The *Drosophila dorsal* Morphogen Represses the *tolloid* Gene by Interacting with a Silencer Element

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The *dorsal* protein (DL) regulates the transcriptional activity of several genes that determine cell fate along the dorsoventral axis of the *Drosophila melanogaster* embryo. DL is present at high levels in ventral nuclei, where it activates some genes (*twi* and *sna*) and represses others (*zen*, *dpp*, and *tld*). DL shows homology to the Rel family of proteins and interacts with specific DNA sequences in the regulatory regions of its target genes. The distal portion of the *zen* gene acts as a silencer that can mediate the repression of a heterologous promoter in ventral regions of the embryo. It contains four DL binding sites which alone are sufficient for activation but not repression. Here we analyze the interaction of DL with another one of its repressed targets, the *tolloid* (*tld*) gene. Approximately 800 bp of 5'-flanking sequences upstream of the *tld* coding region were shown to drive an expression pattern indistinguishable from the wild-type pattern. A 423-bp fragment located within these sequences contains two DL binding sites and was shown to act as a silencer to mediate ventral repression. Point mutations in the sites abolish not only DNA binding but also ventral repression. We discuss a comparison of the DNA sequences from the *zen* and *tld* promoters and the possible mechanisms of transcriptional silencing.

Cell fate along the dorsoventral (DV) axis in early *Drosophila melanogaster* development is determined by the activity of several zygotic genes whose localized expression is regulated by the *dorsal* (*dl*) transcription factor (18, 41). Initially, the *dorsal* protein (DL) is uniformly distributed in the cytoplasm of the egg cell. After fertilization, during cleavage cycle 10 and until cycle 14, DL is transported from the cytoplasm into the nuclei in a regulated manner such that ventral nuclei receive high levels of DL while lateral and dorsal nuclei receive progressively decreasing amounts of DL (42, 46, 56). This regulation involves a signal transduction pathway involving maternal factors that trigger the asymmetric movement of DL into nuclei (11, 43). As a result, a nuclear concentration gradient of DL forms, with the highest concentration in ventral nuclei. DL can thus be described as a maternal morphogen, because it acts in a concentration-dependent manner to set up distinct patterns of zygotic gene expression along the DV axis.

Genetic and biochemical studies revealed that the expression of a number of genes is controlled directly by DL (18). Genes such as *twist* (*twi*) and *snail* (*sna*) are activated by high concentrations of DL in ventral nuclei, where they direct the formation of the mesoderm (20, 23, 39, 59). In lateral regions of the embryo, intermediate amounts of DL can turn on genes, such as *rhomboid* (*rho*), which are involved in the formation of the neuroectoderm (19, 24). DL is also believed to repress the transcription of a set of genes responsible for the differentiation of dorsal tissues, such as the amnioserosa and dorsal ectoderm (16, 17). Three of the zygotic genes in this class have been cloned and characterized: *zerknüllt* (*zen*) (44), *decapentaplegic* (*dpp*) (57), and *tolloid* (*tld*) (50). Transcription of these genes is initiated during cleavage cycle 10 at about the same time as when DL enters nuclei in ventral regions of the embryo. Their tran-

scripts appear only in the dorsal half of the embryo and are never seen in ventral regions. It is thought that DL restricts their expression by preventing expression in ventral nuclei (45, 57).

DL is a sequence-specific DNA binding protein that shows homology to the Rel family of transcription factors (47, 55). It activates or represses target genes by interacting directly with specific binding sites in their regulatory regions (18). The consensus sequence of the DL binding site, GGG (A/T)₄₋₅C₁₋₃, was deduced from several studies and is very similar to the consensus sequence of the most studied member of the family, NF- κ B (27, 33, 47). A number of DL binding sites have been found in promoter regions of the activated genes *twi*, *sna*, and *rho* (19, 20, 23, 39, 59). In most cases, they are clustered in regions which are saturated with binding sites for other transcription factors, implying that a complex set of short-range interactions mediates the spatially restricted expression patterns.

In addition to transcriptional activation, DL also mediates repression. DL interacts with a distal promoter element of the *zen* gene that contains four DL binding sites (17). It has been shown that this ventral repression element (VRE) can act as a negative enhancer to overcome the activity of heterologous promoters (7, 17). It acts independently of its orientation, its distance, and the sequence context of the heterologous promoter and is thus reminiscent of the transcriptional silencers first identified in yeast cells (4). The repression element from the *dpp* gene exhibits characteristics similar to that of the repression element from the *zen* promoter (16).

Not all of the DL binding sites found so far match the consensus sequence, and the sequence variation among individual sites determines their different affinities for DL. It has been shown that the affinity of DL binding sites sets the threshold response of the target genes to different amounts of DL (24). The DL binding sites in the *zen* promoter are 5 to 10 times stronger than those in the *twi* promoter, and these affinities correlate with the differential responses of the *zen*

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and *twi* promoters to the DL gradient (23, 59). In lateral regions of the embryo, *zen* is repressed by low levels of DL, but *twi* cannot be activated (42).

