The *dorsal* gradient morphogen regulates stripes of *rhomboid* expression in the presumptive neuroectoderm of the *Drosophila* embryo

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*rhomboid* (*rho*) encodes a putative transmembrane receptor that is required for the differentiation of the ventral epidermis. It is initially expressed before the completion of cellularization in lateral stripes within the presumptive neuroectoderm. Here, we present evidence that the maternal morphogen *dorsal* (*dl*) acts in concert with basic helix-loop-helix (*b-HLH*) proteins, possibly including *twist* (*twi*), to activate *rho* in both lateral and ventral regions. Expression is blocked in ventral regions (the presumptive mesoderm) by *sna/l* (*sna*), which is also a direct target of the *dl* morphogen. A 300-bp region of the *rho* promoter (the NEE), which is sufficient for neuroectoderm expression, contains a cluster of *dl* and *b-HLH* activator sites that are closely linked to *sna* repressor sites. Mutations in these binding sites cause genetically predicted changes in the levels and limits of *rho* expression. In particular, the disruption of *sna*-binding sites causes a derepression of the pattern throughout ventral regions, providing evidence that *sna* is directly responsible for establishing the mesoderm/neuroectoderm boundary before gastrulation. The tight linkage of activator and repressor sites in the *rho* NEE is similar to the arrangement of binding sites observed in the *even-skipped* stripe 2 element, which is regulated by *bicoid* (*bcd*). This suggests that the *dl* and *bcd* morphogens use a similar mechanism to make stripes in the *Drosophila* embryo.

**[Key Words: dorsal morphogen; rhomboid expression; b-HLH proteins; Drosophila embryogenesis; neuroectoderm]**

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Twelve genes are essential for initiating dorsal–ventral polarity in the early embryo (for review, see Anderson 1987; Levine 1988; Rushlow and Arora 1990; Govind and Steward 1991). Eleven of these genes act indirectly on the embryonic pattern by controlling the activity of the twelfth gene, called *dorsal* (*dl*), which is the final maternal morphogen (Anderson et al. 1985a,b; Roth et al. 1989, Stein et al. 1991). *dl* is related to the vertebrate oncogene *rel* and the mammalian regulatory factor NF-xB (Steward 1987; Ghosh et al. 1990; Kieran et al. 1990). All three proteins are sequence-specific transcription factors that are regulated at the level of nuclear transport. This process leads to the formation of a *dl* gradient, with peak levels of the protein present in ventral regions and progressively lower levels in lateral and dorsal regions [Roth et al. 1989; Rushlow et al. 1989; Steward 1989]. Peak levels of *dl* trigger the differentiation of the mesoderm [Simpson 1983], whereas lower levels in lateral regions coincide with the presumptive neuroectoderm. *dl* also functions as a transcriptional repressor and restricts the expression of dorsal ectoderm determinants to dorsal regions [Rushlow et al. 1987; St. Johnson and Gelbart 1987].

Mounting evidence indicates that *dl* controls development by directly regulating the expression of tissue determinants (for review, see Ip and Levine 1992). The dorsal–ventral limits of these determinants may depend on the affinities and/or numbers of *dl*-binding sites present in their promoters. Interactions between *dl* and its target genes provide a model for investigating how a morphogen gradient establishes distinct territories of gene activity that determine cell fate in the early embryo. Recent studies suggest that *dl* initiates the differentiation of the mesoderm by activating the expression of two regulatory genes, *twist* (*twi*) and *snail* (*sna*) [Thisse et al. 1987, 1991; Jiang et al. 1991; Pan et al. 1991; Ip et al. 1992]. The *twi* and *sna* promoters contain multiple *dl*-binding sites, and mutations in these sites disrupt the expression in early embryos [Jiang et al. 1991; Ip et al. 1992]. *dl*-binding sites have also been identified in the promoter of another target gene, called *zerknüllt* (*zen*), which is negatively regulated by the morphogen. *zen* expression is normally restricted to dorsal regions of early embryos, but it is expressed in both dorsal and ventral regions in *dl* mutants [Rushlow et al. 1987]. The region of the *zen* promoter that is responsible for repressing ventral ex-
pression contains four high-affinity \( dl \)-binding sites [Doyle et al. 1989; Ipf et al. 1991], and mutations in these sites cause a derepression of the pattern similar to that observed in \( dl^- \) mutants [Jiang et al. 1992].

