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Multiple modes of dorsal–bHLH transcriptional synergy in the *Drosophila* embryo

Paul Szymanski and Michael Levine[1]

Department of Biology, Pacific Hall, Center for Molecular Genetics, 9500 Gilman Drive, University of California at San Diego, La Jolla, CA 92093-0347, USA

[1]Corresponding author

Synergistic interactions between the maternal regulatory factor dorsal (dl) and basic helix–loop–helix (bHLH) activators are essential for initiating differentiation of the mesoderm and neuroectoderm in the early *Drosophila* embryo. Here we present evidence that dl–bHLH interactions mediating gene expression in the neuroectoderm and mesoderm are fundamentally distinct. Close proximity of dl and bHLH binding sites is essential for the synergistic activation of gene expression in the lateral neuroectoderm, where there are diminishing levels of the dl regulatory gradient. In contrast, sharp on/off patterns of gene expression in the presumptive mesoderm do not require linkage of these sites. Analysis of minimal and synthetic promoter elements suggests that dl and bHLH activators, such as twist, might interact with different rate-limiting components of the transcription complex. These results are consistent with two distinct modes of dl–bHLH synergy: cooperative binding to DNA (requiring linkage of sites) and synergistic contact of basal transcription factors (not requiring linkage). Finally, the characterization of a 57 bp synthetic minimal stripe unit (MSU) provides evidence for a third tier of dl–bHLH synergy. Tandem copies of the MSU function as a *bona fide* enhancer and can mediate neuroectoderm expression in transgenic embryos even when placed 4.5 kb downstream of a test promoter. Multiple copies of the MSU function synergistically only when linked, but not when separated. We propose that this linkage requirement provides the basis for the evolution of modular promoters composed of discrete, non-overlapping enhancers.

Key words: bHLH proteins/dorsal/*Drosophila* embryo/transcriptional synergy

Introduction

During *Drosophila* embryogenesis, complex patterns of gene expression are often specified by composite promoters containing a series of non-overlapping, autonomous enhancer elements. For example, the segmentation genes *even-skipped* (*eve*) and *hairy* (*h*) both contain several distinct enhancers that control the expression of separate stripes in blastoderm stage embryos (Howard *et al.*, 1988; Goto *et al.*, 1989; Harding *et al.*, 1989; Howard and Struhl, 1990; Pankratz and Jackle, 1990; Riddihough and Ish-Horowicz, 1991). Separate regulatory elements have been identified for other complex patterning genes, including *rhomboid* (*rho*) and *fushi tarazu* (*ftz*) (Hiromi *et al.*, 1985; Ip *et al.*, 1992a). Moreover, the string (cdc25) kinase, which controls patterned domains of mitotic activity, is expressed in a complex pattern by several distinct regulatory elements (Edgar *et al.*, 1994). In the present study we investigate the basis for promoter modularity, in which regulatory information is integrated within a small segment of DNA, by defining minimal enhancer elements in transgenic embryos.

Most enhancers that have been defined in transgenic organisms or through the use of stable transformation assays in homologous cultured cells possess a complex structure, suggesting that they might have arisen through the duplication of simpler elements (Saksela and Baltimore, 1993). Some of these enhancers function from remote 5′ positions, are contained within introns or are located downstream of the associated transcription unit (Singh and Birshtein, 1993). A recurring theme is the presence of multiple binding sites for multiple activators. For example, the *rho* neuroectoderm enhancer (NEE) is located 1.7 kb upstream from the transcription start site (Ip *et al.*, 1992a). It is 300 bp in length and contains binding sites for the maternal regulatory factor dorsal (dl), as well as E box binding sites, which are recognized by basic helix–loop–helix (bHLH) activators (Murre *et al.*, 1989). The NEE mediates gene expression in the lateral neuroectoderm in response to low levels of the maternal dl gradient (summarized in Figure 1). There are four high affinity dl binding sites in the NEE, as well as five E boxes, which interact with at least two distinct classes of bHLH activators (Ip *et al.*, 1992a; Gray *et al.*, 1994). *In vitro* binding assays suggest that cooperative DNA binding interactions between dl and bHLH activators lead to the efficient occupancy of dl binding sites in lateral regions of the embryo, where there are low levels of dl protein (Jiang and Levine, 1993). In principle, this dl–bHLH synergy can activate *rho* in both ventral and lateral regions of the embryo in response to high and low levels of dl. However, expression is excluded from ventral regions, the presumptive mesoderm, by a zinc finger repressor called snail (sna) (Alberga *et al.*, 1991; Kosman *et al.*, 1991, Leptin, 1991); this repression is mediated by four sna sites in the NEE (Ip *et al.*, 1992a). Thus the NEE contains 13 binding sites for at least four different regulatory proteins, including three activators and one repressor.

dl–bHLH interactions are also important for initiation of the embryonic mesoderm. Peak levels of dl are required for the activation of two regulatory genes, *twist* (*twi*) and *sna* (Jiang *et al.*, 1991; Pan *et al.*, 1991; Thisse *et al.*, 1991; Ip *et al.*, 1992b). twi, one of the bHLH proteins that interacts with dl, functions as a transcriptional activator (Thisse *et al.*, 1988), while sna is a zinc finger repressor that establishes the boundary between the presumptive

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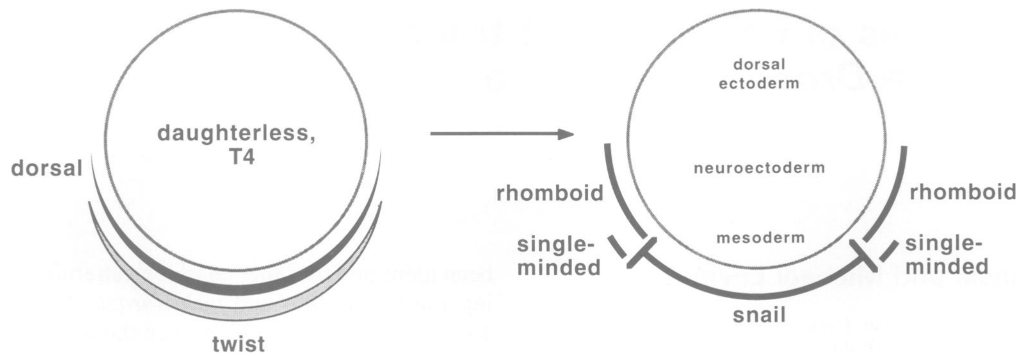

Fig. 1. Summary of dl−bHLH interactions in the mesoderm and neuroectoderm. The circles represent cross-sections through precellular embryos, with dorsal up and ventral down. The left circle shows the broad dl gradient, with peak levels in ventral regions and progressively lower levels in lateral and dorsal regions. dl triggers a steeper pattern of *twist* (*twi*) expression. twi is not the only bHLH protein present in early embryos. Two others, daughterless (da) and T4, are maternally expressed and ubiquitously distributed. The circle on the right shows the output patterns generated by dl and bHLH activators. dl−twi interactions are important for the localized expression of *snail* (*sna*) within the presumptive mesoderm. The sharp lateral limits of the sna pattern coincide with the boundary between the embryonic mesoderm and neuroectoderm. *sim* is expressed in a single row of cells, the presumptive mesectoderm, just beyond the *sna* limits. dl−bHLH interactions are also important for the activation of *rho* in the lateral neuroectoderm, where there are diminishing levels of the dl gradient. Target promoters that contain high affinity dl binding sites and closely linked E boxes can be activated in both the mesoderm and neuroectoderm. However, sna is a zinc finger repressor that helps establish the mesoderm/neuroectoderm boundary by blocking the expression of *sim*, *rho* and other genes that are restricted to the lateral mesectoderm and neuroectoderm. Target promoters and enhancers (such as the *rho* NEE) containing high affinity dl sites, E boxes and sna repressor sites are excluded from the mesoderm and restricted to lateral regions.