The factors that determine whether DL acts as an activator or as a repressor of transcription have yet to be identified. It was suggested that different types of DL binding sites may influence DL behavior (17). However, several lines of evidence rule out this possibility. First, a comparison of DL binding sites from activated versus repressed genes did not reveal two classes of DL binding sites. Second, DL binding sites from the *zen* and *twi* promoters were shown to be interchangeable (25). Third, DL binding sites from the *zen* promoter, when inserted upstream of a minimal heat shock promoter, mediate its activation, implying that DL is by default an activator and works as a repressor only in specific promoter contexts (25, 38). To determine what aspects of these promoters are important for transcriptional repression, we have begun a study to compare the silencer regions from the *zen* and *tld* genes.

The *tld* gene product is required for the normal development of dorsal tissues in the *Drosophila* embryo (50). Like those of *zen* and *dpp*, the initial pattern of expression of *tld* is restricted to the dorsal regions of the embryo (50). Here we analyze the DNA sequences required for the spatial regulation of the *tld* gene. We present evidence that DL directly represses *tld* gene expression in ventral regions of the early embryo by binding to 5'-flanking sequences required in vivo for ventral repression. Our experiments also show that these sequences can behave as a transcriptional silencer to confer repression on a heterologous promoter.

MATERIALS AND METHODS

Expression and purification of DL. The truncated form of DL containing the N-terminal 378 amino acids, which include the entire Rel domain, was fused to the glutathione S-transferase (GST) gene in the pGEX expression vector (Pharmacia, Piscataway, N.J.). The *NdeI-EcoRI* fusion fragment for this subcloning was derived from the pAR *dl*-378 T7 expression plasmid (46) and was blunt ended before insertion into the filled-in *Bam*HI site of the pGEX vector. The transformed cells were grown to an optical density at 600 nm of 0.5 and induced with 0.8 mM isopropyl- β -D-thiogalactopyranoside (IPTG). After 2 h, the cells were harvested and resuspended in a 1/100 volume of buffer Z (100 mM NaCl, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES] [pH 7.9], 12.5 mM MgCl₂, 2 mM dithiothreitol, 0.1% Nonidet P-40, 20% glycerol, 2.5 μ g of leupeptin per ml, 2.5 μ g of pepstatin per ml, 2.5 μ g of aprotinin per ml, 1 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride). Lysozyme was added to 0.2 mg/ml, and the suspension was incubated for 10 min on ice with mixing. The cells were sonicated four times for 15 s each time, and the lysate was spun at 12,000 $\times g$ for 20 min at 4°C. The fusion protein was affinity purified from the clear supernatant by the addition of a 1/10 volume of glutathione-agarose beads (Pharmacia) and incubation for 5 min at room temperature with mixing. The beads were pelleted by centrifugation and washed three times with 20 volumes of PBS buffer (0.14 M NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄ [pH 7.2]) plus 1% Triton X-100. The fusion protein was eluted from the resin by three consecutive washes for 2 min each with one bed volume of freshly prepared 10 mM glutathione-50 mM Tris (pH 8). The eluate was stored at -70°C.

EMSA. For electrophoretic mobility shift assays (EMSA), DNA restriction fragments or oligonucleotides were end

labeled with [γ -³²P]ATP (ICN; 7,000 Ci/mmol) to a specific activity of 10⁷ cpm/ μ g. DL was incubated for 10 min at 20°C with 5 $\times$ 10³ cpm of labeled probe in 20 μ l of 10 mM HEPES (pH 7.9)-50 mM NaCl-1 mg of bovine serum albumin per ml-3 mM MgCl₂-10 mM EDTA-6 mM β -mercaptoethanol-10% glycerol-8 μ g of poly(dI-dC) $\cdot$ poly(dI-dC) per ml. The reaction mixture was then loaded onto a 4% polyacrylamide gel in 25 mM Tris base-190 mM glycine. Electrophoresis was run for 50 min at 4°C at 15 V/cm, and the gel was dried and autoradiographed.

The sequences of the upper strands of the oligonucleotides used as probes were as follows (the numbers represent the distance upstream [-] or downstream [+] from the transcription start site of the *zen* or *tld* gene: Z1 (*zen* -1555), 5' TATCTGGTTTTCCTCGAAAATCTTATGA; *tld* -482, 5' TGAAGGTGGAATTACCGCGAAACGATTTCGTTCAT; *tld* mut -482, 5' TGAAGGTGGAATTCTAGCGAAACGATTCGTTTCAT; *tld* 305, 5' CAAGGTGTGAGCAGGAATACCCAAATCAAATCCATTG; *tld* mut -300, 5' TGTGAGCATGAATTCAAAAATCAAATCC; *tld* +42, 5' CGAGCCCCAGAAATACCCACGGTACC; and *tld* mut +41, 5' ACAGCCCCCAGAATTCAACACGGTAC.

