site. The silencer contains high affinity dl binding sites, as well as negative elements that bind one or more 'corepressors' (Jiang *et al.* 1993; Kirov *et al.* 1993; Lehming *et al.* 1994). dl is intrinsically an activator, but when it binds next to corepressors it is converted into a long-range silencer that can block the ventral expression of various heterologous promoters over long distances. For example, the dl silencer can block the ventral expression of *eve* stripe 2 even when positioned 5 kb away from the closest bcd activator sites in the stripe enhancer (H. Cai & M. Levine, unpublished results). This ventral repression is obviously distinct from the repression mediated by sna, which requires close proximity to the bcd activator sites.

8. CHROMATIN BOUNDARY ELEMENTS

Recent studies suggest that the dl silencer might

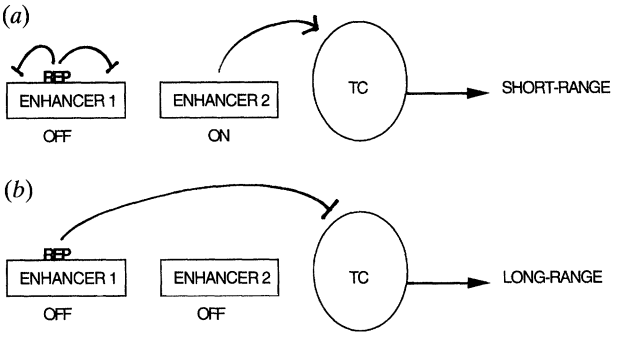

Figure 5. Short-range versus long-range repression. (*a*) Hypothetical promoter containing two non-overlapping enhancers. A short-range repressor, such as sna, inactivates only the enhancer to which it is bound (enhancer 1). The neighbouring enhancer is free to interact with the transcription complex (C). (*b*) The binding of a silencer to a given enhancer leads to the inactivation of all enhancers in the promoter.

work over long distances through a mechanism that is distinct from enhancer–promoter interactions. This conclusion stems from an analysis of chromatin boundary elements in *Drosophila*. Several DNA fragments have been identified that can insulate a transcription unit from neighbouring cis regulatory elements (Kellum & Schedl 1991; Geyer & Corces 1992). For example, a 340 b.p. DNA fragment from the gypsy retrotranspon will block upstream, but not downstream, enhancers when inserted into a complex promoter. Indeed, a simple stripe assay in the early embryo confirms this directional repression. The insertion of the gypsy fragment into the *eve* promoter directionally blocks upstream stripe enhancers (H. Cai & M. Levine, unpublished results). Similar results were obtained with the 900 b.p. scs element located at the boundaries of the hsp70 heat shock locus (Kellum & Schedl 1991).

Although the gypsy boundary element efficiently blocks enhancer–promoter interactions, it does not appear to block the dl silencer. This conclusion is based on the characterization of a fusion promoter that contains the gypsy element located between an upstream dl silencer and downstream stripe 2 enhancer. As discussed above, the silencer blocks the ventral expression of the stripe 2 enhancer. It continues to block this expression even when the gypsy boundary element is interposed between the silencer and downstream stripe 2 enhancer. This observation suggests that the boundary element fails to block the dl silencer. An implication of this observation is that enhancers and silencers work through fundamentally different mechanisms. For example, enhancers are thought to function through a 'looping' mechanism; perhaps silencers alter chromatin structure. Obviously a number of other scenarios can be envisaged.

9. FUTURE PROSPECTS

In conclusion, we note that the dl silencer does not operate in a void. As already discussed, it is associated with the *zen* gene, which is located within the Antennapedia gene complex (ANT-C). The ANT-C spans 350 kb of DNA and includes ten different

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transcription units (see Lindsley & Zimm 1992). Perhaps as much as 100 kb of genomic DNA corresponds to cis regulatory information, including between 20 to 50 different enhancers and silencers. How do the proper enhancers and silencers interact with the right promoters? For example, what protects the neighbouring *proboscipedia* and *Deformed* genes from the dl silencer? Future studies will address this question of 'enhancer trafficking', and identify and characterize potentially novel chromatin boundary elements within the ANT-C.