mesoderm and neuroectoderm (Simpson, 1983; Boulay *et al.*, 1987). A combination of genetic analyses and promoter fusion assays suggests that *sna* is activated by dl and twi, as summarized in Figure 1 (Ip *et al.*, 1992b). The broad dl gradient triggers a steeper pattern of twi expression and then the two proteins function multiplicatively to establish the sharp lateral limits of the *sna* expression pattern within the presumptive mesoderm. As a result of these dl−twi interactions, both peak levels of dl in the ventral-most regions and intermediate levels in ventrolateral regions activate *sna* to the same extent and thereby define a sharp on/off pattern of expression. The *sna* promoter contains a cluster of low affinity dl binding sites, as well as two twi-class E box binding sites (Ip *et al.*, 1992b). The low affinity sites restrict expression to regions containing high levels of dl (the presumptive mesoderm). In contrast, high affinity dl sites in the *rho* NEE permit expression in the lateral neuroectoderm in response to low levels of dl (Ip *et al.*, 1992a; Jiang and Levine, 1993).

Given the importance of dl−bHLH interactions in the specification of the embryonic mesoderm and neuroectoderm, we have characterized a number of synthetic and minimal promoter elements that are regulated by these proteins in transgenic embryos. We present evidence that dl−bHLH interactions mediating gene expression in the lateral neuroectoderm are fundamentally different from the dl−bHLH synergy responsible for establishing the sharp sna expression pattern within the presumptive mesoderm. Neuroectoderm expression depends on close proximity of high affinity dl binding sites and E boxes (within ~50 bp), although there is flexibility in the exact spacing and organization of the sites. In contrast, dl−bHLH interactions underlying mesoderm differentiation do not require proximity of the binding sites. A minimal 57 bp element is identified that can direct lateral stripes of expression when placed in a promoter proximal position. This element, the minimal stripe unit (MSU), contains two high affinity dl binding sites and two E boxes; these latter sites are recognized by both bHLH activators and the sna repressor. Two copies of the MSU, one 5′ and one placed downstream of the transcription unit, function additively to generate a staining pattern that is only slightly more intense than the one obtained with a lone 5′ copy. In contrast, two tandem copies of the MSU work multiplicatively and direct a staining pattern that is virtually indistinguishable from the endogenous *rho* gene. Linked, multiple copies can function as a *bona fide* enhancer, even when positioned nearly 5 kb downstream of the transcription start site. These studies suggest that individual MSUs work multiplicatively only when linked and we suggest that this provides the basis for the evolution of modular promoters composed of discrete enhancers.

Results

dl−bHLH interactions were investigated by manipulating the spacing of dl and bHLH binding sites in various defined and synthetic promoters. The promoters were attached to a *lacZ* reporter gene and analyzed in transgenic (P transformed) embryos by *in situ* hybridization, using a digoxygenin-labeled *lacZ* antisense RNA probe.

Close proximity of dl and twi binding sites is required for neuroectoderm expression

A 440 bp *twi* promoter fragment that includes the proximal element (PE, located between −440 and −180 bp upstream of the *twi* transcription start site) directs expression in the ventral-most 12–14 cells in response to peak levels of the dl protein (Jiang *et al.*, 1991; Jiang and Levine, 1993; see Figure 3A). The PE contains two low affinity dl binding sites that can be efficiently occupied only by high levels of dl protein. Conversion of these sites to high affinity dl binding sites (PEe) results in an expanded pattern of expression spanning 20 cells in the ventral and ventrolateral regions (Jiang and Levine, 1993). The modification of this element to include two twi-class E boxes (PEe-Et)