Although studies on the \( twi, sna, \) and \( zen \) promoters strongly suggest that \( dl \) participates directly in the differentiation of the mesoderm and dorsal ectoderm, substantially less is known about its role in the differentiation of the neuroectoderm. Immunolocalization studies suggest that low levels of \( dl \) protein may extend to the lateral midline, at the boundary between the ventral neuroectoderm and the dorsal ectoderm [Roth et al. 1989; Rushlow et al. 1989; Steward 1989]. As discussed above, these low levels appear to be sufficient to repress \( zen \) and restrict its expression to the presumptive dorsal ectoderm. It has been proposed that \( dl \) regulates neuroectoderm genes indirectly by activating repressors in ventral regions and restricting others to dorsal regions [Irish and Gelbart 1987; Ray et al. 1991]. According to this model, the expression of neuroectoderm determinants is a default state that occurs in lateral regions where there are no repressors. To determine whether low levels of \( dl \) in lateral regions initiate the differentiation of the neuroectoderm directly, we have examined the regulation of a gene called \( rhomboid \) (\( rho \)).

\( rho \) encodes a putative transmembrane protein that may function as a receptor in a cell-signaling pathway and is required for the differentiation of a subset of the ventral epidermal cells that arise from the neuroectoderm [Mayer and Nüsslein-Volhard 1988; Bier et al. 1990]. It is one of several genes that display localized expression in the presumptive neuroectoderm before the onset of gastrulation [i.e., Kosman et al. 1991; Leptin 1991; Rao et al. 1991]. Among these, \( rho \) is the best candidate for a direct target of the \( dl \) morphogen because it displays the earliest restricted expression. Here, we show that a 300-bp sequence [called the neuroectoderm element (\( NEE \))] in the \( rho \) promoter is sufficient to direct early neuroectodermal expression. The \( NEE \) contains four \( dl \)-binding sites, and mutations in these sites virtually abolish expression in the early embryo. Activation of the \( NEE \) also depends on two closely linked E-box sequences [Murre et al. 1989] that are recognized by the \( twi \) protein. The ventral border is defined by \( sna \), which encodes a zinc finger protein [Boulay et al. 1987] that mediates repression by binding near \( dl \) and \( twi \) activator sites within the \( NEE \). The arrangement of activator and repressor sites in the \( rho \) \( NEE \) is similar to that observed for the \( even-skipped \) (\( eve \)) stripe 2 element, which directs a segmentation stripe of expression in response to the \( bicoid \) (\( bcd \)) morphogen gradient [Stanojevic et al. 1989, 1991; Small et al. 1991]. Thus, it appears that the \( bcd \) and \( dl \) morphogens use an analogous mechanism to generate stripes of gene expression in the early embryo.

**Results**

Previous localization studies have shown that the \( rho \) expression pattern is highly dynamic, with transient expression in a variety of tissues, including the neuroectoderm, mesectoderm, peripheral sensory organs, tracheal pits, and amnioserosa [Bier et al. 1990]. Early expression in the neuroectoderm correlates with the reduction in ventral epidermis seen in \( rho^- \) mutants. A genomic DNA fragment containing 2.2 kb of \( rho \) 5'-flanking sequence was found to be sufficient to rescue defects in the ventral epidermis observed in \( rho^- \) embryos [Bier et al. 1990]. When this \( rho \) promoter region is attached to a \( lacZ \) reporter gene [summarized in Fig. 1], it directs many aspects of the normal \( rho \) pattern, including early expression in the presumptive neuroectoderm [Fig. 2A,B]. Expression was visualized by in situ hybridization with a digoxigenin/UTP-labeled antisense RNA probe [Tautz and Pfeifle 1989; Kosman et al. 1991]. Staining is first detected during nuclear cycle 13 and is restricted to the presumptive neuroectoderm by the onset of cycle 14 [Fig. 2A]. This pattern persists during gastrulation and the rapid phase of germ-band elongation [Fig. 2B]. By the completion of cellularization, the homogenous stripes of expression become nonuniform and show crude pair-rule modulations along the anterior–posterior axis. Neuroectoderm expression is lost following elongation and reappears later in the differentiating mesectoderm. During segmentation and germ-band shortening, mesectodermal expression becomes quite intense and staining also becomes visible in the tracheal pits [see Fig. 2D]. There is a 1- to 2-hr delay in the onset of expression in the mesectoderm relative to the endogenous \( rho \) gene [Bier et al. 1990].

**Identification of a minimal \( NEE \)**

To identify regulatory sequences responsible for the early neuroectoderm pattern, various pieces of the \( rho \) promoter were attached to the \( lacZ \) reporter (summarized in Fig. 1). A \( rho-lacZ \) fusion gene containing 2.0 kb of 5'-flanking sequence directs a pattern that is quite similar to the 2.2-kb fusion, although the levels of expression are reduced [data not shown]. The 1.8-kb \( rho-lacZ \) fusion gene displays normal expression during germ-band shortening, including intense staining in the mesectoderm and tracheal pits. In contrast, early expression is grossly abnormal, with sharply reduced levels and a derepressed pattern that extends throughout ventral regions that will form the mesoderm [Fig. 2C]. This ectopic staining pattern is similar to the derepression of the \( rho \) pattern observed in \( sna^- \) mutants [Kosman et al. 1991; Rao et al. 1991]. The 1.6-, 1.2-, 0.7-, 0.4-, and 0.2-kb \( rho-lacZ \) fusion genes failed to direct any aspects of the normal \( rho \) pattern [data not shown].