DNase I footprinting. DNase I footprint analyses were carried out as previously described (54). The binding reaction mixtures contained 5 $\times$ 10⁴ cpm of end-labeled probe (specific activity, 10⁷ cpm/ μ g) in 50 μ l of binding buffer [10 mM HEPES (pH 7.9), 50 mM NaCl, 5 mM EDTA, 10% glycerol, 8 μ g of poly(dI-dC) $\cdot$ poly(dI-dC) per ml, 6 mM β -mercaptoethanol, 0.1 mg of bovine serum albumin per ml]. After incubation at room temperature for 10 min, the probe was placed on ice for another 10 min. Fifty microliters of 10 mM MgCl₂-5 mM CaCl₂ was added to the reaction mixture, and then 10 ng of freshly diluted DNase I was added. The reaction was allowed to proceed for 5 min on ice and then was stopped by the addition of 100 μ l of stop buffer (1% sodium dodecyl sulfate [SDS], 20 mM EDTA, 20 μ g of tRNA per ml). The samples were extracted once with phenol-chloroform (1:1) and once with chloroform, ethanol precipitated, and electrophoresed on 8% polyacrylamide-7 M urea gels. The G+A chemical sequencing ladder was produced as previously described (35).

Methylation interference. Methylation interference assays were performed as previously described (52). Partial methylation of ³²P-labeled probes was achieved with dimethyl sulfate (DMS) as described by Maxam and Gilbert (35). About 10 ng of DNA (specific activity, 10⁷ cpm/ μ g) was incubated in 200 μ l of 50 mM sodium cacodylate-10 mM MgCl₂-1 mM EDTA (pH 8) with 1 μ l of DMS for 1 min at 20°C. The reaction was stopped by the addition of 50 μ l of 1 M β -mercaptoethanol-1.5 M sodium acetate (pH 7)-100 μ g of tRNA per ml. After precipitation and a wash with 70% ethanol, the DNA was dried and resuspended for the binding reaction. The binding reaction with the DL-378-GST fusion protein and electrophoresis on a native polyacrylamide gel are described above. Free and bound forms of DNA were visualized by autoradiography of the wet gel for several hours at 4°C and then excised, and DNA was eluted from the crushed gel slices with 0.5 M ammonium acetate-10 mM magnesium acetate-1 mM EDTA-0.1% (wt/vol) SDS-10 μ g of tRNA per ml. After phenol-chloroform extraction and ethanol precipitation, the purified DNA was cleaved with 1 M piperidine for 30 min at 90°C and lyophilized. It was electrophoresed on an 8% polyacrylamide-7 M urea sequencing gel.

In vitro mutagenesis of DL binding sites. The oligonucleotides used to create mutant DL binding sites are listed above.

FIG. 1. Expression of the *tld* promoter-*lacZ* fusion gene. Whole-mount preparations of transgenic embryos were hybridized to digoxigenin-11-UTP-labeled antisense *lacZ* probes. All embryos are oriented so that the anterior is to the left and dorsal is up, except in panel D, which shows a more dorsal view. (A) Precellular embryo in early cleavage cycle 14. A dorsal-on/ventral-off pattern that includes the anterior and posterior poles is observed as early as cleavage cycle 10 and persists until early cleavage cycle 14. (B) Embryo undergoing cellularization in mid-cycle 14. The signal has been lost from the poles and is weaker in bands along the anteroposterior axis. (C) Embryo derived from a *dl*⁻ female about the same age as that shown in panel B. Note that transcripts are present in both dorsal and ventral regions of the embryo. (D) Embryo undergoing gastrulation. Bands of expression persist in dorsolateral regions but not in the most dorsal region. (E) Embryo just beginning germ band elongation. Bands of expression are still visible. (F) Embryo approaching the end of the rapid phase of germ band elongation. A patch of expression in the head remains. (G) Stage 8 embryo. A new pattern of expression that is segmentally repeated emerges. Arrowheads highlight the strongest staining in the gnathal segments. (H and I) Stage 11 embryos. The patches of cells that express the fusion gene (and the endogenous *tld* gene) are located near the boundary of the neurogenic ectoderm and the dorsal ectoderm. The embryo in panel I shows a slightly more ventral view to point out the bilateral rows of segmental expression (arrows in panels H and I). cf, cephalic furrow.

Single-stranded DNA was prepared from a BlueScript (Stratagene, La Jolla, Calif.) plasmid containing 800 bp of *tld* promoter DNA that included the three identified DL binding sites. In vitro mutagenesis was performed with a Mutagene kit (Bio-Rad, Melville, N.Y.).