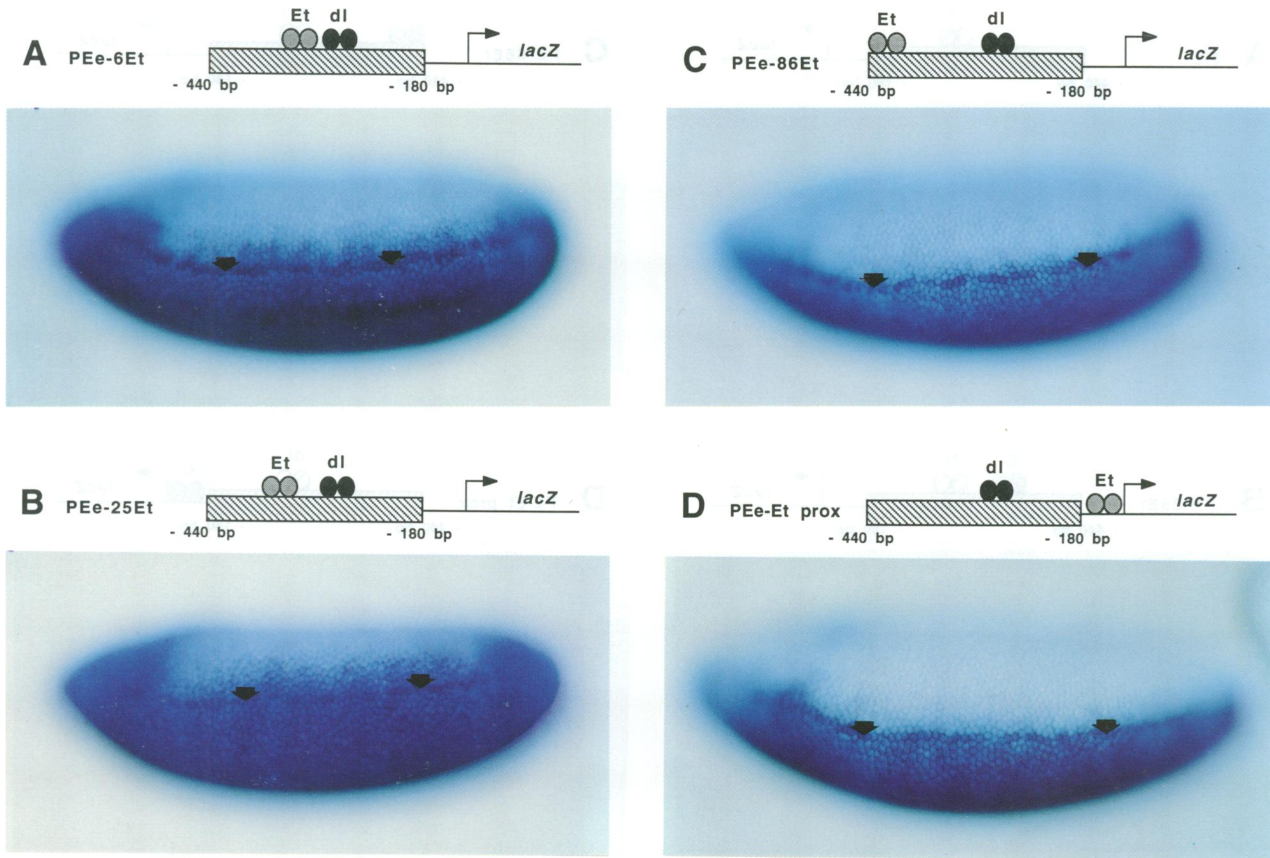

Fig. 2. Neuroectoderm expression requires close linkage of dl and bHLH binding sites. Cellularizing, transgenic embryos are oriented with anterior to the left and dorsal up. The staining patterns were visualized by *in situ* hybridization using a mixture of digoxygenin-labeled *sim* and *lacZ* antisense RNA probes. The arrowheads indicate the position of the sim line (the presumptive mesectoderm), which is located just beyond the limits of the sna expression pattern. (**A**) Staining pattern directed by the PEe-6Et fusion gene. The limits of the *twi* PE element are indicated by the stippled bar (it extends from −440 to −180 bp relative to the *twi* transcription start site). The two twi-class E boxes (Et) are located 6 bp upstream of the two high affinity dl binding sites (indicated by the filled circles). The PEe-6Et−*lacZ* fusion gene directs expression throughout the mesoderm and extends laterally through the mesectoderm and into ventral regions of the neuroectoderm. The staining ends about five cells beyond the mesoderm/neuroectoderm boundary. (**B**) As (A) except that the promoter was modified in order to separate the twi and dl binding sites by 25 bp. The staining pattern is virtually identical to that shown in (A). (**C**) As (A) and (B) except that the twi and dl binding sites are separated by 86 bp within the PE element. This spacing results in a narrowing of the expression pattern, so that staining extends only one to two cells beyond the mesoderm/neuroectoderm boundary. (**D**) Staining pattern obtained when two twi E boxes were placed proximal to the initiation site, ~200 bp downstream of the dl binding sites. Staining is restricted to the mesoderm and mesectoderm and only occasionally extends into the neuroectoderm.

results in a further expansion of the pattern, so that staining extends through both the ventral and lateral regions (the presumptive mesoderm and neuroectoderm; see Figure 2A). The dorsoventral limits of the expression pattern were determined by hybridizing transgenic embryos with a mixture of *lacZ* and *single minded* (*sim*) RNA probes. *sim* is expressed in the presumptive mesectoderm, which is just a single cell in width on either side of the presumptive mesoderm (Crews *et al.*, 1988; Thomas *et al.*, 1988). The *lacZ* reporter gene is expressed about five cells beyond the *sim* lines, indicating a total expression pattern of ~30 cells (Figure 2A).

The high affinity dl sites and Et E boxes are separated by 6 bp in the PEe-Et promoter (also called PEe-6Et) shown in Figure 2A. This spacing corresponds to the natural separation of dl and twi sites within the *rho* NEE (Ip *et al.*, 1992b; see below). To determine whether there is a stringent spacing requirement for dl−twi interactions, these sites were separated by various distances. The first experiment of this sort involved placing the two twi E boxes 25 bp upstream of the high affinity dl sites (PEe-25Et; Figure 2B). This promoter directs an expression pattern that is indistinguishable from the PEe-6Et promoter. In both cases the lateral limits of the pattern extend about five cells beyond the *sim* lines.

It is conceivable that the helical phasing of the binding sites is important for dl−bHLH synergy. To test this possibility, the binding sites were also separated by 11 and 20 bp, which should position the dl and twi proteins on opposite sides of the DNA helix relative to the 6 and 25 bp organizations. Both the PEe-11Et and PEe-20Et promoters direct expression in both the ventral and lateral regions, with the dorsoventral limits extending about five cells beyond the sim lines (data not shown). Together these results indicate considerable flexibility in the arrangement of binding sites; separating the two classes of sites by 6, 11, 20 or 25 bp has no discernible effect on the dorsoventral limits of expression.

Additional spacing experiments suggest that there are limits to this flexibility and that dl−twi synergy depends on close proximity of the binding sites. When the twi E boxes are placed 86 bp upstream of the high affinity dl

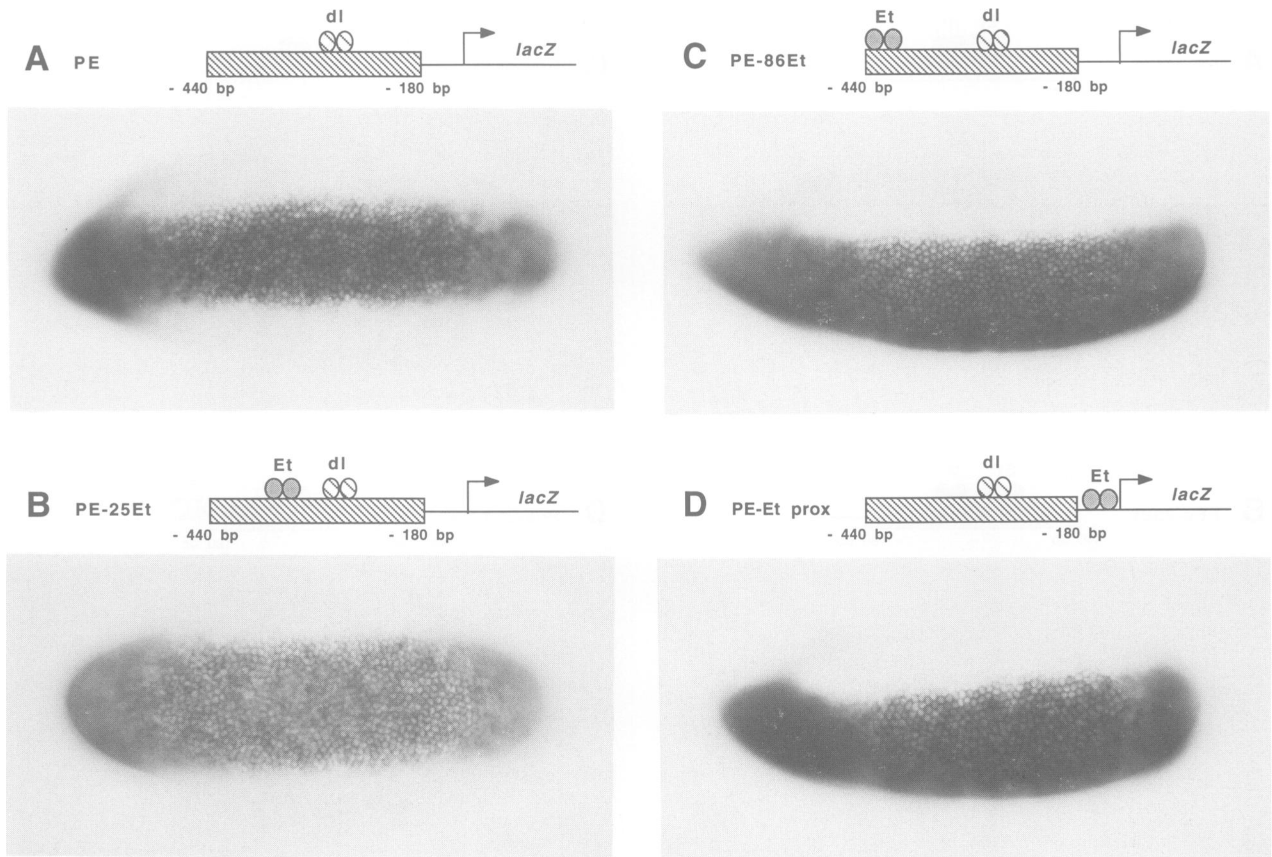

Fig. 3. Mesoderm expression does not depend on linked dl and bHLH binding sites. Cellularizing, transgenic embryos are oriented with anterior to the left. Staining patterns were visualized by hybridization with a digoxygenin-labeled *lacZ* antisense RNA probe. (**A**) Ventral view of an embryo containing a truncated twi promoter containing the first 440 bp of 5′ flanking sequence. The native PE sequence contains two low affinity dl binding sites, indicated by the striped circles. This twi–*lacZ* fusion gene directs expression in the ventral-most 12–14 cells, where there are peak levels of dl. The lateral limits of the pattern are rather fuzzy. (**B**) As (A) except that two twi binding sites were placed 25 bp upstream of the low affinity dl sites. The expression pattern is expanded as compared with (A). Staining now includes the entire presumptive mesoderm and abruptly ends near the mesoderm/neuroectoderm boundary. The lateral limits of expression are not quite as sharp as those observed for the endogenous sna pattern. (**C**) Lateral view of an embryo containing the PE-86Et fusion gene, whereby the twi E boxes were placed 86 bp upstream of the low affinity dl sites. The staining pattern is as broad as that shown in (B) and includes sharp lateral limits. (**D**) As (C) except that the PE-Et promoter contains the two twi E boxes proximal to the initiation site, ~200 bp downstream of the low affinity dl sites.

