The *dorsal* morphogen gradient regulates the mesoderm determinant *twist* in early *Drosophila* embryos

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A gradient of the maternal morphogen *dorsal* (*dl*) initiates the differentiation of various tissues along the dorsal–ventral axis of early *Drosophila* embryos. *dl* is a sequence-specific DNA-binding protein that is related to the mammalian regulatory factor NF- κ B. Previous studies suggest that *dl* can function as a transcriptional repressor. To determine how *dl* functions as an activator we have examined the promoter of the mesoderm determinant gene *twist* (*twi*). Genetic studies suggest that peak levels of *dl* protein in ventral regions of early embryos initiate *twi* expression. Using a combination of promoter fusion–P-transformation assays, and *in vitro* DNA-binding assays coupled with site-directed mutagenesis, we establish a direct link between *dl*-binding sites and *twi* expression in the early embryo. We also present evidence that the dorsal–ventral limits of *twi* expression depend on the number and affinity of *dl*-binding sites present in its promoter. A comparison of *twi* with a second *dl* target gene, *zen*, suggests a correlation between the affinities of *dl*-binding sites and response to different thresholds of *dl* morphogen.

[Key Words: *twist*; *dorsal*; *Drosophila* embryos; dorsal–ventral pattern]

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The establishment of the two primary body axes of the early *Drosophila* embryo depends on four maternally active regulatory genes that encode products present in the unfertilized egg. Three of these genes [*bicoid* (*bcd*), *nanos*, and *torso*] are responsible for the anterior–posterior axis [for review, see Nüsslein-Volhard et al. 1987], whereas only one, *dorsal* (*dl*), is required for the dorsal–ventral axis [Roth et al. 1989; Rushlow et al. 1989; Steward 1989]. The best characterized maternal morphogen is *bcd*, which encodes a homeo domain protein that binds DNA and functions as a transcriptional activator [Driever et al. 1989; Struhl et al. 1989]. It is expressed in a broad gradient with peak levels at the anterior pole [Driever and Nüsslein-Volhard 1988] and regulates gene expression in a concentration-dependent manner [Driever and Nüsslein-Volhard 1989; Struhl et al. 1989]. *bcd* is thought to control cell fate through a “French flag” mechanism, whereby target genes are restricted to discrete domains in response to distinct threshold levels of the morphogen [Wolpert 1969]. Although not studied to the same extent as *bcd*, *dl* offers several advantages for an in-depth analysis of how target genes differentially respond to a common morphogen gradient. In particular, *dl* is the only morphogen known to be required for dorsal–ventral polarity, and it is easy to manipulate the *dl* gradient, so that uniformly high, low, or intermediate levels can be expressed in all embryonic nuclei [Roth et al. 1989].

The *dl* protein is distributed in a concentration gradient along the dorsal–ventral axis of early embryos, with peak levels in the ventral-most regions and progressively lower levels in lateral and dorsal regions [Steward et al. 1988]. This concentration gradient is established by selective nuclear transport [Roth et al. 1989; Rushlow et al. 1989; Steward 1989] and is responsible for initiating the differentiation of the mesoderm, neuroectoderm, and dorsal ectoderm. Peak levels of *dl* initiate the expression of regulatory genes [*twist* (*twi*) and *snail* (*sna*)] responsible for the differentiation of the mesoderm in ventral regions [Simpson 1983; Boulay et al. 1987; Thisse et al. 1987, 1988], whereas lower levels may control the expression of regulatory factors (such as AS-C) required for the formation of the neuroectoderm in lateral regions [Kosman et al. 1991]. *dl* also influences cell fate by repressing gene expression and restricting the expression of regulatory genes, such as *zerknüllt* (*zen*) and *decapentaplegic* (*dpp*), to dorsal regions where they are responsible for the differentiation of dorsal ectodermal derivatives, including the amnioserosa and dorsal epidermis [Rushlow et al. 1987; St. Johnston and Gelbart 1987].

dl is a sequence-specific DNA-binding protein that shares extensive homology with the oncoprotein *rel* and the mammalian transcriptional regulator NF- κ B [Steward et al. 1987; Ghosh et al. 1990; Kieran et al. 1990]. All three of these proteins are regulated by selective nuclear transport, and at least in the case of *dl* and NF- κ B, the

proteins must enter nuclei to regulate gene expression and influence cell fate. Studies on the target gene *zen* suggest that *dl* may function as a transcriptional repressor, which is responsible for keeping *zen* expression off in ventral regions of early embryos (Ip et al. 1991). Repression is mediated by a distal region of the *zen* promoter, called the ventral repression (VR) element (Doyle et al. 1989). The VR element has the properties of a silencer sequence, in that it can act over long distances to mediate the VR of heterologous promoters (Doyle et al. 1989; Ip et al. 1991). *dl* is likely to mediate long range repression as the VR element contains several high-affinity *dl*-binding sites.

Genetic studies and sequence similarities with NF- κ B suggest that *dl* may also function as an activator, which turns on genes in ventral regions of early embryos. Peak levels of *dl* initiate the expression of two target genes called *twi* and *sna*, which encode regulatory factors responsible for the differentiation of the ventral mesoderm (Boulay et al. 1987; Thisse et al. 1987; 1988). *twi*⁻ and *sna*⁻ embryos display a similar failure of ventral furrow formation and loss of the mesoderm and its derivatives (Leptin and Grunewald 1990). The present study is concerned with how *dl* activates the expression of *twi*, to complement our previous studies on the *zen* promoter. *twi* and *zen* appear to differ in two important ways with respect to their regulation by the *dl* morphogen gradient. First, *twi* is activated, whereas *zen* is repressed. Second, *zen* is sensitive to lower levels of the morphogen than *twi*. For example, in mutants where *dl* is expressed at uniformly low levels in all nuclei, *twi* is not activated but *zen* is completely repressed (Roth et al. 1989).