Preparation of P transposons. The *tld* promoter-*lacZ* fusion gene was prepared by inserting a 796-bp *Bam*HI-*Kpn*I fragment that includes the *tld* sequences from -728 to +68 into a *lacZ* AUG transformation vector (2). The minimal stripe element (MSE) P transposon contains the 480-bp stripe 2 element from the *eve* promoter attached to a basal *eve-lacZ* fusion gene (53). The MSE-*lacZ* fusion gene was inserted into the CaSpeR injection vector, which contains the *white* gene as a selectable marker (60). The chimeric promoters were obtained by insertion of various *tld* promoter fragments into the *Eco*RI site at the 5' end of the MSE (25) (see Fig. 6). The 645-bp fragment is a *Bst*YI fragment that lies between -728 and -81 bp upstream of the transcription start site. The 423-bp fragment is a *Hin*PI-*Bst*YI fragment that lies between -505 and -728 bp upstream of the start site. The fragments were placed upstream of the MSE in an orientation opposite from that of the endogenous *tld* gene. A single copy of the 645-bp fragment (wild type and mutant) and two copies of the 423-bp fragment (wild type and mutant) were assayed in the P transformation repression assay.

P transformation and in situ hybridization. P transposons were introduced into the *Drosophila* germ line by injection of *white*⁻ [Df (1)w67c23] embryos by standard methods (1a). At least three lines were studied for each construct. *dl*⁻ embryos were derived from Df(2L) *dl* H/Df(2L)119 females. The expression patterns directed by the fusion promoters were analyzed in transgenic embryos by whole-mount in situ hybridization with an antisense *lacZ* RNA probe labeled with digoxigenin-11-UTP (Boehringer Mannheim, Indianapolis, Ind.). Photography was done with Nomarski optics on a Nikon FXA microscope. Composite figures were prepared by use of 35-mm color slides of individual embryos scanned into the computer program Adobe Photoshop (Macintosh) by an RFS2035 scanner (Kodak, Rochester, N.Y.) and printed with a Kodak XL7700 printer.

RESULTS

A total of 800 bp of promoter sequences is sufficient to direct a normal *tld* pattern. *tld* transcripts can first be detected in precellular cleavage cycle 10 embryos in a broad dorsal-on/ventral-off pattern of expression (50). This temporal and spatial pattern is very similar to that seen for the *zen* and *dpp* genes. The lack of expression of these genes in ventral regions of the embryo is under the control of the *dl* morpho-

gen (1). It is known that DL interacts with the distal region of the *zen* promoter and the second intron of the *dpp* gene to mediate ventral repression (16, 17). To determine whether DL also directly interacts with the *tld* promoter, it was necessary to delineate the regulatory sequences that define a wild-type expression pattern.

The *tld* transcription unit is 3.5 kb (50). The start of transcription was accurately determined by primer extension analysis (data not shown). Two primer extension products were observed; one product was 4 nucleotides longer than the other and shared the same start site as the cDNA. Genomic DNA fragments that contain the transcription unit plus 5 kb of 5'-flanking (promoter) sequences were shown to rescue *tld* mutants in P-element-mediated transformation studies (50). For the present study, different lengths of these promoter sequences were fused to the *lacZ* reporter gene and tested for their ability to drive a normal *tld* expression pattern. Figure 1 shows a series of transformant embryos that carry one of the fragments, containing approximately 800 bp of 5'-flanking sequences. The embryos were hybridized with digoxigenin-labeled antisense *lacZ* RNA. The *lacZ* expression pattern is essentially indistinguishable from that of the wild type (50). During precellular stages, transcripts are detected on the dorsal side of the embryo and wrap around both the anterior and the posterior poles (Fig. 1A). During cellularization, transcripts are rapidly lost from the poles, and changes in intensity occur along the anterior-posterior axis, such that bands of expression are observed by the time that gastrulation begins (Fig. 1B). In contrast, transformant embryos derived from *dl*⁻ females exhibit staining around the entire DV axis, although modulation along the anterior-posterior axis is not affected (Fig. 1C). During germ band extension, two bands of expression persist but then fade by the completion of the rapid phase of extension (stage 8) (Fig. 1D to F). As is the case for the endogenous *tld* gene, the late pattern was seen to emerge in stage 8 or 9 embryos. Three patches of cells located within the presumptive gnathal segments appeared (Fig. 1G, arrowheads), and then additional patches in more posterior segments appeared. The most intense expression was seen during stage 11 in segmentally repeated patches of epidermal cells located ventral to each tracheal pit on both sides of the embryo (Fig. 1H and I). This pattern disappeared during germ band retraction.