sites (PEe-86Et) there is a substantial narrowing of the pattern, so that staining extends only about one cell beyond the *sim* lines (Figure 2C). This staining pattern is only slightly broader than that observed for the PEe promoter, which completely lacks twi E box sequences (Jiang and Levine, 1993; data not shown). A similar pattern is obtained when the twi E boxes are placed ~200 bp downstream of the dl sites (Figure 2D). Staining is restricted to the mesoderm and mesectoderm and encompasses just 20 cells in the ventral and ventrolateral regions. These results suggest that synergistic activation of target promoters in the lateral neuroectoderm depends on close proximity, but not exact spacing, of high affinity dl sites and twi E boxes.

Spacing is not important for dl–twi interactions in the presumptive mesoderm

As noted earlier, the unmodified twi PE promoter sequence contains two low affinity dl binding sites and mediates expression in the ventral-most 12–14 cells, which corresponds to a subdomain of the presumptive mesoderm where there are peak levels of dl protein (Figure 3A). In addition, the lateral limits of the pattern are not sharp. When two twi E box sequences are placed either 25 or 86 bp upstream of these two low affinity dl sites (PE-25Et or PE-86Et), the modified promoter directs uniformly strong expression in the entire presumptive mesoderm and the staining pattern spans ~18–20 cells (Figure 3B and C). Moreover, the lateral limits are quite sharp, such that staining abruptly ends at the boundary between the presumptive mesoderm and neuroectoderm (Figure 3C). A similar expression pattern is observed when the twi E boxes are placed 200 bp downstream of the low affinity dl binding sites (Figure 3D). Again, the staining pattern encompasses the entire presumptive mesoderm and includes sharp lateral borders at the mesoderm/neuroectoderm boundary. Staining does not extend into lateral regions, the presumptive neuroectoderm, where there are low levels of dl protein. These results suggest that the dl–twi synergy underlying the sharp *sna* expression pattern does not depend on linkage of dl and twi binding sites.

twi facilitates dl-mediated activation

Analyses of dl–twi interactions have involved complex promoter elements that are likely to contain binding sites for various unidentified regulatory factors (see, for

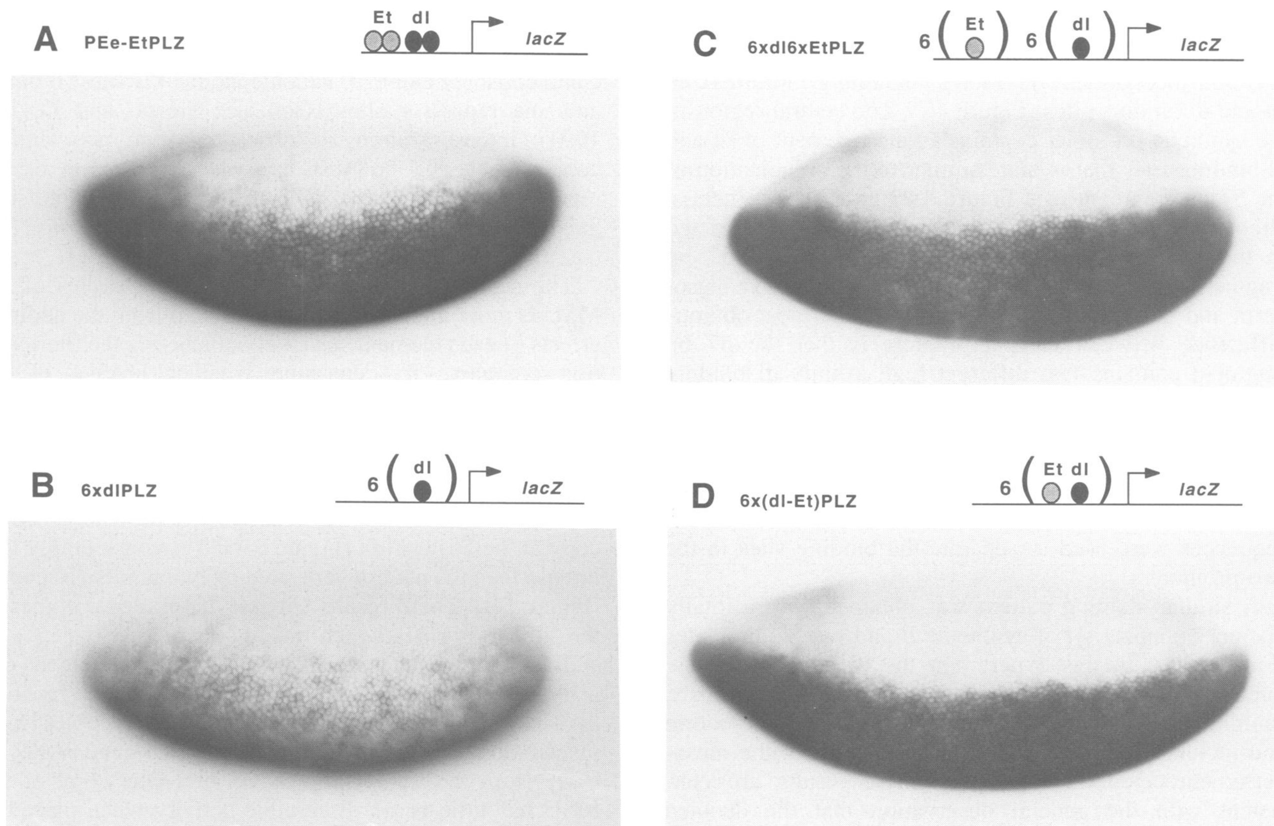

Fig. 4. dl and twi are sufficient for mesoderm expression. Cellularizing, transgenic embryos are oriented with anterior to the left and dorsal up. Staining patterns were visualized after hybridization with a digoxygenin-labeled *lacZ* antisense RNA probe. (**A**) Embryo containing a minimal 57 bp region from the modified PEe-6Et twi promoter (see Figure 2A) attached to a minimal P-element basal promoter. This element contains two twi E boxes placed 6 bp upstream of two high affinity dl sites. It directs a broad pattern of expression, including the presumptive mesoderm, mesectoderm and weak staining extending to ventral regions of the neuroectoderm. This pattern is quite similar to that observed for the entire twi PEe-6Et, which includes an additional ~390 bp of the twi promoter region. (**B**) Expression pattern obtained with six tandem copies of a high affinity dl binding site attached to the P-element promoter. Staining extends throughout the presumptive mesoderm, but is weak and erratic. (**C**) Expression pattern obtained with six copies of a twi E box placed upstream of six dl sites. Staining is considerably more intense and uniform as compared with (B). The pattern extends throughout the entire presumptive mesoderm and mesectoderm and includes relatively sharp lateral limits. (**D**) As (C) except that the fusion gene contains a different arrangement of twi E boxes and dl sites. The staining pattern is virtually indistinguishable from the one shown in (C).

example, Figures 2 and 3). We have further examined these interactions in the context of synthetic promoters composed solely of multimerized dl and twi binding sites. An indication that dl and twi may be sufficient for robust expression was suggested by the staining pattern obtained with a minimal fusion gene containing just a 57 bp sequence from the modified PEe-6Et promoter (Figure 4A; compare with Figure 2A). The full-length PEe-6Et promoter is 440 bp in length and includes promoter proximal elements that participate in the activation of *twi* expression (Pan *et al.*, 1991). A 57 bp fragment containing the two high affinity dl binding sites and two twi Et sites was placed directly upstream of a minimal P-element promoter−*lacZ* reporter gene (Wharton and Crews, 1993). The P-element promoter is truncated to position −42 and is little more than a TATA box and initiation site. The resulting fusion gene mediates staining throughout the presumptive mesoderm and also extends to ventral regions of the lateral neuroectoderm (Figure 4A).