Here we show that a relatively small region (1.2 kb) of *twi* 5'-flanking sequence is sufficient to generate an essentially normal *twi* pattern when it is attached to a *lacZ* reporter gene and expressed in P-transformed embryos. DNA-binding studies reveal a total of four *dl*-binding sites in the promoter, and site-directed mutagenesis establishes a direct link between these sites and expression in vivo. The *dl* sites present in the *twi* promoter possess lower affinities than those in *zen*, which might account for the different sensitivities of the two promoters to the *dl* gradient.

Results

As a first step toward identifying potential *dl* response elements in the *twi* promoter, we attached different 5'-flanking sequences to the bacterial *lacZ* reporter gene, as summarized in Figure 1. The arrow indicates the position of the transcription start site, based on studies by Thisse et al. (1988). *twi* 5'-flanking sequences were isolated from a genomic DNA library by using a cDNA fragment containing the first 340 bp of the protein-coding sequence. We determined the nucleotide sequence of a 3-kb region, extending from -2.9 kb upstream of the transcription start site to the initiating ATG (not shown). A partial restriction map of the *twi* promoter is presented in Figure 1.

The promoter fusions that were examined include the

Figure 1. Summary of *twi-lacZ* P transposons. The top horizontal bar (solid region) represents a restriction map of a 3-kb region of the *twi* promoter, extending from -2.9 kb upstream of the transcription start site (arrow) to +160 bp at the initiating AUG. A synthetic *Bam*HI site was created at position +160 to facilitate cloning (see Materials and methods). The *twi* sequences were placed at the initiating AUG of the *lacZ*-coding sequence (represented by the open portion of the bar). A series of truncated promoters were prepared, with the smallest including just 180 bp of 5'-flanking sequence. The levels and limits of expression obtained with each construct are summarized at right. The 2.9-, 2.0-, and 1.2-kb constructs gave patterns of expression comparable to the authentic *twi* pattern (+++). In contrast, the smaller promoters gave narrower limits and lower levels of expression (++ or +). The CA1 $\times$ DE and CA2 $\times$ DE constructs contain one or two copies of a 400-bp *twi* fragment (from -1.2 kb to -800 bp) placed upstream of the minimal 180-bp *twi-lacZ* fusion. Asterisks (*) indicate deviations from the wild-type patterns after cellularization. Nonetheless, the CA2 $\times$ DE construct gives a broader initial pattern than the CA1 $\times$ DE construct.

twi basal promoter, as well as 160 bp of untranslated leader sequences. The initial experiments involved a set of seven truncated promoters, with the largest containing 2.9 kb of 5'-flanking sequence and the smallest including the first 180 bp of proximal promoter sequences. The *twi-lacZ* fusions were introduced into embryos with the CasPer transformation vector (Thummel et al. 1988) and standard P-transformation methods (see Materials and methods). The expression of the *lacZ* reporter gene was monitored by in situ hybridization with an RNA hybridization probe and whole-mount preparations of P-transformed embryos (Tautz and Pfeifle 1989; Kosman et al. 1991). This procedure is quite sensitive and permits detection of reporter gene expression in early

embryos, comparable to the time when the endogenous *twi* gene is regulated.

5'-Flanking sequence of 1.2 kb generates an authentic *twi* pattern

The first *twi-lacZ* fusion that we tested contains 2.9 kb of 5'-flanking sequence (see Fig. 1), which directs an expression pattern that is indistinguishable from the endogenous *twi* gene (Fig. 2). Reporter gene expression is detected as early as nuclear cycle 12 (data not shown), comparable to the time when *twi* RNAs are first observed (Thisse et al. 1987; Leptin and Grunewald 1990). By cycle 14, intense expression is observed along the ventral surface and extends through the anterior and posterior poles (Fig. 2A). The lateral limits include an average of ~20 cells, with ~10 cells on either side of the ventral midline (Fig. 2B). The lateral borders appear to be quite sharp, with reduced levels of expression spanning just two or three cells. Reporter gene expression persists after the invagination of the ventral furrow and is detected during germ-band elongation (Fig. 2C). The dorsal patch of staining that is occasionally seen (Fig. 2A, arrow) is not due to *twi* promoter sequences but, rather, results from the P-transformation vector (J. Jiang, Y.T. Ip, and S. Small, unpubl.).

Similar expression patterns were obtained with truncated *twi-lacZ* fusion genes containing 2 or 1.2 kb of 5'-flanking sequence (see Fig. 1). In both cases, expression extends along the ventral surface and encompasses the poles (Fig. 3A). The lateral limits span the entire presumptive mesoderm and appear to extend into the mesectoderm (Fig. 3B). Expression persists during germ-band elongation (not shown).

Five of six independent P-transformed lines containing a 1-kb truncated promoter (see Fig. 1) showed a pattern similar, but somewhat narrower, than those obtained with the 2.9-, 2.0-, and 1.2-kb promoters (cf. Fig. 3C,D with A,B and Fig. 2). Expression is observed in ~16–18 ventral cells, whereas the larger promoters yield patterns encompassing ~20 cells. This observation suggests that sequences located between –1.2 and –1 kb are important for expression in lateral regions containing low levels of the *dl* morphogen. One of the lines containing the 1-kb fusion gene displayed a discontinuous staining pattern, with gaps in anterior and posterior regions (data not shown). A similar abnormal pattern is observed for *twi* in *dl/+*, *twi/+* double heterozygotes (Kosman et al. 1991).