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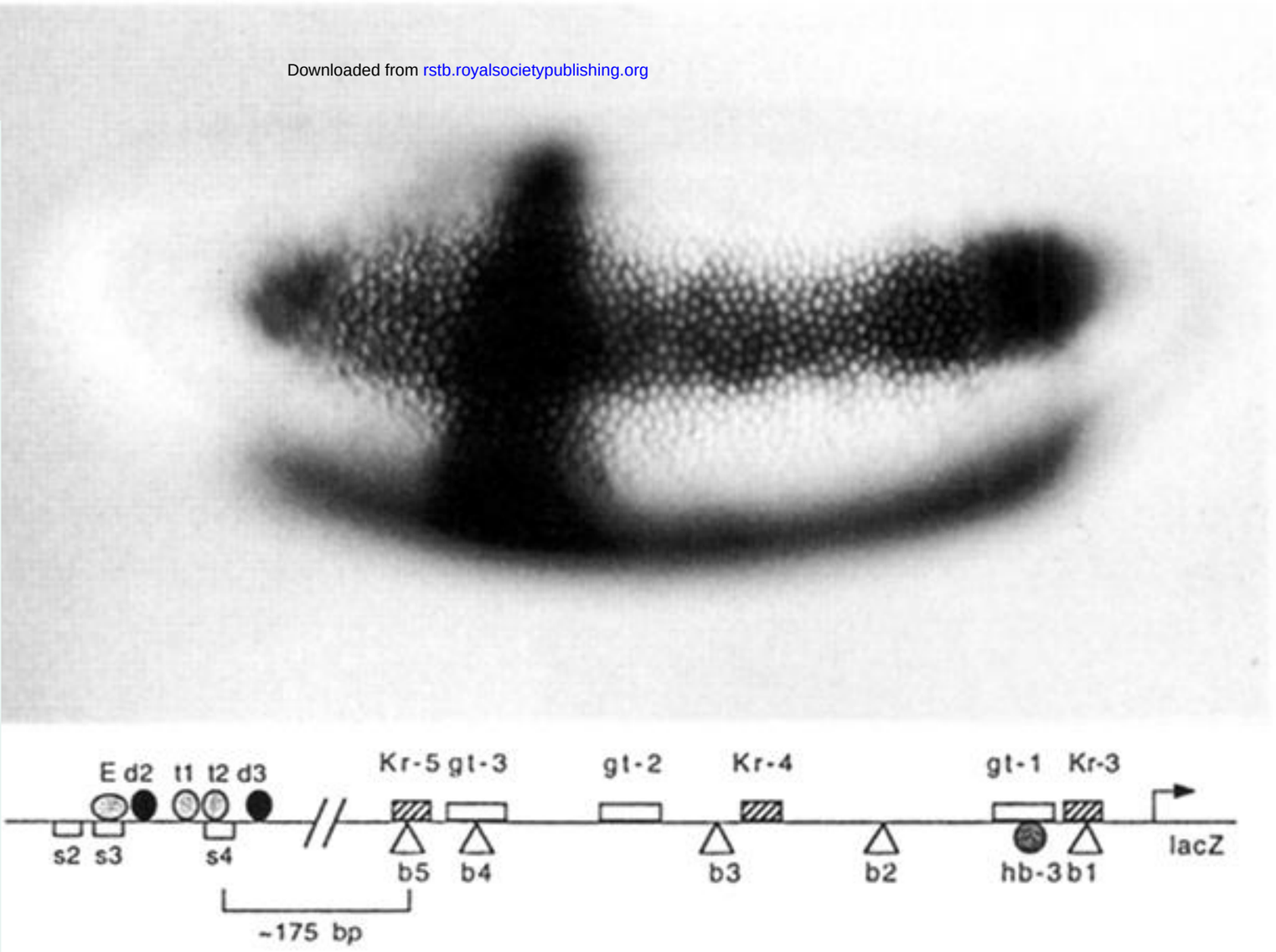

gure 4. Enhancer autonomy and additive expression tterns. Lateral view of a transgenic embryo that contains usion promoter with a minimal *rho* NEE and the *eve* stripe nhancer (see diagram below the embryo). The embryo is ented with anterior to the left; it is undergoing llularization (*ca.* 3 h after fertilization). The two enhancers nction is a completely additive fashion, to yield a composite ttern (an *eve* stripe + the *rho* lateral stripes). This result ggests that repressors bound to one enhancer do not teract with activators bound to the neighbouring enhancer. r example, sna bound to the NEE does not interfere with d activators in the stripe 2 enhancer since they are arated by 175 b.p., which is beyond the range of efficient a repression.

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