Either three or four tandem copies of a high affinity dl binding site mediate weak and erratic expression of a reporter gene in ventral regions of precellular transgenic embryos (Jiang *et al.*, 1991; Pan *et al.*, 1991). Similar staining patterns were obtained with six copies of a high affinity dl binding site placed upstream of the P-element promoter (Figure 4B). Variable expression is observed throughout the presumptive mesoderm, although the pattern is patchy and lacks distinct lateral limits. It would appear that dl is inherently a weak transcriptional activator and requires additional factors to mediate optimal expression in early embryos. twi may be an even poorer activator than dl. Six tandem Et twi E box recognition sites fail to activate the *lacZ* reporter gene (data not shown).

A combination of dl and twi binding sites results in synergistic expression of the *lacZ* reporter gene throughout the presumptive mesoderm of precellular embryos (Figure 4C and D). In the first experiment of this sort, six tandem copies of the twi Et binding site were placed immediately upstream of six tandem copies of the high affinity dl binding site (Figure 4C). This organization of binding sites generates strong, nearly uniform staining in the entire mesoderm. In contrast to the situation observed for the dl binding sites alone, the combination of dl and twi sites directs distinct, fairly sharp lateral borders. Double staining experiments with the *sim* probe indicate that the lateral limits extend to the single row of mesectodermal cells (data not shown), similar to the pattern obtained with the PE-Et series presented in Figure 2. There are several

potential explanations for the different staining patterns obtained with the 57 bp PEe-EtPLZ fusion gene (Figure 4A) and the synthetic promoter containing multimerized Et and dl binding sites (Figure 4C). The central region of the synthetic promoter contains an arrangement of Et and dl binding sites that is quite similar to the organization of the 57 bp fragment (see Figure 4A and C). Nonetheless, although expression of the synthetic promoter is restricted to the presumptive mesoderm (Figure 4C), the 57 bp fragment directs expression in both the presumptive mesoderm and neuroectoderm (Figure 4A). The most obvious difference between these promoters is that the 57 bp fragment contains two different high affinity dl binding sites, TD4e and TD5e, which possess distinct core recognition sequences (Jiang and Levine, 1993). In contrast, the synthetic promoter contains multiple copies of just a single dl binding site (TD5e). In addition, dl−bHLH interactions might be influenced by promoter context, in that different sequences were used to separate the binding sites in the two promoters.

A similar staining pattern was obtained with a totally distinct organization of synthetic dl and twi binding sites (Figure 4D). In this experiment the binding sites were interspersed (dl, twi, dl, twi, etc.). As seen previously, staining encompasses the entire presumptive mesoderm and includes reasonably sharp lateral limits at the mesoderm/neuroectoderm boundary. These results are consistent with the general observation that the detailed organization of dl and twi sites is not essential for synergistic interactions.

Reconstruction of a minimal rhomboid enhancer

A 650 bp *rho* NEE can generate lateral stripes in transgenic embryos even when placed in the opposite orientation 5 kb downstream of the transcription start site (S.Gray and M.Levine, unpublished results). It is conceivable that unidentified factor binding sites in the NEE are responsible for long-range interactions with basal components at the transcription start site. Alternatively, perhaps dl−bHLH complexes bound to the enhancer are sufficient for these long-range interactions. To investigate this issue we sought to identify the minimal sequence requirements for enhancer activity.

Various minimal NEE fragments were analyzed in transgenic embryos (Gray *et al.*, 1994; data not shown). The smallest element that mediates neuroectodermal expression corresponds to a synthetic 57 bp fragment derived from the modified PEe-Eds *twi* promoter sequence described by Jiang and Levine (1993). This fragment is similar to the 57 bp fragment used in Figure 4A, except that it contains two Eds E boxes in place of the twi-class Et E boxes. The Eds sequences bind ubiquitous maternal bHLH proteins [daughterless (da) and T4], but possess at least 10-fold lower affinity for twi than the Et E box (Ip *et al.*, 1992a). In addition, the Eds sequence binds the sna repressor and, consequently, modified twi PE promoters containing high affinity dl sites and Eds E boxes direct lateral stripes of expression in the presumptive neuroectoderm (Jiang and Levine, 1993).

One copy of the synthetic 57 bp element was placed upstream of the minimal P-element promoter (80 bp from the initiation site) driving the *lacZ* reporter gene. This fusion gene directs weak lateral stripes in transgenic embryos (Figure 5A). The staining pattern is similar to that obtained with a minimal mammalian kappa light chain enhancer element, which contains κB sites, E boxes and sna repressor sites (Gonzalez-Crespo and Levine, 1994). Intense staining is observed when two tandem copies of the 57 bp MSU are placed upstream of the reporter gene (Figure 5B). This pattern is virtually indistinguishable from that observed for the full-length *rho* NEE (see Ip *et al.*, 1992a).

The staining obtained with two tandem copies of the MSU is more intense than that predicted from the additive effects of each element separately contacting the transcription complex. To determine whether linkage of the elements is important for this multiplicative activation, one copy of the MSU was placed at the 5′ position and another copy was placed downstream of the *lacZ* reporter gene (Figure 5C). The resulting staining pattern is only slightly more intense than that observed with a single copy at the 5′ position (Figure 5A); it is considerably less intense than the pattern generated by two tandem 5′ copies (Figure 5B). These results suggest that multiple copies of the MSU work best when linked (see below).

To determine whether the minimal 57 bp element can activate transcription from a distance and thereby function as a *bona fide* enhancer, one, two or four copies were placed downstream of the *lacZ* reporter gene, ~4.5 kb away from the transcription start site. One copy of the MSU has little or no discernible activity when placed at the 3′ position (data not shown). This could indicate that the element lacks factor binding sites required for long-range enhancer−promoter interactions. Alternatively, the dl and bHLH activators might be inherently 'weak' and can work over long distances only when multimerized. In this regard we note that the full-length *rho* NEE contains four dl sites and five E boxes. Two tandem copies of the 57 bp MSU direct weak lateral stripes, comparable with the staining obtained with one copy of the element at the 5′ position near the TATA box (Figure 6A; compare with Figure 5A). Four tandem copies of the MSU at the 3′ position direct lateral stripes of intermediate intensity (Figure 6B), comparable with the staining pattern obtained with the full-length *rho* NEE at the 3′ location (data not shown). These results suggest that dl−bHLH complexes within the *rho* NEE possess the intrinsic ability to communicate with basal components at the transcription start site over long distances.