Further truncations of the *twi* promoter cause even more substantial disruptions in expression. Fusion genes containing either 800 or 440 bp of 5'-flanking sequence display similar abnormal patterns. In both cases, expression is lost from the poles (Fig. 3E) and there is a substantial narrowing in the lateral limits (Fig. 3F). Staining does not encompass the entire presumptive mesoderm, but instead includes an average of ~14 cells in the ventral-most regions. Furthermore, the lateral borders of expression appear to be less sharply defined than those seen for the larger *twi-lacZ* fusions and the endogenous *twi* RNA. Thus, it appears that the removal of sequences

Figure 2. Expression pattern of the “full-length” *twi-lacZ* fusion gene. Embryos were collected from P-transformed lines containing the 2.9-kb *twi-lacZ* fusion gene. Whole-mount preparations were hybridized with *lacZ* antisense RNA probes to visualize reporter gene expression. Embryos are oriented with anterior to the left, and dorsal up. (A) Cellularized embryo. Staining extends through the anterior pole and the ventral half of the posterior pole. The arrow indicates a site of expression that is not a property of the *twi* promoter but is due to sequences in the P-transformation vector. (B) Cellularized embryo tilted ventrally to show lateral limit of expression (arrowheads). The lateral border is quite sharp (comparable to the endogenous *twi* RNA pattern) and is located 10–11 cells dorsal-lateral from the ventral midline. This coincides with the region of the fate map near the boundary between the mesectoderm and neuroectoderm (Hartenstein et al. 1985). (C) Embryo undergoing germ-band elongation. Staining extends throughout the extent of the invaginated mesoderm and persists until after the completion of elongation and the onset of muscle differentiation. (ec) Ectoderm; (ms) mesoderm.

between –1.0 kb and –800 bp causes erratic and narrower limits of expression. Removal of sequences between –440 and –180 bp (see Fig. 1) results in the complete loss of expression in ventral regions, indicating the importance of this region in mediating activation by peak levels of the *dl* morphogen.

Figure 3. Expression patterns obtained with truncated *twi-lacZ* fusion genes. Embryos were collected from P-transformed lines and hybridized with a *lacZ* antisense RNA probe. (A,C,E) Lateral views, with anterior to the left and dorsal up. (B,D,F) Ventral views, with anterior to the left. (A,B) Cellularizing embryos containing the 1.2-kb *twi-lacZ* fusion gene. The expression pattern appears normal and is comparable to that obtained with the larger *twi* promoters (i.e., cf. with Fig. 2). The ventral-lateral limits include ~20 cells, and the borders appear sharp (arrowhead in B). (C,D) Cellularizing embryos expressing the 1.0-kb *twi-lacZ* fusion. The ventral-lateral limits of expression appear to be slightly narrower (from 20 to ~18 cells). (E,F) Nuclear cycle 14 embryos expressing the 800-bp *twi-lacZ* fusion. There is a marked narrowing of the ventral-lateral limits (~14 cells), and the lateral borders are not as sharp as those obtained with the larger promoters (arrowhead; cf. with B and D). In addition, expression is completely lost from the anterior and posterior poles. (pc) Pole cells.

The twi promoter contains separable distal and proximal elements

The results obtained by progressively truncating the *twi* promoter suggest that optimal expression depends on two regulatory elements, a distal element (DE) located between –1.2 kb and –800 bp, and a proximal element (PE) located between –440 and –180 bp. To determine whether the DE can function autonomously we prepared the 1 × DE and 2 × DE fusion genes summarized in Figure 1. The 1 × DE construct contains one copy of the DE attached to a minimal 180-bp *twi-lacZ* fusion, whereas 2 × DE contains two tandem copies of the DE. Both fusion genes direct intense expression of the *lacZ* reporter in ventral and polar regions of early embryos (Fig. 4). One copy of the DE gives an initial pattern of expression that is quite similar to fusion genes containing 2.9, 2.0, or 1.2

kb of 5'-flanking sequence. Expression extends along the entire ventral surface and includes both poles (cf. Fig. 4A with Figs. 2 and 3A–D). The lateral limits of expression encompass the presumptive mesoderm and mesectoderm and span an average width of ~20 cells (Fig. 4B). However, after cellularization, crude pair-rule modulations in expression appear, whereby the normal pattern becomes stripy, owing to reduced expression in inter-stripe regions (Fig. 4B).

The initial staining pattern obtained with two tandem copies of the DE is substantially more intense and broader than the wild-type pattern and encompasses ~26 cells (cf. Fig. 4C,D with A,B). After cellularization the expression pattern includes quite distinctive pair-rule stripes, with interstripe regions being narrower by about three cells as compared with the lateral limits of the stripes (Fig. 4D). The origin of the interstripes is not

Figure 4. Autonomous action of the DE and the role of *twi* autoregulation. P-transformed embryos containing the 1 $\times$ DE (A,B) and 2 $\times$ DE promoter fusions (C,D). Embryos were stained and oriented as described in the legends to Figs. 2 and 3. (A) Early cycle 14 embryo expressing the 1 $\times$ DE fusion. An essentially normal pattern of expression is observed, with staining extending through the anterior and posterior poles. Staining is uniform along the ventral surface. (B) A late cycle 14 embryo (ventral view) expressing the 1 $\times$ DE fusion. The pattern is becoming discontinuous along the ventral surface (the arrow indicates one of the crude stripes). (C) Early cycle 14 embryo expressing the 2 $\times$ DE fusion. Intense staining is detected along the ventral surface and extends through the anterior and posterior poles. At this early stage there is no evidence of discontinuities in the staining pattern. (D) A cellularizing embryo expressing the 2 $\times$ DE fusion. At this later stage there are clear pair-rule modulations in the staining pattern (i.e., arrow), resulting from the repression of expression in interstripe regions. The lateral limits of the interstripes are recessed by about three cells relative to the stripes. Initially, in earlier embryos, the ventral-lateral border is homogenous and coincides with the edges of the stripes (not interstripes) seen in this older embryo. (E) A wild-type cellularizing embryo showing the endogenous *twi* RNA pattern. Expression extends through the poles, and the ventral-lateral limits include ~ 20 cells (cf. Figs. 2B and 3B). (F) The *twi* RNA pattern in an embryo lacking *twi* protein (*twi*LD96/*twi*LD96 homozygote). The ventral-lateral limits are narrower than those seen in wild type, and there are pair-rule modulations in staining along the ventral surface. The lateral limits of each stripe includes 14 cells; the interstripe regions include only 10 cells.