DL binds to at least three sites in the *tld* promoter. Since the 800-bp fragment responded to regulation by *dl* (Fig. 1C), we initiated a systematic search for DL binding sites that might be located within it. We first scanned the region by EMSA with overlapping restriction fragments from the 800-bp region (see map in Fig. 2). The fragments were end labeled with ³²P, incubated with a bacterially produced truncated form of DL which contains the N-terminal 378-amino-acid Rel domain fused to GST, and run on native polyacrylamide gels. Previous experiments with oligonucleotides containing DL binding sites from the *zen* promoter showed that the presence of the GST tag does not impair DL binding and actually facilitates it by improving the solubility of fused DL (29a). The results of the EMSA are shown in Fig. 2. The lanes of the gel are marked with the fragment number from the restriction map. Three of the fragments produce specific band shifts when incubated with DL (Fig. 2, lanes 3, 5, and 9) but not when incubated with the control protein expressed from the pGEX vector alone (Fig. 2, lanes 3', 5', and 9').

To further localize DL binding sites within the restriction fragments, we performed DNase I footprinting assays with end-labeled fragments that cover the potential binding sites.

FIG. 2. DL binds to specific sites in the promoter of the *tld* gene. The upper part of the figure shows a partial restriction map of 800 bp of the *tld* promoter. The transcription start site (+1) was determined from the sequence of a full-length cDNA (50) and primer extension analysis (data not shown). The letters (restriction endonuclease sites) and numbers above the line indicate the DNA fragments used for the EMSA shown below. The letters below the line indicate sites used in the footprint analysis shown in Fig. 3 (see the text). Lanes: 1 to 9: ³²P-labeled DNA fragments incubated with affinity-purified DL-378-GST fusion protein; 1' to 9', the same labeled DNA fragments but incubated with control protein (extracts from IPTG-induced cells bearing the pGEX vector); Z1 and Z1', control ³²P-labeled oligonucleotides containing the most distal DL binding site from the *zen* promoter incubated with DL-378-GST protein and control protein, respectively. The arrow points to the DL DNA-specific complexes. The *tld* fragment shifts are of a lower intensity than those of the control *zen* oligonucleotide (compare lane 1 with lanes 3, 5, and 9). The lower amount of radioactivity in the *tld* band shifts is probably due to the fact that more labeled DNA is present in the lanes containing the Z1 oligonucleotide. Abbreviations: A, *AvrII*; Asp, *Asp718*; B, *BamHI*; D, *DdeI*; Hf, *HinfI*; HP, *HinPI*; T, *TaqI*.

A 378-bp *AvrII*-*TaqI* fragment labeled at the *AvrII* end and a 169-bp *AvrII*-*DdeI* fragment labeled at the *DdeI* end were used for scanning the most distal DL binding site, and the footprints are shown in Fig. 3A. *DdeI*-*HinfI* and *DdeI*-*XbaI* fragments were used in delineating the second and third, most proximal sites, respectively (data not shown). The assay revealed three regions of protection located at -479, -298, and +46 bp relative to the transcription start site. The protected sequences for all three sites are shown in Fig. 3B. They closely match the derived core recognition consensus sequence for DL, but the actual regions protected from DNase I are much longer and span between 21 and 23 bp. The core recognition sequence is centered in the protected regions. As DL most likely binds to its site as a dimer (12, 21), we conclude that DL binds symmetrically to the half-sites of the weakly palindromic core sequence.

Methylation interference experiments gave us additional proof that the identified sites are indeed the points of direct contact with DL. Figure 4 shows the methylation interference pattern for the most distal DL binding site. Methylation of all the outer G residues interfered with the binding. Similar results have been obtained for the other two DL binding sites in the *tld* promoter (data are summarized in Fig. 3B), for the DL binding sites in the *zen* promoter (17), and for the binding sites of other Rel family proteins, such as NF- κ B (34).

FIG. 3. Identification of DL binding sites in the *tld* promoter by DNase I footprinting. (A) DNase I footprint of the most distal DL binding site. A 378-bp *AvrII*-*TaqI* fragment (–536 to –158 bp upstream of the transcription start site) was labeled at the *AvrII* site for the upper strand, and a 169-bp *AvrII*-*DdeI* fragment (–536 to –367 bp) was labeled at the *DdeI* site for the lower strand (see map in Fig. 2). The labeled probes were incubated with buffer only (lanes 2 and 6), with 4 μ l (lanes 3 and 7) or 20 μ l (lane 8) of affinity-purified bacterial extracts from IPTG-induced cells carrying the pGEX expression vector, and with 4 μ l (lanes 4 and 9) or 20 μ l (lane 10) of affinity-purified DL-378-GST fusion protein. The incubation mixtures were digested with DNase I, and the purified DNA was resolved on an 8% polyacrylamide–7 M urea gel. Lanes 1 and 5 represent the chemical cleavage of the DNA fragments at G+A. The core recognition DL binding sequence is marked with asterisks. (B)