Discussion

We have presented evidence that the proximity of dl and bHLH binding sites is important for the synergistic activation of gene expression in the lateral neuroectoderm of early *Drosophila* embryos in response to low levels of dl protein. Linkage does not require a fixed organization of sites, in that different arrangements work equally well in mediating neuroectoderm expression. This observation indicates that certain enhancers, such as the kappa light chain enhancer (Saksela and Baltimore, 1993) and the *rho* enhancer (Ip *et al.*, 1992a), may possess flexible organizations. dl−twi interactions in the presumptive mesoderm do not require linkage of sites, suggesting that these proteins might independently contact the transcription complex. Analysis of synthetic promoters composed

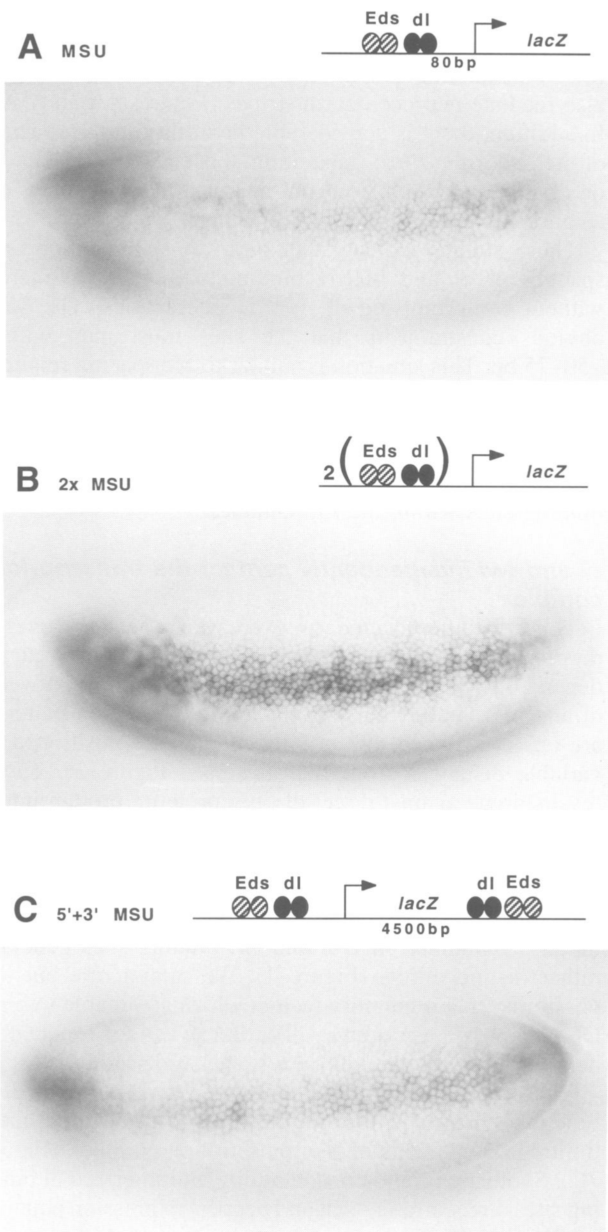

Fig. 5. Linked copies of a minimal regulatory element work better than unlinked copies. Cellularizing, transgenic embryos are oriented with anterior left and dorsal up. They were stained with a digoxygenin-labeled *lacZ* antisense RNA probe. (**A**) Expression pattern obtained with a *lacZ* fusion gene containing a synthetic 57 bp MSU attached to the P-element basal promoter. The MSU was derived from a modified twi PE promoter sequence containing two E boxes (Eds) placed 6 bp upstream of two high affinity dl sites. The E boxes are different from the twi-class E boxes used in the previous experiments. The Eds E boxes bind ubiquitous, maternal bHLH proteins such as da and T4 (see Figure 1 summary). One copy of the MSU mediates weak lateral stripes in the presumptive neuroectoderm when placed 80 bp upstream of the start site. (**B**) As (A) except that two tandem copies of the MSU were placed at the 5′ position. Intense staining is observed in the lateral neuroectoderm. (**C**) As (B) except that the two MSUs were separated, with one placed at the 5′ position and the other positioned downstream of the *lacZ* reporter gene. The staining pattern is quite weak, only slightly stronger than the pattern obtained with just a single MSU located at the 5′ position (A).

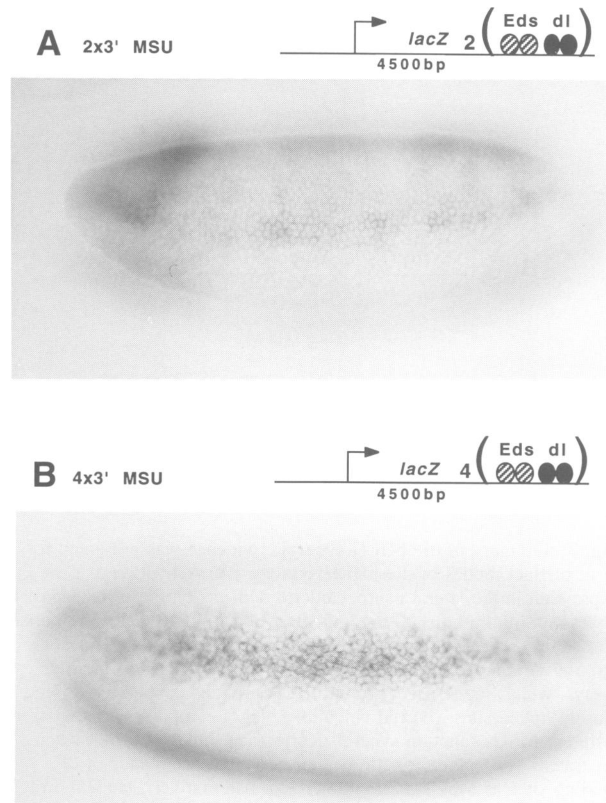

Fig. 6. Tandem copies of the MSU function like an enhancer over long distances. Cellularizing, transgenic embryos were stained with a digoxygenin-labeled *lacZ* antisense RNA probe. (**A**) Expression pattern obtained with a *lacZ* fusion gene containing two tandem copies of the MSU located downstream of the *lacZ* reporter gene (~4.5 kb from the transcription start site). Weak staining is observed in the lateral neuroectoderm, similar to the staining pattern obtained with one copy of the MSU at the 5′ position (compare with Figure 5A). (**B**) As (A) except that the fusion gene contains four tandem copies of the MSU. The staining pattern includes strong lateral stripes in the presumptive neuroectoderm. The pattern is quite similar to that obtained when the full-length 650 bp *rho* NEE is placed at the 3′ position (S.Gray and M.Levine, unpublished results).

of simple multimers of dl and twi sites reinforces the notion that these factors might interact with different rate-limiting components of the complex. A synthetic 57 bp element containing just two dl sites, two E boxes and two sna sites is sufficient to direct lateral stripes in transgenic embryos. Tandem multimers of this minimal element can function as a *bona fide* enhancer and work over long distances to activate a heterologous promoter. Multiple copies of this minimal element function synergistically when linked, but not when separated. We propose that this linkage requirement provides the basis for the evolution of complex, modular promoters containing discrete, autonomous enhancers.

Enhancers as integrating pattern elements

This study provides evidence for three distinct modes of dl−bHLH transcriptional synergy: (i) cooperative interactions between closely linked high affinity dl sites and E boxes (Figure 7A); (ii) synergistic interactions among tandem arrays of minimal dl−bHLH complexes (Figure 7B); (iii) independent contact of different rate-limiting components of the transcription complex (Figure 7C).