known; however, the pattern of *twi* expression observed in *twi* mutants suggests that they might reflect a property of *twi*⁺ gene activity. Embryos homozygous for a *twi* protein null mutation show reduced and stripy expression of the *twi* RNA, suggesting that optimal expression depends on some type of autoregulation process (cf. Fig. 4F with the wild-type *twi* RNA pattern in Fig. 4E).

The *twi* promoter contains *dl*-binding sites

Systematic gel-shift assays were done to determine whether there are any *dl*-binding sites within the DE or PE regions of the *twi* promoter. This analysis involved digesting DNA fragments encompassing these regions into 60- to 150-bp pieces, labeling them with ³²P, and incubating the DNAs with *dl* protein made in bacteria.

Electrophoresis on native gels revealed that three of the fragments contain *dl*-binding sites, including a 147-bp *Hinf*I fragment (located between -398 and -251 bp in the PE), a 93-bp *Eco*RI-*Taq*I fragment (-876 to -783 bp in the DE), and a 63-bp *Bst*NI fragment (-1.16 to -1.10 kb in the DE).

Previous DNA-binding studies identified four *dl*-binding sites in the *zen* promoter (Ip et al. 1991). These sites share a common consensus sequence: GGG(A/T)_nCCA. There are either 4 or 5 central nucleotides, usually A's and T's, that separate the GGG and CCA half-sites. To determine the core recognition sequences in the *twi* promoter we scanned the DNA fragments identified in the gel-shift assays for matches to the *zen* consensus. The distal-most fragment located at about -1.1 kb includes the sequence GGGCAAACCC (TD2; see Fig. 6, below),

Figure 5. The *dl* protein binds to specific sites in the *twi* promoter. (A) Gel-shift assay comparing the relative affinities of the *zen* oligo B sequence (Ip et al. 1991) with the TD2 and TD3 *dl*-binding sites in the DE. The *zen* oligo B sequence (lane 1), as well as oligonucleotides that encompass the TD2 (lane 2) and TD3 (lane 3) sequences, were 32 P-labeled, incubated with a truncated *dl* protein made in bacteria, and run on a native polyacrylamide gel. The three oligonucleotides were labeled to the same extent, so that the relative intensities of the protein–DNA complexes (arrow) represent an approximate measure of binding affinity. The *zen* oligo B sequence has at least 5–10 times higher affinity than the TD2 site and is >20 times stronger than the TD3 site. (B) DNase I footprint assays with a wild-type (lanes 1–3) and mutagenized (lanes 4–6) DNA fragment spanning the PE region of the promoter (for details, see Materials and methods). The locations of these binding sites within the *twi* promoter are summarized in Fig. 6. The fragment was labeled with 32 P, incubated with increasing amounts of *dl* protein, partially digested with DNase I, and electrophoresed on a polyacrylamide–urea gel. Lane 1 is a control showing the DNase I digestion pattern without added protein; lanes 2 and 3 contain increasing amounts of the *dl* protein (fivefold increases). Increasing amounts of *dl* protein result in an extended region of protection, spanning ~30 bp. The diagram at right shows the nucleotide sequence of the binding sites (located ~300 bp upstream from the transcription start site). Increasing amounts of *dl* protein fail to fill the mutagenized TD4/TD5 sites (arrow, lanes 4–6). Lanes 7–9 show the DNase I digestion pattern of a 400-bp fragment from the *zen* promoter, located between approximately –1300 bp to –900 bp upstream from the transcription start site. This fragment contains three *dl*-binding sites (at –1270, –1200, and –1150 bp). Site 2 at –1200 bp corresponds to the oligo B sequence. The same increasing amounts of *dl* protein were used as in lanes 1–6. Note that bands of comparable intensity in the *twi* TD4/TD5 region and the *zen* oligo B site (arrows) are not protected to the same extent with equivalent amounts of *dl* protein. The level of protection seen in lane 3 for *twi* is comparable to that observed in lane 8 for *zen*, indicating that fivefold more protein is needed to give equivalent binding to the *twi* site.

which is similar to the consensus-binding site present in the *zen* promoter. The second *dl* site in the DE is located at –820 bp and contains the sequence GGGGAATC-CA (TD3; see Fig. 6). Oligonucleotides were synthesized for each site and used in gel-shift assays (Fig. 5A). Both oligonucleotides bind the *dl* protein, and the binding can be specifically competed with an oligonucleotide that contains a high-affinity *dl* site from the *zen* promoter (oligo B; data not shown). Comparison of the TD2 and TD3 oligonucleotides with the *zen* oligo B sequence indicates that the *zen* site has at least a 5- to 10-fold higher affinity for the *dl* protein than either TD2 or TD3 (Fig. 5A).