Knowing the sequences with which DL interact, it was possible to prepare oligonucleotides bearing point mutations in the core recognition sequence and to show that these totally eliminated DL binding. As can be seen in Fig. 5, the mutations that changed the essential G residues for other nucleotides (see Materials and Methods) abolished DL binding (compare lanes 2, 4, and 6 with lanes 3, 5, and 7). In the experiments shown in Fig. 5, equal molar concentrations of oligonucleotides were used, and the intensities of the band shifts, including the *zen* band shift, were approximately the same. It appears that the relative affinities of DL binding to the *tld* promoter are similar to the affinities of the strong DL binding sites of the *zen* promoter.

A total of 423 bp of the *tld* promoter can act as a VRE. DL is thought to mediate repression by interacting first with silencer sequences and then along with other factors, blocking the activity of the transcription apparatus. These other factors may also interact with silencer sequences and function as corepressors. There is evidence for this type of interaction on the *zen* promoter (22, 30), although the nature of the corepressor and whether it interacts directly with DL is unknown. A comparison of the *zen* VRE with the VREs from the *tld* and *dpp* genes might elucidate the common features of VREs that are important for the mechanism of silencing. Therefore, we tested *tld* promoter fragments for their ability to repress the transcription of a heterologous promoter. The repression assay involves a well-defined promoter element of 480 bp from the *even-skipped* (*eve*) gene that directs the expression of *eve* stripe 2 (25). This element was fused to the *eve* basal promoter and a *lacZ* reporter gene (53) (diagrammed in Fig. 6) and transferred into flies by P-element-mediated transformation. It directs a broad pattern of expression in the anterior half of the embryo that eventually is refined into a stripe by the time that cellularization is completed (53). An example is shown in Fig. 7A. Transgenic embryos were hybridized with digoxigenin-labeled antisense *lacZ* RNA probes. Intense staining was observed for stripe 2 in both dorsal and ventral regions of the embryo. Weak staining of other stripes was sometimes seen in heavily stained embryos.

The *tld* promoter fragments that were tested in the repression assay are schematized in Fig. 6. Initially, a 645-bp fragment that lies between –728 and –81 bp upstream of the transcription start site and that includes two DL binding sites was tested for its ability to confer ventral repression of *eve* stripe 2. Figure 7B shows one such transgenic embryo. Only half a stripe was observed because of repression in ventral regions. To show that this repression is dependent on DL, transgenic males were crossed to *dl*[–] females, and their embryos were hybridized to *lacZ* probes. Figure 7C shows that a full stripe appeared in the absence of DL. A smaller fragment of 423 bp (–505 to –728) that includes the same two DL binding sites was also shown to act as a VRE and was also dependent on DL (Fig. 7D and E). In this case, the fragment was duplicated. To prove that the DL binding sites are directly involved in mediating the observed ventral

tld DNA sequences that footprint with DL. The protected sequences are delineated for the upper and lower strands. The shift of 2 or 3 nucleotides toward the 3' end for each does not reflect any asymmetry in cutting by DNase I, because the attacked phosphodiester bonds of the two strands are nearest neighbors in space but appear to lie a few bonds apart when plotted in two dimensions (8). The methylated residues that interfere with DL binding are highlighted with asterisks (see Fig. 4).

FIG. 4. Identification of the points of contact of DL with its cognate binding sites by a methylation interference assay. A 169-bp *AvrII-DdeI* DNA fragment containing the most distal DL binding site was end labeled on the upper or lower strand. The purified DNA was partially methylated with DMS, incubated with the DL-378-GST fusion protein, and electrophoresed on a preparative 4% native polyacrylamide gel. The free DNA and protein-DNA complexes were eluted separately from the gel and cleaved with piperidine. The DNA was then fractionated on an 8% polyacrylamide-7 M urea gel. M, cleavage at G+A; F, free DNA; B, DNA bound to DL. The nucleotides contained within the footprint shown in Fig. 3A are delineated, and the nucleotides at the sites of contact are highlighted with asterisks. The results of assays performed with fragments that contain each of the DL binding sites are summarized in Fig. 3B.

repression, we introduced mutations in the two DL binding sites (the same point mutations as those described above) of the VRE and tested the 423-bp mutant VRE for the repression of stripe 2. An example of a transgenic embryo carrying mutant DL binding sites is shown in Fig. 7F. Stripe 2-directed expression was no longer repressed in ventral nuclei, a result of the failure of DL to bind to the mutated sites, as has been shown in vitro with mutated oligonucleotides (Fig. 5).