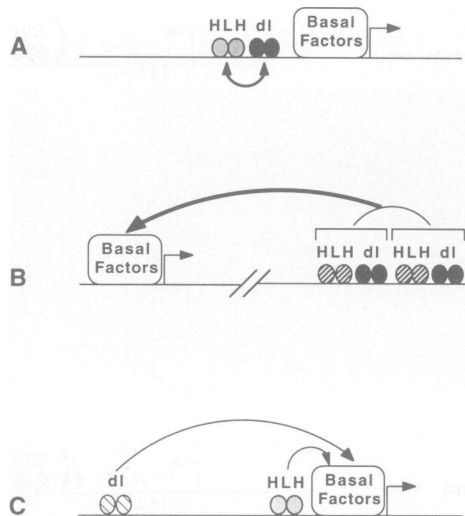

Fig. 7. Summary of dl−bHLH synergy. Evidence was presented for three distinct modes of dl−bHLH synergy. (**A**) Activation of gene expression in the lateral neuroectoderm depends on close linkage of E boxes and high affinity dl binding sites. Separating the sites by either 86 or 200 bp nearly abolishes neuroectoderm expression. This proximity requirement is consistent with previous *in vitro* binding assays, which suggest that dl and bHLH proteins cooperatively bind closely linked sites. (**B**) Individual dl−bHLH complexes contained in different MSUs function synergistically when linked, but not when separated. This might arise from more efficient occupancy of dl binding sites as MSUs are multimerized. Alternatively, tandem arrays of dl−bHLH complexes might be more effective at recruiting adaptors or basal factors as compared with unlinked complexes. (**C**) Strong, uniform expression of target genes in the presumptive mesoderm depends on synergistic interactions between low affinity dl sites and unlinked twi-class E boxes. The fact that these sites can be separated by large distances suggest that dl and twi might interact with distinct basal factors in the transcription complex. This view is reinforced by the analysis of synthetic promoters containing multimerized dl and twi binding sites (see Figure 4).

Previous *in vitro* DNA binding assays demonstrated that dl and various bHLH proteins can cooperatively occupy closely linked sites (Jiang and Levine, 1993). The demonstration that neuroectoderm expression depends on closely linked dl and twi binding sites provides *in vivo* support for cooperative binding. Separating the sites by 86 or 200 bp might disrupt these interactions, so that the low levels of dl protein present in the lateral neuroectoderm fail to occupy even high affinity dl sites.

Characterization of the mammalian interferon β promoter suggests that individual factor binding sites, including those recognized by NF-κB, IRF-1 and ATF-2, must be rigidly organized for optimal expression (reviewed by Tjian and Maniatis, 1994). This stereotyped arrangement appears to be imposed by HMG I(Y), which binds to multiple sites and sets a higher order structure. Such a rigid spacing requirement would place severe constraints on the evolution of enhancers, which often contain multiple binding sites for several different regulatory factors.

This study provides evidence that at least some promoter elements mediating combinatorial regulation may possess a rather flexible organization. Several entirely distinct arrangements of dl and bHLH binding sites can mediate neuroectoderm expression. For example, both a minimal mammalian kappa light chain enhancer and the *rho* enhancer mediate expression in the neuroectoderm (Ip *et al.*, 1992a; Gonzalez-Crespo and Levine, 1994), yet they contain distinct organizations of dl and HLH (E box) binding sites. Moreover, a synthetic 57 bp element (the MSU) containing yet another arrangement of sites can also mediate neuroectoderm stripes (Figures 4A and 5A). In addition, there is considerable flexibility in the spacing of neighboring sites; separating the sites by 6, 11, 20 or 25 bp has no effect on neuroectoderm expression (Figure 2).

These studies indicate that both the organization and spacing of dl and bHLH binding sites can be altered without compromising dl−bHLH interactions. The only obvious constraint is that the sites must map within ~50–75 bp. This situation is similar to the spacing requirements for the sna repressor. Efficient repression is observed when the sna sites map within ~100 bp of dl activator sites (Gray *et al.*, 1994). Thus there appears to be substantial flexibility in the organization of the factor binding sites within the *rho* enhancer.

dl and twi independently contact the transcription complex

In contrast to neuroectoderm expression, the sharp, mesoderm-restricted *sna* expression pattern does not appear to depend on cooperative DNA binding interactions between dl and bHLH activators. Comparable expression patterns are observed when low affinity dl sites are positioned at variable distances from twi sites (see Figure 3). These results argue against direct dl−twi protein−protein interactions within the *sna* promoter. It is conceivable that additional, unknown DNA binding proteins mediate indirect cooperative contact of dl and twi bound to distant sites. However, this seems unlikely, since synthetic promoters containing only dl and twi binding sites generate rather distinct limits (Figure 4). We cannot rule out the possibility that regulatory factors which are unable to bind DNA might be recruited by dl and/or twi and subsequently help the latter proteins form a higher order complex.

We propose that unlinked dl and twi binding sites function synergistically by interacting with different rate-limiting components of the transcription complex (Figure 7C). Synthetic promoters containing multimerized dl binding sites direct erratic, 'salt and pepper' expression patterns in the presumptive mesoderm (see Figure 4A). Similar patterns were observed for sensitized *sna−lacZ* fusion genes (Ip *et al.*, 1992b). By itself, dl may be a weak activator, because it contacts a basal factor that does not ensure the assembly of a complete transcription complex. Consequently, dl-mediated activation might be stochastic. Multimerized twi binding sites are insufficient to mediate transcriptional activation, although these function synergistically with dl sites to activate synthetic promoters in the presumptive mesoderm (Figure 4). We propose that dl interacts with a basal factor required for the initial assembly of the complex, such as the TATA binding protein (TBP), while twi interacts with a basal factor that is brought into the complex at a later point in assembly or elongation. This would explain why dl is sufficient to initiate erratic expression, whereas twi facilitates dl-mediated activation, but fails to work as an activator when alone.

The slope of the dl gradient might account for the distinct modes of dl−twi synergy (Figure 7A and C). As discussed above, neuroectoderm expression depends on high affinity dl sites and closely linked twi E boxes, while

mesoderm expression is mediated by low affinity sites and unlinked E boxes. The dl gradient appears to be exponential, with a steep drop in lateral regions (Roth *et al.*, 1989; Rushlow *et al.*, 1989; Steward, 1989). Low affinity dl sites might be efficiently occupied in ventral regions, where there are plateau levels of dl protein. In ventrolateral regions, site occupancy might be only slightly diminished (30–50% maximum occupancy), due to the slight reduction in the dl gradient. In these regions, activation by dl alone might be somewhat erratic. However, independent interactions of dl and twi with the transcription complex might augment expression in these regions, as discussed above. Interestingly, closely linked low affinity dl sites and twi E boxes appear to mediate a slight expansion of the pattern (into the mesectoderm) prior to the rapid decline in the dl gradient. The PE-6Et and PE-25Et promoters appear to yield slightly broader patterns than either PE-86Et or PE-Et prox (see Figure 3). In contrast, site occupancy in lateral regions might require both high affinity dl sites and cooperative interactions with linked twi E boxes.

Reconstruction of a minimal rho enhancer

To our knowledge, the 57 bp MSU (Figures 5 and 6) represents the smallest regulatory sequence that can integrate multiple activators and repressors to generate a tissue-specific expression pattern in a transgenic metazoan. Consequently, this element provides a basis for determining the minimal requirements for long-range enhancer activity. A single copy of the 57 bp element mediates only very weak stripes when placed just upstream of a heterologous TATA box. A *lacZ* fusion gene containing one 5′ copy and one 3′ copy directs slightly stronger expression. It would appear that the 5′ and 3′ copies work in an additive fashion when separated. In contrast, two tandem copies at the 5′ position direct intense staining comparable to endogenous *rho* expression. These results suggest that there are two tiers of dl−bHLH synergy. First, there are local, cooperative interactions between dl and bHLH proteins bound to neighboring sites within the MSU (Figure 7A). Second, different dl−bHLH complexes bound to separate MSU elements can work synergistically to activate transcription (Figure 7B). It would appear that tandemly arrayed MSUs are more efficient at recruiting adaptors and/or basal transcription factors than individual, unlinked copies (Figure 7).