The nucleotide sequence of the 147-bp *Hinf*I fragment within the PE region of the *twi* promoter that tested positive in the gel-shift assays includes six copies of the *zen* half-site sequence, but no obvious match with the full consensus sequence. DNase I footprint assays were done to identify the sequence recognized by the *dl* protein. A 465-bp *Dra*I–*Xho*I DNA fragment that extends from –649 bp to –184 bp and encompasses the poten-

tial binding sites was 32 P-labeled at the *Xho*I site, incubated with increasing amounts of the truncated *dl* protein, and electrophoresed in a polyacrylamide/urea gel after digestion with DNase I (Fig. 5B). The highest concentration of *dl* protein results in an extended region of protection, spanning ~30 bp. The nucleotide sequence of the protected region is shown to the right of the autoradiogram. It includes two tandem sequences (TD4 and TD5) that are related, but not identical, to the *dl* consensus discussed above. Smaller DNA fragments containing either one of these sequences failed to form detectable complexes with the *dl* protein in gel-shift assays (data not shown). This observation suggests that either sequence alone binds with only low affinity, but the juxtapositioning of the sites might allow cooperative binding of the *dl* protein. Comparison of the TD4 and TD5 sites with those present in the *zen* promoter indicates that the *twi* sites possess at least four- to fivefold lower affinities for the *dl* protein than the *zen* oligo B site (Fig. 5B, cf. lanes 2 and 3 with 8 and 9).

dl-binding sites mediate activation in vivo

To determine whether *dl* regulates *twi* expression di-

rectly, we mutagenized the *dl*-binding sites present in the *twi* promoter. Site-specific changes were made in each of the four sites by using mutagenic oligonucleotides, as summarized in Figure 6. In each case, the GGG motif that is conserved in all *dl*-binding sites was substituted with T's or A's. Gel-shift and DNase I footprint assays indicate that these mutations either reduce or abolish *dl* binding (i.e., see Fig. 5B, lanes 4–6).

The importance of the *dl* sites in the DE was examined by expressing a *twi-lacZ* fusion gene that contains the mutagenized TD2 and TD3 sites in the context of an otherwise normal 1.2-kb construct (see Figs. 1 and 3A,B). The mutagenized *twi* promoter shows an abnormal pattern of expression (Fig. 7A,B) that is similar to the *twi* pattern seen in *dl/+;twi/+* double heterozygotes (Kosman et al. 1991), and to one of the lines containing 1 kb of 5'-flanking sequence (data not shown; see Figs. 1 and 3D). Most notably, there are gaps in the pattern near the head and tail, narrower limits of expression in ventral regions (from ~20 to ~18 cells), and reduced expression at the anterior and posterior poles (Fig. 7A). These results suggest that the *dl*-binding sites present in the DE are essential for normal *twi* expression.

The role of the *dl*-binding sites in the proximal element was investigated by examining the expression of a truncated *twi-lacZ* fusion gene containing the mutagenized TD4 and TD5 sites (see Fig. 6 summary). The first 440 bp of *twi* 5'-flanking sequence was used, which

normally directs expression in the ventral-most 14 cells containing peak levels of the *dl* morphogen (Fig. 7C). Disruptions in the TD4 and TD5 sites result in a marked reduction in expression (Fig. 7D), although there are variations in the levels that are obtained, ranging from the complete loss of expression to about a fivefold reduction as compared with the wild-type promoter. This observation indicates that the TD4 and TD5 sites directly mediate activation of the *twi* promoter in vivo.

Discussion

We have presented evidence that the number and affinities of *dl*-binding sites determine the dorsal-ventral limits of target gene expression in the early embryo. The *twi* promoter contains two *cis* regulatory elements (summarized in Fig. 8): The PE mediates expression in the ventral-most regions of the embryo where there are peak levels of the *dl* morphogen, whereas the DE is responsible for expression in lateral regions containing lower levels of the morphogen. The PE and DE each contain two *dl*-binding sites, and mutations in these sites reduce or abolish expression in the embryo. Multimerization of the intact DE results in expanded limits of expression, which extend into the presumptive neuroectoderm where there are only very low levels of *dl* protein. We have also shown that the highest-affinity *dl*-binding sites present in the *twi* promoter have substantially lower affinities for the *dl* protein as compared with the highest-affinity sites in the *zen* promoter. This observation may explain how the *dl* concentration gradient differentially regulates the two genes in early embryos.

The *dl* concentration gradient differentially regulates *twi* and *zen*

The role of *dl* in tissue differentiation provides an excellent paradigm for understanding the so-called French flag model of morphogenesis, whereby a concentration gradient of a morphogen controls cell fate by regulating gene expression in a threshold-dependent manner (Wolpert 1969). Previous studies have shown that the expression of *twi* and *zen* depends on different threshold levels of the *dl* morphogen (Roth et al. 1989). Low levels of *dl* that are not sufficient to trigger *twi* expression are adequate for the complete repression of *zen*. These different thresholds might reflect the quality of the *dl*-binding sites present in the two promoters. The region of the *zen* promoter that is responsible for mediating repression contains four closely linked high-affinity *dl*-binding sites, which might be filled by even low levels of the protein (Ip et al. 1991). In contrast, the binding sites present in the *twi* promoter possess relatively low affinities and might be filled only by peak levels of the *dl* protein present in the ventral-most regions of early embryos. Direct comparison of the *zen*- and *twi*-binding sites in gel-shift and footprint assays suggests that the strongest *zen* sites possess at least a higher affinity than the *twi* sites (see Fig. 5).