The above results suggest that the first 2.2 kb of the \( rho \) promoter contains two regulatory elements: one responsible for early expression in the presumptive neuroectoderm and another that directs late expression in the mesectoderm and tracheal pits. In an effort to identify the minimal \( rho \) promoter element that is responsible for neuroectodermal expression, we analyzed the activities of four different heterologous promoter fusions [Fig. 1]. A 600-bp region of the \( rho \) promoter, located between −2.2 and −1.6 kb, was attached to a minimal hsp70
Figure 1. Summary of rho–lacZ gene fusions. The uppermost horizontal line corresponds to a restriction map of the rho 5' flanking sequences and extends from a HindIII site at -2.2 kb upstream from the putative rho transcription start site (Bier et al. 1990) to an SspI site at +90 bp. The progressively smaller horizontal bars represent truncated rho promoter fragments that were inserted in the pCaSpere–AUG P-transformation vector, which includes the entire bacterial lacZ-coding sequence. The numbers to the left of each fusion indicate the distance (in kb) from the transcription start site. The four horizontal bars at the bottom represent heterologous fusion promoters, whereby 5' pieces of the rho promoter were attached to the pWHL P-transformation vector. This vector contains the minimal hsp70 promoter. The first two of these heterologous fusions include the region from -1.7 to -1 kb, and -1.7 to -1.4 kb (the midline element). The bottom two fusions correspond to NEEs; the larger one includes a 600-bp region (-2.2 to -1.6 kb); the smaller one is 300 bp in length (-2 to -1.7 kb). Abbreviations: (A) AvaII; (Bgl) BglII; (Bs) BstEII; (E) EcoRI; (H) HindIII; (N) NdeI; (S) StyI; (Ssp) SspI; (X) XhoI; (Xmn) XmnI; HSP 70, minimal promoter of the heat shock 70 gene; lacZ bacterial β-galactosidase-coding region; (NEE) neuroectoderm element; (MLE) midline element.

promoter. This fusion promoter displayed nearly normal expression in the presumptive neuroectoderm (Fig. 2E). Staining was detected before cellularization and persisted during gastrulation and early germ-band elongation. A similar pattern was obtained with an internal 300-bp fragment (-2.0 to -1.7 kb), although weak staining is detected in ventral regions (Fig. 2F). Hereafter, we refer to the 600-bp promoter sequence as the 600-bp NEE, and the 300-bp sequence as the 300-bp NEE. A heterologous fusion gene that contains rho promoter sequences between -1.7 and -1 kb directs all aspects of the late rho pattern obtained with the “full-length” 2.2-kb rho–lacZ gene, including expression in the mesectoderm and tracheal pits (Fig. 2D). The 300-bp sequence between -1.7 and -1.4 kb also directs expression in the midline but gives only weak expression in the tracheal pits (data not shown).

dl, twi, and sna protein-binding sites in the NEE

Previous genetic studies suggest that the ventral limits of the early rho pattern depend on sna, which functions as a repressor that keeps rho off in the presumptive mesoderm (Kosman et al. 1991; Rao et al. 1991). In sna mutants there is a derepression of the rho pattern throughout ventral regions. The activation of rho is somewhat less certain, although expression depends on dl because the early pattern is completely abolished in dl mutants (D. Kosman, unpubl.). In addition, there is a delay in the onset of rho expression in twi mutants, suggesting the possibility that dl and twi act in concert to initiate the early rho pattern (Kosman et al. 1991). To determine whether any of these genes might act directly on the rho promoter, we performed systematic DNase I protection assays with dl, twi, and sna fusion proteins.
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using DNA fragments that span the entire NEE. The fusion proteins were prepared in the pGEX vector system (Smith and Johnson 1988) and purified on glutathione affinity beads (see Materials and methods).

The NEE contains a total of 10 high-affinity *dl*-, *twi-* and *sna-*binding sites. An example of the DNA-binding assays is shown in Figure 3A. In this experiment a StyI–EcoRI fragment of ~300 bp, which corresponds to the 300-bp NEE (see Figs. 1 and 2F), was 32P-labeled at the EcoRI site, incubated with increasing amounts of *dl* (lanes 2–4), *twi* (lanes 6–8) or *sna* (lanes 10–12) proteins, digested with DNase I, and electrophoresed on a polyacrylamide–urea gel. This fragment contains all of the high-affinity *dl*-, *twi-* and *sna*-binding sites that are present in the 600-bp NEE, none were identified in the regions located between -2.2 and -2.0 kb or -1.7 and -1.6 kb (data not shown). The locations of the highest affinity binding sites are summarized in Figure 3B. The most striking finding of the DNA-binding studies is the clustering of *dl*-, *twi-* and *sna*-binding sites. For example, there are three *sna*-binding sites located between the distal-most *dl* site at -1960 bp (d1) and the next (more proximal) site at -1820 bp (d2). Furthermore, one of the *sna* sites [at -1770 (s4)] directly overlaps a *twi* site [Fig. 3A, lanes 8,12].