DISCUSSION

Here we have shown that 780 bp of DNA upstream of the transcription start site of the *tld* gene is sufficient to direct a pattern of expression indistinguishable from that of the wild type when fused to a *lacZ* reporter gene. We have also shown that a 423-bp fragment derived from these sequences can act as a VRE to mediate DL-dependent repression of a heterologous promoter. This fragment contains two DL binding sites, which reside 183 bp apart and which bind to DL with a similar affinity. In vivo relevance for these sites was obtained by changing the residues which contact DL and demonstrating that they could no longer mediate ventral repression in the context of the 423-bp VRE. Thus, like *zen* and *dpp*, *tld* appears to be directly repressed by DL via regulatory elements that can act as silencers. We have

FIG. 5. Specificity of DL binding. The end-labeled oligonucleotides (see Materials and Methods for their sequences) were incubated with affinity-purified DL-378-GST fusion protein, and the incubation mixtures were run on a native 4% polyacrylamide gel. Lanes: 1, oligonucleotide containing a DL binding site from the *zen* promoter; 2 and 3, oligonucleotides containing the native and mutated distal DL binding sites, respectively; 4 and 5, oligonucleotides containing the native and mutated second most proximal DL binding sites, respectively; 6 and 7, oligonucleotides containing the native and mutated third most proximal DL binding sites, respectively.

shown that the *tld* VRE can function at a distance of about 500 bp from the transcription start site and in an orientation opposite the native orientation.

A comparison of the VREs from *zen* and *tld* has revealed that the only obvious thing that they have in common are DL binding sites. The sites are not at similar positions with respect to each other or to the transcription start site. The sites themselves are not identical. The sites in the *zen* VRE are GGGAAAACC and GGGTTTCTCCC (17), and the *tld* VRE sites are GGAATTACC and GGAATACCC. The third DL binding site, located in the 5'-untranslated region of the *tld* gene (GGGTATTTC), is weaker and was not used in the P transformation experiments; at present we do not know whether it is functional. The *tld* VRE contains two DL binding sites 183 bp apart; the proximal site is located 283 bp upstream of the transcription start site. The *zen* VRE contains four sites; three of them lie in a region of 140 bp, with the most proximal site being about 1 kb upstream from the transcription start site. The *dpp* VRE lies within the second intron and contains multiple DL binding sites scattered in a region of several hundred base pairs (16). From studies of the *zen* and *dpp* VREs, it is known that they also carry enhancer functions and can drive weak expression of heterologous promoters in the dorsal part of the embryo. Likewise, the *tld* VRE appears to direct weak dorsal *lacZ* staining outside the *eve* stripe 2 expression domain, especially in younger embryos (data not shown), suggesting that it also contains an enhancer(s).

What is it about the VRE that allows DL to become a repressor? Do other factors bind to the VRE and interact with DL? Protein-protein interactions could have different effects on transcriptional activity, such as increasing the affinity of a protein for its binding site or influencing the function of a protein. For example, protein interactions have been shown to play an important role in the activation of the *rho* promoter, in which binding sites for basic helix-loop-helix proteins reside near DL binding sites (19, 24). These proteins bind cooperatively in the presence of one another and activate *rho* expression. The result of this type of protein interaction is an increased sensitivity of the promoter to DL.

FIG. 6. Summary of promoter fusions used in the P transformation assays. The arrow corresponds to the transcription start site in the *eve* basal promoter. The hatched bar represents 3 kb (75%) of the *lacZ* coding sequence. The closed bar represents the 480-bp minimal *eve* stripe 2 element. The open bar corresponds to different forms of the *tld* promoter, and the circles represent the DL binding sites (closed are wild-type and open are mutant binding sites). (A) MSE heterologous promoter. (B) Wild-type 645-bp *tld* promoter fragment fused to MSE. (C) Wild-type 423-bp fragment fused to MSE. (D) Mutant 423-bp fragment fused to MSE.

Thus, low levels of DL are able to activate transcription in lateral regions of the embryo. Protein-protein interactions have not yet been shown to occur on any of the VREs, but there is evidence that such interactions probably allow DL to function as a repressor rather than as an activator. It is known that DL mediates transcriptional repression in a promoter context-dependent manner because, when it is bound to its cognate binding sites in front of a minimal promoter, it brings about activation (25, 38). This result suggests that there are additional DNA sequences in the VRE that mediate the binding of proteins, which then interact with DL to make a repressor. We previously delineated a 55-bp DNA fragment from the *zen* VRE, a minimal VRE, that contains only one DL binding site and the short A+T-rich sequence adjacent to it (30). When triplicated, it can mediate the repression of the *eve* stripe 2 heterologous promoter (30). Point mutations in either the DL binding site or the A+T-rich sequence abolish ventral repression of the *lacZ* reporter gene fused to the VRE in vivo. Moreover, mutations in the A+T-rich sequence cause ectopic expression in ventral regions, indicating that the minimal silencer has been converted to an enhancer. Similar results have been obtained by Jiang et al. with a longer fragment from the *zen* VRE that includes the described minimal VRE plus an additional DL binding site and adjacent sequences (22). We have not yet isolated a protein which can bind to the A+T-rich sequence and can act as a corepressor together with DL, causing ventral repression. A careful examination of the sequences surrounding the DL binding sites in the *tld* VRE revealed one short stretch of DNA that resembles the A+T-rich sequence in the *zen* VRE (30). It lies 9 bp downstream of the site located at -466 in the *tld* promoter. However, there are no putative A+T-rich sequences near either of the other two DL binding sites in the *tld* promoter.