Figure 6. Summary of the sequences and locations of *dl*-binding sites in the *twi* promoter. The horizontal line represents a restriction map of the *twi* promoter. The arrow at +1 indicates the transcription start site. The DE region is shown as a solid bar and extends from -1.2 kb to -800 bp. It contains the TD2 and TD3 *dl*-binding sites. The PE region extends from -440 to -180 bp and contains the TD4- and TD5-binding sites. The nucleotide sequences of the four *dl*-binding sites are shown below the map. The horizontal arrows above the sequences indicate the orientation of the binding sites relative to the transcription start site. mTD2, mTD3, mTD4, and mTD5 show the mutagenized sites, which involved substituting highly conserved G residues with Ts and As. These substitutions reduce or abolish binding to the *dl* protein and were used for the P-transformation experiments shown in Fig. 7.

Figure 7. The *dl*-binding sites are important for *twi* expression. P-transformed embryos were hybridized to show the RNA patterns of the *lacZ* reporter gene. (A,B) P-transformed embryos expressing the 1.2-kb *twi-lacZ* fusion gene containing point mutations in the two *dl*-binding sites (TD2 and TD3) in the DE. (C,D) P transformants expressing the normal (C) and mutagenized (D) 440-bp *twi-lacZ* fusion. (A) A lateral view of a cycle 14 embryo showing the expression of the mutagenized 1.2-kb *twi* promoter. Mutations in the TD2 and TD3 sites cause reduced expression at the poles and slight gaps in the pattern near the head and tail (arrowheads). This pattern is similar to the expression of *twi* RNA pattern in *dl/+;twi/+* double heterozygotes (Kosman et al. 1991). (B) A ventral-lateral view of an embryo similar to the one shown in A. (C) Lateral view of a mid-cycle 14 embryo showing the expression of the normal 440-bp *twi-lacZ* fusion gene. Staining is homogenous along the ventral surface, but expression is lost from the poles (as compared with the normal *twi* pattern). The arrow indicates weak staining in a dorsal patch due to the P-transformation vector. (D) Lateral view of a late cycle 14 embryo showing the expression of the 440-bp *twi-lacZ* fusion gene containing point mutations in the TD4 and TD5 *dl*-binding sites. Ventral expression is essentially abolished. Expression in the dorsal head patch (arrow) indicates that the embryo was stained long enough to reveal any expression specified by the mutagenized *twi* promoter.

Comparison of the expression limits obtained with the $1 \times$ DE versus $2 \times$ DE *twi-lacZ* fusions (see Fig. 4) is consistent with the notion that the number and quality of *dl*-binding sites determine the threshold response. One copy of the DE drives normal ventral-lateral limits of expression (~ 20 cells). In contrast, two copies of the DE (containing four rather than two *dl*-binding sites) drive a broader pattern of expression, including a total of 26 cells. It would appear that the additional *dl* response elements permits expression in lateral regions containing low levels of the *dl* protein, which are normally insufficient to activate the *twi* promoter. Similar results were obtained with *bcd* response elements in the *hunchback* (*hb*) promoter. As these were multimerized, expression was obtained in progressively more posterior regions containing diminishing levels of *bcd* (Driever et al. 1989; Struhl et al. 1989). One interpretation of these results is that multimerization of *bcd* and *dl* response elements facilitates cooperative binding to DNA, so that low levels of the morphogen can interact productively with the target promoters.

The expanded limits seen for the $2 \times$ DE promoter extend beyond the mesoderm and mesectoderm into ventral regions of the presumptive neuroectoderm, suggest-

ing that there may be a direct link between the *dl* morphogen and the activation of regulatory genes responsible for the differentiation of the neuroectoderm. In principle, a target promoter containing numerous high-affinity *dl*-binding sites could be activated directly by the *dl* morphogen in the presumptive neuroectoderm.

During the final stages of preparing this discussion Thisse et al. (1991) published a study on *dl* response elements in the *twi* promoter that were identified on the basis of transient cotransfection assays. These investigators identified two clusters of *dl*-binding sites, and there is a good correlation between the locations of these clusters and the limits of the DE and PE sequences identified in our study. However, the earlier study identified a total of eight *dl*-binding sites in the *twi* promoter, three in the PE, and four in the DE (the eighth maps just proximal to the PE). Two of the three sites in the PE correspond to the TD4 and TD5 sites identified here, but none of the four sites identified previously corresponds to our TD2 and TD3 sites in the DE. The TD2 and TD3 sites are located on opposite ends of the DE (see Fig. 8) and bracket the cluster described by Thisse et al. (1991). We believe that the four sites identified here represent the highest-affinity sites present in the *twi* promoter and

Figure 8. Summary of *twi* promoter elements. To generate a normal pattern of expression, 1.2 kb of *twi* 5'-flanking sequence appears to be sufficient. We have presented evidence that this region contains two ventral activation elements, the DE located between -1.2 kb and -800 bp and the PE located between -440 and -180 bp. The DE and PE each contain two *dl*-binding sites. The PE is responsible for activating the *twi* promoter in the ventral-most cells of early embryos in response to peak levels of the *dl* morphogen. The DE is responsible for three aspects of the normal *twi* pattern. First, it is required for expression in lateral regions containing low levels of the *dl* morphogen. Second, the DE mediates expression at the anterior and posterior poles. Finally, it causes a slight narrowing of the normal pattern in the region of the presumptive cephalic furrow. It is possible that all three aspects involve cooperative interactions between the *dl* and *twi* proteins. We propose that the DE contains *twi* response elements and one or more binding sites for a repressor that is expressed near the cephalic furrow.

have demonstrated their importance in regulating *twi* expression in the embryo. It is conceivable that one or more of the low-affinity sites (some of which we fail to detect in our binding assays) identified previously act in concert with the high-affinity sites to drive optimal expression.