The nucleotide sequences of the high-affinity *dl*-, *twi-* and *sna*-binding sites are summarized in Figure 3B. The four *dl* sites contained within the NEE are closely related to those identified in the *twi*, *sna*, and *zer* promoters (Ip et al. 1991, 1992; Jiang et al. 1991; Pan et al. 1991; Thisse et al. 1991). Three of the four sites contain a GGG half-site, and all four include a second half-site with 2 C residues; the half-sites are separated by 5 central nucleotides that tend to be A-T rich. The *twi* protein contains a basic helix-loop-helix (b-HLH) motif and appears to bind DNA as a homomultimer (Fig. 3A, lanes 6–8). The sequences that are protected by the *twi* fusion protein include two 6-bp conserved motifs, CANNTG,
may correspond to divergent E boxes [Lenardo et al. 1987], which are binding sites for the prototypic b-HLH proteins E12 and E47 [Murre et al. 1989]. However, the core sequences contained within the twi sites, CA or TA, are unusual for E boxes, most of which include central GG or CC residues. The two twi sites located between −1780 and −1770 bp are bounded by dl-binding sites at −1820 bp (d2) and −1760 bp (d3). This close linkage between dl and twi sites is consistent with previous evidence for cooperative interactions between the two proteins (see Discussion; Kosman et al. 1991).

The sna protein contains five zinc fingers [Boulay et al. 1987], and the four sna sites present in the NEE contain the consensus sequence C-A/C-A-C-T-T-G-C (see Fig. 3B). Interestingly, the sna consensus is related to the E-box motif and includes the sequence CA − TT/G. In fact, two of the sna sites, one located at −1830 bp (s3) and another at −1775 bp (s4), contain an E box [CACCTTG and CACATG], and the sna site at −1775 bp directly overlaps the proximal-most twi site (t2; see Fig. 3B). The similarity of the sna consensus sequence with the E-box motif suggests that the sna protein may function as a repressor by competing with b-HLH activators for common binding sites.

**In vivo relevance of factor-binding sites**

The expression patterns obtained with the 1.8- and 1.6-kb rho–lacZ fusion genes suggests that some or all of the dl-, twi-, and sna-binding sites identified within the NEE...
might be important for early expression in the presumptive neuroectoderm. The 1.8-kb fusion gene lacks two of the $dl$-binding sites, one $twi$ site, and three $sna$ sites (see Fig. 3B summary) and directs a highly abnormal pattern of expression that extends throughout ventral regions (see Fig. 2C; cf. with A). The 1.6-kb $rho$-lacZ fusion lacks the remaining $dl$, $twi$, and $sna$ sites and is completely inactive in the presumptive neuroectoderm. To establish a definitive link between the binding sites and localized expression of the NEE, individual sites were disrupted by in vitro mutagenesis.

The first set of experiments involved disrupting $dl$-binding sites. The conserved GGG half-sites in the $d2$, $d3$ and $d4$ $dl$-binding sites were substituted [see Materials and methods], and in vitro DNase I footprint assays show that the binding is totally abolished (data not shown). A 600-bp NEE–$lacZ$ fusion gene containing mutations in the $d3$ and $d4$ sites directs substantially weaker expression than the 600-bp NEE, low levels of staining are occasionally observed when the three mutated $dl$-binding sites were introduced into the 300-bp NEE (Fig. 4F; cf. with E). The embryo in Figure 4F shows the strongest residual staining among those carrying this fusion gene, most lack expression entirely. These results provide strong evidence that $dl$ contacts the $rho$ NEE directly and mediates transcriptional activation.

Similar experiments were done to examine the role of the two $twi$-binding sites within the NEE. There is a marked reduction in the levels and limits of expression obtained with the 600-bp NEE containing mutations in these sites [Fig. 4C, cf. with A]. The slight derepression of staining that is sometimes observed in ventral regions is probably the result of mutations in the $twi$ $t2$ site, which also abolishes binding of the $sna$ repressor to the overlapping $s4$ site (see below). A 600-bp NEE containing mutations in both the $d2$, $d3$, and $d4$ $dl$-binding sites and the two $twi$-binding sites is completely inactive in the presumptive neuroectoderm (data not shown). However, there may be additional $dl$- and $twi$-binding sites located outside of the 300-bp NEE because mutations in these five binding sites do not completely abolish the expression of an otherwise normal 2.2-kb $rho$–$lacZ$ fusion gene [Fig. 4G; cf. with Fig. 2A]. It should be noted that this embryo shows the strongest residual staining among those that were examined.