The characterization of the 57 bp MSU suggests that enhancers might evolve from the multimerization of a simple upstream activator element. A single MSU placed just upstream of TATA functions like a typical promoter proximal element. It fails to mediate expression when placed far from the promoter (data not shown). In contrast, either two or four tandem copies of the MSU can mediate long-range activation of a heterologous promoter from the 3′ position. Synergy among linked MSUs (Figure 7B) might provide the basis for the evolution of complex enhancers. Separate copies of a duplicated proximal element can function as a long-range enhancer only when they remain linked and not if they are dispersed.

Materials and methods

Site-directed mutagenesis

The pGEM7Zf+ derivatives pGHX and pGHXe, containing the *twi* PE and PEe promoter fragments respectively (Jiang and Levine, 1993) were used as templates to prepare single-stranded DNA. Pairs of twi E boxes (CATATG) were introduced by *in vitro* mutagenesis (Sambrook *et al.*, 1989) at positions −354 to −349 and −343 to −338 bp of the *twi* promoter to create PE-25Et and PEe-25Et and at positions −416 to −411 and −405 to −400 bp to create PE-86Et and PEe-86Et. To make PE-Et prox and PEe-Et prox, the plasmid pGXP, which contains twi promoter sequences from −180 bp to +300 bp (Jiang *et al.*, 1991) was used to prepare a single-stranded template. A pair of twi E boxes was introduced at positions −86 to −81 and −75 to −70 bp. The 5 bp spacing between E boxes corresponds to the natural spacing found between the twi E boxes of the *rho* NEE (Ip *et al.*, 1992a). In all constructs the dl sites are located at positions −312 to −302 and −294 to −285 bp. The identities of all modified promoters were confirmed by DNA sequencing prior to subcloning into P-element vectors as described below.

Plasmid constructions

Modified *twi* promoters were transferred from the phagemid vectors into derivatives of the P transformation vector pCaSpeR-AUG which carry *twi* promoter sequences (Jiang and Levine, 1993). For PE-25Et, PE-86Et, PEe-25Et and PEe-86Et, a 260 bp *Eco*RI−*Xho*I fragment consisting of the modified *twi* PE (−440 to −180 bp) was subcloned into a plasmid carrying *twi* promoter sequences from −180 to +160 bp. For PE-Et prox and PEe-Et prox, a 320 bp *Xho*I−*Bam*HI fragment (−180 to +160 bp) was subcloned into plasmids carrying the *twi* PE and PEe (−440 to −180 bp) respectively.

PEe-EtPLZ was constructed by annealing partially overlapping oligonucleotides and extending with Klenow DNA polymerase to generate an 80 bp DNA fragment of sequence cggcggtaccgagCTCG**CATATG**TTGAG**CATATG**TTT<u>GGGGATTTTCCC</u>AAATCGA<u>GGGAAAACCC</u>AAgg atccgccg. This fragment contains a 57 bp sequence from the PEe-Et promoter (upper case, −339 to −283 bp) plus flanking sequences for subcloning (lower case) and contains two twi E boxes (boldface) and two high affinity dl sites (underlined). The 80 bp fragment was digested with *Kpn*I and *Bam*HI and the resulting 66 bp fragment was inserted between the *Kpn*I and *Bam*HI sites of the P transformation vector C4PLZ, which contains the P-element promoter and transposase N-terminal sequence fused in-frame with the *lacZ* gene (Wharton *et al.*, 1993). The MSU plasmid was constructed in a similar manner, except the E box sequence CACCTG was used instead of CATATG. The identities of PEe-EtPLZ and the MSU were confirmed by DNA sequencing.

To make 2× MSU, the 80 bp blunt fragment was ligated to *Eco*RI linkers and digested with *Eco*RI and the resulting 90 bp fragment was inserted into the *Eco*RI site of the MSU plasmid (located in the 5′ polylinker of C4PLZ, upstream of the *Kpn*I site). The identity of the 2× MSU construct was confirmed by DNA sequencing. To make 5′+3′ MSU, 2×3′ MSU and 4×3′ MSU, the 2× MSU plasmid was partially digested with *Eco*RI and completely with *Hin*dIII (located downstream of the *Bam*HI site). The DNA was blunted with Klenow, ligated to *Bgl*II linkers and digested with *Bgl*II. A 206 bp fragment containing two copies of the MSU was inserted at high multiplicity into the *Bgl*II site of C4PLZ (located in the 3′ polylinker) to make 2×3′ MSU and 4×3′ MSU, which were identified by restriction digestion. A 116 bp fragment containing one copy of the MSU was inserted into the *Bgl*II site of the MSU plasmid to produce 5′+3′ MSU.

To construct 6× dlPLZ and 6× EtPLZ, synthetic oligonucleotides containing a high affinity dl site (TD5e; see Jiang and Levine, 1993) or twi E box were annealed, ligated at high multiplicity to generate multimers and digested with *Bam*HI and *Bgl*II to eliminate head-to-head and tail-to-tail ligations. The species corresponding to six copies were gel purified and inserted into the *Bam*HI site of C4PLZ. The sequences of the synthetic dl oligonucleotides were 5′-gatcctGGGAAAACCCga and 5′-gatctcGGGTTTTCCCag. The TD5e dl sites are indicated by upper case letters. Multimerization results in 8 bp of DNA between each of the dl sites. The spacing between the dl sites in the twi PE is 7 bp. The sequences of the synthetic twi E box oligonucleotides were 5′-gatcCATATGa and 5′-gatctCATATG. The E boxes are in capitals. Multimerization results in 5 bp of DNA between each of the E boxes, the same spacing as in the *rho* promoter (Ip *et al.*, 1992a) and in the modified twi PE constructs of Figures 2 and 3. To make 6× dl 6× EtPLZ, the 6× Et fragment was inserted into the *Bam*HI site of 6× dlPLZ, which was regenerated only on the upstream side of the dl sites. This results in a 7 bp spacing between the last dl site and the first twi E box.

To construct 6× (dl-Et)PLZ, two synthetic oligonucleotides, 5′-**CATATG**CTTTG<u>GGGAAAACCC</u>gagctg and 5′-tc<u>GGGTTTTCCC</u>-

CCAAGCATATGcagc, were annealed to obtain a double-stranded fragment with non-palindromic 3′ overhangs consisting of 22 bp of DNA sequence derived from the PEe-Et promoter (upper case) plus flanking sequences for subcloning (lower case). This fragment was ligated at high multiplicity to generate head-to-tail multimers with 6 bp between each of the dl sites (underlined) and twi E boxes (boldface). Synthetic *Not*I adapters (5′-GGCCGCTG) and *Bam*HI adapters (5′-GATCCAGC) were then ligated to the multimers and the DNA was digested with *Not*I and *Bam*HI to remove unwanted ligation products. The species corresponding to six copies was gel purified and inserted between the *Not*I and *Bam*HI sites of C4PLZ. The identity of all synthetic multimer constructs was confirmed by DNA sequencing.

P transformation and whole mount in situ hybridization

P transposons carrying the *lacZ* reporter gene fusions were introduced into the *Drosophila* germline using standard methods (Spradling and Rubin, 1982). Multiple independent transgenic lines were examined for each construct. *lacZ* gene expression was visualized by whole mount *in situ* hybridization using a digoxygenin–UTP-labeled antisense RNA probe (Tautz and Pfeifle, 1989; Jiang *et al.*, 1991). In Figure 2 an antisense *sim* probe was included in the hybridization to visualize the limits of the presumptive mesoderm.

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