Cooperative interactions between *dl* and *twi*

The DE is responsible for expression in lateral regions where there are diminishing levels of the *dl* protein. Gene-dosage studies suggest that this might involve cooperative interactions between the *dl* and *twi* proteins. The *twi* expression pattern is highly abnormal in *dl*^{-/+}, *twi*^{-/+} double heterozygotes, including a catastrophic reduction in lateral and polar regions, and gaps near the head and tail (Kosman et al. 1991). It is conceivable that the *dl* and *twi* proteins both interact with the DE, as a 1.2-kb *twi-lacZ* fusion gene containing point mutations in the TD2 and TD3 *dl*-binding sites shows an abnormal pattern that is similar to the *twi* RNA pattern observed in *dl*^{-/+}, *twi*^{-/+} double heterozygotes. The simplest interpretation of these observations is that the DE contains both *dl* and *twi* response elements which, together, drive optimal expression in lateral regions. We have shown that this region contains two *dl*-binding sites, but it is not known whether there are also *twi*-binding sites present. The *twi* protein contains a helix-loop-helix motif (Murre et al. 1989a) that is preceded by a basic region. On the basis of DNA-binding studies with other such proteins it is likely that *twi* is also a sequence-specific DNA-binding protein (Lassar et al. 1989; Murre et al. 1989a,b).

Activation vs. repression

It is not clear how *dl* acts as both an activator of *twi* and a repressor of *zen* in the same cells of early embryos. One possibility is that the binding sites present in the *zen* promoter mediate repression, whereas the binding sites in *twi* mediate activation. Although the *dl* sites present in the two promoters are clearly related there may be important differences. All eight binding sites in the two promoters contain the GGG half-site (i.e., see Fig. 6), but there is considerable variation in the sequence and spacing of the second half-site. By analogy to NF- κ B, perhaps *dl* forms a heteromeric complex with one or more as yet unknown *rel*-related subunits (Ghosh et al. 1990; Kieran et al. 1990). The *dl* subunit might interact with the conserved GGG half-site, whereas a different subunit contacts the distinct half-sites present in the *zen* versus *twi* *dl* recognition sequences. Thus, one form of the complex may recognize the *zen* sites and repress transcription, whereas another form binds the distinct sites in the *twi* promoter and activates expression. A prediction of this model is that there are additional *rel*-related proteins active in the early embryo. An alternative possibility is that unknown "cofactors" interact with *dl* to mediate either transcriptional activation or repression. According to this model, the *dl* sites present in the *zen* promoter may map near binding sites for unknown factors that act in concert with *dl* to mediate repression. In contrast, the *twi* promoter might contain sites for a different unknown factor that interacts with *dl* to activate transcription.

Materials and methods

Construction of *twi-lacZ* fusion genes

The *twi* promoter was isolated by screening a genomic DNA library (kindly provided by D. Goldberg) with a 340-bp fragment from the 5' end of a full-length *twi* cDNA. The cDNA was obtained from the 0- to 4-hr library prepared by Brown and Kafatos (1988), which was screened with a 500-bp DNA fragment within the *twi*-coding region (kindly provided by Dr. Perrin-Schmidt). The nucleotide sequence of a 3-kb region of the *twi* promoter, extending from -2.9 kb upstream of the transcription start site to the initiating AUG, was determined by using double-stranded DNA and the Sequenase kit purchased from U.S. Biochemical (Cleveland, OH).

The pCaSpeR-AUG P-transformation vector (Thummel et al. 1988) was used for all *twi-lacZ* fusions. This vector uses the *white* gene as a marker and contains the bacterial *lacZ*-coding sequence with an in-frame AUG codon. A polylinker containing a unique *EcoRI* and *BamHI* site was placed just upstream of the AUG. To subclone *twi* promoter sequences into the vector we used site-directed mutagenesis to create a *BamHI* site at the initiating AUG of the *twi* gene. The desired *twi* fragments were obtained by digesting with *BamHI* (3' limit) and a series of restriction sites in the upstream region. The fragments were cloned into the *BamHI* and *EcoRI* sites within the polylinker of the vector. The cloning was facilitated by abolishing the *BamHI* located at -10 bp of the wild-type gene by creating a 1-nucleotide substitution (GGATCC to AGATTC).

The 1 $\times$ DE and 2 $\times$ DE constructs were made by isolating a 400-bp *HindIII-EcoNI* fragment, located between -1.2 kb and

–800 bp. The fragment was blunted with Klenow and ligated into the unique *EcoRV* site of the pBluescript SK+ vector (Promega, Madison, WI) at a high insert/vector molar ratio. Recombinants containing one or two copies of the 400-bp insert in the correct orientation were isolated and excised from the vector by digestion with *SmaI* and *XhoI*. These fragments, containing either one or two copies of the original 400-bp fragment, were mixed with a 340-bp *XhoI*–*BamHI* fragment containing 180 bp of 5′-flanking sequence and the untranslated leader. These fragments were ligated together into the CaSpeR–AUG vector cut with *BamHI* and *EcoRI*.

The 1.2-kb *twi*–*lacZ* fusion containing point mutations in the TD2 and TD3 *dl*-binding sites was prepared with a 1-kb *HindIII*–*XhoI* *twi* DNA fragment bearing the two mutagenized sites (see below). This fragment was ligated with the 340-bp *XhoI*–*BamHI* fragment containing the *twi* minimal promoter and leader sequence, and inserted into the P-transformation vector cut with *BamHI* and *EcoRI*. This construct is identical to the wild-type 1.2-kb-*twi* fusion, except for the point mutations in the TD2 and TD3 *dl*-binding sites. A similar approach was used to obtain the 440-bp *twi*–*lacZ* fusion gene that contains mutations in the TD4 and TD5 sites.