Mutations in $sna$-binding sites cause a dramatic derepression of the $rho$ pattern in ventral regions, similar to that observed in $sna^-$ mutants [Kosman et al. 1991]. Disruptions in the $sna$ $s1$, $s3$, and $s4$ sites were done in a way that did not interfere with the activities of nearby $dl$ and $twi$ activator sites. In particular, mutagenesis of the $sna$ $s4$ site did not interfere with the binding activity of the $twi$ $t2$ site (see Materials and methods, Fig. 3B summary). The inactivation of the three $sna$ sites causes an otherwise normal 2.2-kb $rho$–$lacZ$ fusion gene to be expressed at high levels in both lateral and ventral regions (Fig. 4H). A similar result was obtained when these sites were mutagenized in the context of the 600-bp NEE (Fig. 4D).

**Discussion**

We have presented evidence that the $dl$ morphogen acts in concert with the protein products encoded by two of its target genes, $twi$ and $sna$, to direct lateral stripes of $rho$ expression within the presumptive neuroectoderm (summarized in Fig. 5). $dl$ and $twi$ activate $rho$, whereas $sna$ excludes its expression from the mesoderm. A 300-bp region of the $rho$ promoter, the NEE, is sufficient for neuroectoderm expression. It contains a cluster of closely linked $dl$, $twi$, and $sna$ sites, and a direct link has been established between these binding sites and the lateral stripes of $rho$ expression. The demonstration that $sna$-binding sites repress $rho$ provides evidence that $sna$ is directly responsible for establishing the boundary between the mesoderm and neuroectoderm before gastrulation. The arrangement of binding sites in the NEE is remarkably similar to the organization of the $eve$ stripe 2 element [Stanojevic et al. 1989, 1991; Small et al. 1991]. We conclude that the $bcd$ and $dl$ morphogens employ an analogous mechanism to make stripes in the early embryo [Fig. 6].

**Establishment of the mesoderm–neuroectoderm boundary**

Previous double staining studies suggest that the lateral limits of $sna$ expression coincide with the boundary between the mesoderm and neuroectoderm. In wild-type embryos the expression patterns of neuroectodermal regulatory genes such as $rho$, single-minded ($sim$), $T3$ (AS-C), and $m7$ [$Espl$], appear to abut, but not cross, the $sna$ border [Romani et al. 1987; Kosman et al. 1991; Rao et al. 1991]. Each of these genes is derepressed in $sna^-$ embryos, such that expression extends into ventral regions that would normally form the mesoderm. These observations prompted the proposal that $sna$ is required for the establishment of the mesoderm–neuroectoderm boundary. The absence of the ventral furrow and mesoderm in $sna^-$ mutants might result from the derepression of neuroectodermal genes normally restricted to lateral regions. Thus, one of the first steps in the specification of the mesoderm appears to be the activation of $sna$, which functions as a repressor to exclude an alternative pathway of development [neuroectoderm].

The results presented in this study suggest that $sna$ participates directly in the formation of the mesoderm–neuroectoderm boundary. High-affinity $sna$-binding sites were identified in the $rho$ NEE, and disrupting these sites causes a derepression of the pattern into the presumptive mesoderm, similar to that observed in $sna^-$ mutants [see Fig. 4H]. It is conceivable that $sna$ acts directly on $rho$ by regulating the expression of one or more intermediate genes, which in turn are responsible for repression in the presumptive mesoderm. However,
this explanation seems unlikely because the putative intermediates must possess the same binding activities as sna. In addition, the timing of rho derepression observed in sna− mutants is consistent with a direct effect. There is no more than a 20- to 30-min lapse between the time when the sna protein is first detected in wild-type embryos and when rho is derepressed in sna− mutants (Alberga et al. 1991; Kosman et al. 1991; Leptin 1991). D-
present in the NEE. It is not known how many of the dl and twi activator sites are blocked by the binding of sna, but gel-shift assays indicate that sna and twi cannot co-occupy the $s_4$ and $t_2$ sites [R. Park, unpubl.]. sna protein is not present in lateral regions that will form the neuroectoderm, and consequently, none of the repressor sites within the NEE are occupied in these regions. There are diminishing levels of $dl$ and twi proteins, which appear to be sufficient to fill most or all of the activator sites. Communolocalization studies indicate that the twi protein can be detected in five to six cells beyond the $sna$ border [Kosman et al. 1991], although it has not been detected in the dorsal-most two to three cells of the presumptive neuroectoderm. Perhaps these cells contain low levels that escaped detection, or other b-HLH proteins can recognize the twi-binding sites and act in concert with $dl$ to activate expression (see Discussion).

Mechanism of repression

The NEE contains six $dl$ and twi activator sites that are closely linked to four sna repressor sites [summarized in Fig. 3B]. Most of the activators map within 50 bp of a repressor site. This close linkage of activators and repressors suggests a short range mechanism of transcriptional repression. A competition mechanism is indicated by the failure of the twi and sna proteins to co-occupy the $t_2$ and $s_4$ sites [R. Park, unpubl.]. The similarity of the twi and sna consensus-binding sites is reminiscent of the situation observed for the bcd activator and the Krüppel (Kr) repressor, which compete for overlapping binding sites in the eve stripe 2 promoter element [Small et al. 1991]. There are other examples of zinc finger proteins that function as transcriptional repressors by competing with activators for overlapping sites [e.g., Keller and Maniatis 1991]. $sna$ may repress $dl$ through a short-range quenching mechanism, whereby local protein–protein interactions between neighboring $dl$ and sna sites block the ability of the $dl$ activator to contact the basal transcription complex [Levine and Manley 1989]. This mechanism would be similar to the interaction of the a2 repressor with the MCM1 activator in yeast [Keleher et al. 1989]. The dorsal border of the rho pattern may be defined by limiting amounts of the $dl$ and/or twi activators, which are not detected beyond the lateral midline.

**Figure 4.** $dl$, twi-, and sna-binding sites regulate the expression of the rho NEE. Precellular P transformants are oriented with anterior to the left and dorsal up. The diagrams above each embryo show the $dl$, twi-, and sna-binding sites that were mutagenized in the context of otherwise normal rho–lacZ fusion genes. (A) Staining pattern obtained with the 600-bp NEE (–2.2 to –1.6 kb) attached to the hsp70 minimal promoter. Expression is restricted to two ventral–lateral bands that extend along the length of the embryo. (B) The 600-bp NEE–lacZ fusion containing point mutations in the $d_3$ and $d_4$ $dl$-binding sites. There is a marked reduction in the level of expression compared with the wild-type fusion [A]. In addition, the expression limits include just 3–5 cells in width, rather than 8–10 cells. (C) The 600-bp NEE–lacZ fusion containing point mutations in both twi-binding sites. Mutations in the $t_2$ site also disrupt the $s_4$ sna-binding site. There is a weakening and narrowing of the pattern, such that the ventral–lateral bands are only 2–3 cells in width, rather than 8–10 cells (see A). (D) The 600-bp NEE–lacZ fusion containing point mutations in three sna-binding sites. Disruption of the sna $s_4$ involved only a single nucleotide substitution and does not disrupt binding of the twi protein to the overlapping $t_2$ site. There is a dramatic derepression of the staining pattern, which extends throughout the presumptive mesoderm in ventral regions. (E) The wild-type 300-bp rho NEE (–2.0 to –1.7 kb) attached to the minimal hsp70 promoter. There is strong expression in the presumptive neuroectoderm, as well as weak expression in the ventral regions. (F) The 300-bp NEE–lacZ fusion containing mutations in the $d_2$, $d_3$, and $d_4$ $dl$-binding sites. There is a marked reduction in the levels of expression, and the pattern is substantially narrower as compared with wild-type [E]. (G) The 2.2-kb rho–lacZ fusion gene containing mutations in the $d_2$, $d_3$, and $d_4$ $dl$-binding sites, and in the $t_1$ and $t_2$ twi sites. There is at least a 10-fold reduction in the levels of expression as compared with the wild-type 2.2-kb rho–lacZ fusion [see Fig. 2A,B]. (H) The 2.2-kb rho–lacZ fusion gene containing point mutations in the three sna-binding sites. There is a dramatic derepression of the pattern, which extends throughout the presumptive mesoderm in ventral regions.

**Figure 5.** Summary of NEE regulation. The diagram shows the approximate limits of $dl$, twi, and sna expression in a cross section of a precellular embryo. The broad $dl$ gradient activates twi, which is restricted within narrower limits. $dl$ and twi act in concert to specify sharp lateral borders of sna expression, which coincide with the boundary between the mesoderm and neuroectoderm [Kosman et al. 1991; Ip et al. 1992]. sna is expressed at uniform levels throughout the presumptive mesoderm. In the ventral-most 18 cells, the rho promoter is inactive owing to the binding of sna protein to the four binding sites.
functions redundantly with other b-HLH proteins. Previous genetic studies are consistent with the possibility of specific \(dl\)--\(twi\) interactions. For example, the simultaneous reduction of both \(dl\) and \(twi\) gene activities (double heterozygotes) causes severe disruptions in \(twi\) and \(sna\) expression [Kosman et al. 1991].

The requirement of both \(dl\) and E-box recognition sequences suggests that interactions between \(dl\) and b-HLH proteins (possibly \(twi\)) are important for transcriptional activation of the \(rho\) NEE. Perhaps such interactions facilitate the binding of \(dl\) to the \(rho\) NEE in lateral regions where there are limiting amounts of the protein. Similar interactions appear to be important for the expression of lymphoid enhancers in mammals because several of these contain closely linked NF-kB and E12/E47-binding sites and mutations in just one class of sites cause dramatic reductions in expression [Sen and Baltimore 1986; Lenardo et al. 1987; Murre et al. 1989].