In vitro mutagenesis

Base-pair substitutions were made with mutagenic oligonucleotides using the Muta-gene kit (Bio-Rad, Richmond, CA) as described previously (Jiang et al. 1991). The two *dl*-binding sites present in the DE were mutagenized by preparing a single-stranded DNA template containing a *HindIII*–*XhoI* *twi* fragment (from –1.2 kb to –180 bp) within the pGem-7Zf(+) vector (Promega). Mutagenic oligonucleotides that change three crucial G residues in the binding sites to either A's or T's were synthesized (see Fig. 5). The substitutions create *DraI* restriction sites, which facilitated the screening for mutagenized templates. The same template was used to prepare mutations in the TD4- and TD5-binding sites present in the PE.

P-transformation and whole-mount *in situ* hybridization

P-transposons containing *twi*–*lacZ* fusion genes were introduced into the *Drosophila* germ line by injection (Rubin and Spradling 1982). *white*[−] embryos homozygous for the *w*⁶⁷ allele were used for all injections. P-transposons were coinjected with the Δ2,3 helper (kindly provided by Dr. F. Laski). Multiple independent transformed lines were generated for all of the *twi*–*lacZ* fusions, and in each case, the expression patterns were determined for at least three independent lines.

Whole-mount *in situ* hybridization was used to detect the expression pattern of the *lacZ* reporter (Tautz and Pfeifle 1989). The procedure that was used is similar to the one reported by Kosman et al. (1991), except that antisense RNA probe was used in place of DNA probes. RNA probes were labeled with a dig-U uracil analog, exactly as described by the manufacturer (Boehringer Mannheim, Germany). Hybridizations were done at 55°C for 36 hr in a buffer containing 50% deionized formamide, 5× SSC, 100 mg/ml of sonicated salmon sperm DNA, 50 mg/ml of heparin, and 0.1% Tween 80. Washes, treatment with anti-dig-U antibody, and histochemical staining were done as described previously (Tautz and Pfeifle 1989; Kosman et al. 1991).

To prepare *lacZ* antisense RNA, the pBluescript KsII⁺ vector was used containing the 2.5-kb *lacZ*-coding sequence (kindly provided by J. Treisman and C. Desplan). The plasmid was linearized by digestion with *PstI* and was transcribed in a buffer containing 40 mM Tris (pH 7.5), 6 mM MgCl₂, 10 mM NaCl, 2 mM spermidine-HCl, 1 mM each of ATP, GTP, CTP, and 0.6

mM UTP (pH 7.5), 0.4 mM digoxigenin-11-UTP (Boehringer Mannheim), 5 mM DTT, 5 U/μl of RNasin (Promega), and 4 U/μl of T3 RNA polymerase (Promega). One microgram of linearized DNA template was added to the reaction mixture in a total volume of 10 μl. The transcription reaction was done at 37°C for 2 hr, and RNAs were hydrolyzed by adding 15 μl of DEPC-treated H₂O and 25 μl of 2× carbonate buffer [120 mM Na₂CO₃ and 80 mM NaHCO₃ (pH 10.2)], and heating at 65°C for 40 min. The reaction was terminated by adding 50 μl of stop buffer [0.2 M NaOAc and 1% acetic acid (pH 6.0)], 10 μl 4 M LiCl, and 5 μl of *Escherichia coli* tRNA (20 μg/μl). The RNA probe was precipitated with ethanol, dissolved in 150 μl of hybridization buffer, and stored at −20°C. The probe was heated at 80°C for 3 min before use.

Preparation of *dl* protein and DNA-binding assays

A truncated *dl* protein (containing the amino-terminal 378 amino acid residues) was overexpressed in *E. coli* with a T7 vector, as described previously (Ip et al. 1991). Crude extracts from induced bacteria were used for both gel-shift and DNase I footprint assays. Gel-shift assays were done as described by Ip et al. (1991).

TD2 and TD3 probes were prepared by annealing complementary 19-base oligonucleotides, which were synthesized according to the sequence of the *twi* promoter. The sequence of TD2 is GGGACAGGGCAAACCCCTG, and the sequence of TD3 is AACCGAGGGGAAGTCCACG. The probes were end-labeled with γ-³²P-labeled ATP and polynucleotide kinase, filled with Klenow, and purified from native polyacrylamide gels. The specific competitor oligo B (TGATTGGGTTTCTCCAGT) contains one of the strong *dl*-binding sites present in the *zen* repression element (Ip et al. 1991), and the nonspecific competitor Zen3A (TCGGGAAACCAGATACTG) is a mutated version of a different strong *dl* site in the *zen* promoter (which lacks a central nucleotide separating the two core half-sites). The mutagenized TD2 and TD3 sites were tested in gel-shift assays with a 63-bp *BstNI* fragment and a 93-bp *EcoRI*–*TaqI* fragment, respectively.

DNase I footprint assays were done essentially as described previously (Heberlein et al. 1985). Binding reactions were done with 5 ng of ³²P-labeled DNA probe in 50 μl of a binding buffer containing 10 mM HEPES (pH 7.9), 50 mM NaCl, 5 μg of BSA, 0.5 μg of poly[d(I-C)], 6 mM β-mercaptoethanol, 5 mM EDTA, and 10% glycerol. After incubating at room temperature for 15 min, 50 μl of 10 mM MgCl₂ plus 5 mM CaCl₂ was added to the reaction mix, followed by freshly diluted DNase I (purchased from Worthington). The DNase I digestion was done at room temperature for 45 sec and quenched by adding 90 μl of stop buffer (1% SDS plus 20 mM EDTA). The samples were extracted with phenol/chloroform (1 : 1), ethanol-precipitated, and electrophoresed in 8% polyacrylamide/7.5 M urea gels.

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Note added in proof

Similar results are reported by Pan et al. (this issue); they provide a detailed characterization of the PE region of the *twi* promoter.

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