An analogous mechanism for stripe formation

Recent studies have established that \(eve\) stripe 2 is regulated by the \(bcd\) morphogen [Small et al. 1991, 1992; Stanoevic et al. 1991]. \(bcd\) activates the expression of \(hunchback\) \((hb)\) [Driever et al. 1989; Struhl et al. 1989], and the two genes act in concert to define a broad domain in the anterior half of the embryo where stripe 2 is initiated. \(bcd\) also activates the gap gene \(giant\) \((gt)\), which defines the anterior border of the stripe [summarized in Fig. 6B]. There are striking parallels with the regulation of the \(rho\) lateral stripes (Figs. 5 and 6). \(dl\) defines a broad region, covering the ventral half of the embryo, where \(rho\) can be activated. It is conceivable that this activation depends on \(twi\) or another b-HLH protein that is regulated by \(dl\). The ventral limit of the \(rho\) pattern is specified by the \(sna\) repressor, which is also regulated by \(dl\). \(eve\) stripe 2 and the \(rho\) lateral stripes may differ by the number of repressors that define their patterns. The posterior border of \(eve\) stripe 2 depends on the gap gene \(Kr\). In contrast, it appears that the dorsal limits of the \(rho\) stripes are specified by limiting amounts of the \(dl\) activator, although we cannot rule out the possibility that this involves one or more repressors that emanate from dorsal regions. In certain cases, limiting amounts of \(bcd\) have been found to be sufficient to define the posterior border of \(eve\) stripe 2, similar to the dorsal limits of \(rho\) [Small et al. 1992].

In addition to the obvious parallels in the \(eve\) and \(rho\) genetic circuits, in both cases the regulatory proteins encoded by the genes interact with 300- to 500-bp promoter elements containing tightly linked activator- and repressor-binding sites. The regulatory proteins that govern the \(eve\) and \(rho\) patterns are largely different. \(bcd\) contains a homeo box and activates transcription with the \(hb\) zinc finger protein. In contrast, \(dl\) contains the REL domain and probably activates \(rho\) in concert with b-HLH proteins. The \(gt\) repressor contains a basic leucine zipper motif, whereas the \(sna\) repressor contains zinc fingers. Despite these differences, both sets of proteins produce stripes of gene expression. We propose that

An analogous mechanism for stripe formation

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the clustering of factor-binding sites observed in the eve stripe 2 element and the rho NEE might reflect weak protein–protein interactions among the stripe regulators. Perhaps these weak interactions can work to coordinate the activation and repression of transcription only when the proteins are brought into close proximity.

Materials and methods

Promoter fusions and P-transformation assays

Most of the rho–lacZ fusions involved the use of a HindIII–Sacl genomic DNA fragment, which includes the region from −2.2 kb to +600 bp of the rho promoter. This fragment was inserted into the phleulescript vector (Stratagene), and its nucleotide sequence was determined by subcloning various subfragments, ranging in size from 300 to 650 bp, into the pGem7Zf + vector (Promega). Sequencing was done with a T7 kit (Pharmacia) and the Sequenase kit (U.S. Biochemical) on double stranded DNA templates.

A series of truncated promoters were constructed with a HindIII–Sphi genomic DNA fragment, spanning from −2.2 kb to +90 bp relative to the putative rho transcription start site (Bier et al. 1990). This fragment was bluntend with Klenow and ligated into the blunt HindIII site of the pGem7Zf + vector. Promoter fragments were obtained by double digestion with KpnI (located in the polynucleotide of the vector) at the 3′ end, and the 5′ sites were selected as shown in Figure 2. The fragments were cloned into the pCaSper–AUGI3-gal P-transformation vector (Thummler et al. 1988) after digestion with KpnI and BamHI. The heterologous promoter fusions were prepared by cloning appropriate rho fragments into the Stul site of the pWHL P-transformation vector, which contains a minimal hsp70 promoter (Drieve et al. 1989).

P-element plasmids were injected into a strain carrying a white null mutation (w1118), together with the 82,3 transposable helper plasmid. At least two, and usually three, independent transformed lines were used for embryo staining. Appropriate staged embryos were fixed and hybridized with a digoxigenin-UTP labeled lacZ antisense riboprobe as described previously (Jiang et al. 1991).

Expression of fusion proteins

A dl-glutathione S-transferase (GST) fusion protein was prepared from bacterial extracts with the pGEXKX expression vector (Smith and Johnson 1988). The dl-pGEX fusion gene was made with a 1.2-kb Ndel–Sacl dl cDNA fragment, which was obtained from the pARl-378 T7 expression plasmid described by Ip et al. (1991). This DNA fragment encodes the amino-terminal 378-amino-acid residues of the dl protein, which includes the entire rel/NF-κB homology region. The fragment was blunt end with T4 DNA polymerase and inserted into the Smal site of the pGEX vector.

The pGEX–twl fusion protein was prepared by inserting a 1.6-kb Ndel–EcoRI twl cDNA fragment into the expression vector. This fragment was obtained from a PAR 3040 expression plasmid containing the entire twl-coding sequence (D. Kosman, unpubl.). An Ndel restriction site was created at the initiating ATG by in vitro mutagenesis, and the fragment was cloned into the PAR vector at the unique Ndel and EcoRI sites. The twl fragment that was obtained from this recombinant was blunt and inserted into the Smal site of the pGEX vector.

The pGEX–sna fusion protein was prepared from a plasmid that contains the GST sequences fused to amino acids 177–390 of the sna protein. This carboxy-terminal region includes all five putative zinc fingers. The plasmid was constructed by first inserting a 1.2-kb Ndel fragment, which contains the full-length sna cDNA from the pAR–sna expression plasmid into the Smal site of the pGEX vector. An internal BamHI fragment was then removed, and the plasmid was religated.

Fusion proteins were prepared according to the method of Smith and Johnson (1988), with the following modifications. Induced bacteria were pelleted by centrifugation and resuspended in 1/10 volume of lysis buffer (25 mM HEPES [pH 7.5], 20 mM KCl, 2.5 mM EDTA, 1% Triton X-100, 1 mM DTT, 0.5 mM PMSF, 1 μg/ml of pepstatin, 1 μg/ml of leupeptin). Resuspended bacteria were subjected to three freeze–thaw cycles and then sonicated for 30 sec. A 1/10 volume of 5 M NaCl was added and placed on ice for 15 min. The lysate was spun at 12,000g for 15 min at 4°C. Fusion proteins were affinity purified by adding 1/10 volume of glutathione agarose beads (Sigma) and incubating for 1 hr at 4°C. The beads were pelleted by centrifugation and washed three times with 20 volumes of lysis buffer and once with 20 volumes of 50 mM Tris [pH 8]. Fusion proteins were eluted from the beads by the addition of 1 volume of 50 mM Tris [pH 8], 10 mM glutathione. The glutathione was removed by dialyzing three times against 250 volumes of buffer B (25 mM HEPES [pH 7.5], 50 mM KCl) 1 mM DTT 1 mM EDTA, 6 mM MgCl2, 10% glycerol, and the protein was stored at −70°C.

DNA-binding assays

DNase I footprinting was done essentially as described by Hoey et al. (1988). A 519-bp HindIII–EcoRI DNA fragment, located between −2.2 and −1.7 kb upstream from the rho transcription start site, was 32P-labeled with Klenow and then redigested with Styl. A 318-bp Styl–EcoRI fragment was purified on a 6% polyacrylamide gel and eluted by incubating overnight in 0.5 M NH4OAc, 10 mM MgOAc, 0.1% SDS, and 1 mM EDTA at 30°C. The fragment was ethanol precipitated and washed in 80% ethanol. Binding reactions were done with pGEX fusion proteins and 100,000 cpm of labeled DNA in 50 μl of footprinting buffer (25 mM HEPES [pH 7.5], 50 mM KCl, 3 mM MgCl2, 1 mM EDTA 0.5% NP-40, 10 μg/ml of poly[dI–dC], 10% glycerol). DNase I digestions, ethanol precipitation of protein–DNA complexes, and gel electrophoresis were done exactly as described (Hoey et al. 1988).

Site-directed mutagenesis

The pGem7Zf + plasmid containing the HindIII–Sphi fragment from the rho promoter (see above) was used to transform the CJ236 strain of Escherichia coli to prepare uracil-containing single-stranded DNA. Mutagenesis was done with the Muta-Gene kit (Bio-Rad) by annealing this template with various mutant oligonucleotides, as suggested by the manufacturer. The in vitro-synthesized double-stranded DNA was transformed into the DH5α strain of E. coli. The identities of mutagenized templates were confirmed by nucleotide sequence analysis.

In general, mutant oligonucleotides contained 26–36 nucleotides, with at least 10 nucleotides on either side of the mutated sequence. The relevant sequences of the oligonucleotides are shown below. The boldface and underlined letters correspond to nucleotide substitutions [cf. with Fig. 3B: d2, CGTCAGTAAA; d3, TCTAGATTAT; d4, AGGGCTGGCCT; t1, CGCGCAAGCTT; t2, AGGGCTGTT; s1, CAGAGCTC; CCG, s3, CCCGGCGGCT, s4, CCGATTGTT. Note that the s mutation contains only one nucleotide substitution, and twi binding to this mutated sequence is preserved while sna binding is virtually abolished (R. Park, unpubl.).
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