Evidence that protein interactions are involved in transcriptional repression has been obtained by Saha et al. (48), who isolated an *Escherichia coli* sequence which encodes a

very basic polypeptide that, when attached to the DNA binding domain of the GAL4 activator, converts the fusion protein into a repressor. In vitro and in vivo studies with yeast cells have shown that this polypeptide represses transcription driven by a variety of activators, including DL (48). One possible explanation is that the positively charged peptide works by interacting with DL bound nearby and prevents it from stimulating transcription. An alternative mechanism is that it interacts directly with the basic transcriptional machinery.

There is an increasing list of proteins known to act as activators and repressors (10). Examples include the *Drosophila* homeobox protein *Ultrabithorax* (*Ubx*) (31), mammalian protein E2F (65), and yeast proteins RAP1 (51, 58), MCM1 (3, 28), SPT13/GAL11 (9), SIN3 (61, 62, 66), and SIN4 (26). Some of the proteins with a dual function, like RAP1, MCM1, UBX, and EF2, recognize specific sequences in the regulatory elements of their target genes (3, 5, 28, 31, 65). Others, like SIN3, do not bind DNA and are recruited in the vicinity of the regulatory sequences via stable protein interactions with accessory DNA binding proteins (63, 64). We do not know which *Drosophila* protein(s) together with DL provides a silencing function for the *tld*, *zen*, and *dpp* VREs, and its discovery is a challenging task for the future. It is also unknown whether this putative corepressor is a general repressor of transcription, as is the case for SSN6 and TUP1, which interact with the MCM1- α 2 complex (29), or whether it specifically modifies DL activity.

The *tld* VRE appears to use a long-range mechanism of repression because it acts at a distance in *cis* to overcome the function of the *eve* stripe 2 promoter, as shown in Fig. 7. The VREs from the *zen* and *dpp* genes and a regulatory element from the *Ubx* gene (36) behave similarly, classifying them as DNA regulatory elements acting as silencers (4). These *cis* regulatory DNA elements have been studied mostly with yeast cells (32), and there is evidence that silencers also negatively regulate mammalian genes (6, 13,

FIG. 7. *tld* promoter fragments can function as VREs. Whole-mount preparations of P-transformed embryos were hybridized to digoxigenin-11-UTP-labeled antisense *lacZ* probes. All embryos are in mid-nuclear cycle 14 and oriented with anterior to the left and dorsal up. (A) Embryo containing the MSE-*eve-lacZ* fusion gene (schematized in Fig. 6A). The pattern is refined into a stripe with equal levels of expression in dorsal and ventral regions. (B) Embryo containing the *tld* 645-MSE-*eve-lacZ* fusion gene (see Fig. 6B). A half stripe is observed because expression is restricted to dorsal and lateral regions and covers about 45% of the DV circumference. (C) Embryo derived from a *dl*⁻ female carrying the same fusion gene as the embryo in panel B. Repression is not observed because of the lack of a DL repressor. (D) Embryo containing the *tld* 423-MSE-*eve-lacZ* fusion gene (see Fig. 6C). A half stripe is observed because of repression in ventral regions, similar to that seen with the 645-bp fragment. (E) Embryo derived from a *dl*⁻ female carrying the same fusion gene as the embryo in panel D. Repression is not observed, and a full stripe appears. (F) Embryo containing the *tld* 423mut-MSE-*eve-lacZ* fusion gene (see Fig. 6D). Repression is not observed, and a full stripe appears because of the elimination of the DL binding sites.

37, 40, 49). However, the mechanism of silencing remains obscure. It is different from the mechanism of repression, which requires short-range interactions between transcriptional regulators. Competition between activators and repressors for overlapping binding sites on the *eve* stripe 2 promoter has been invoked as a mechanism for its spatial regulation along the anteroposterior axis of the early *Drosophila* embryo (54) as well as for the regulation of the *Drosophila* gap gene *Krüppel* (14). In many other cases, transcription factors which possess DNA binding domains but lack activation domains compete with activators for the same DNA binding sites and thereby block activation (10). Another short-range "quenching" mechanism regulates the expression of *rho*: the repressor (*sna* protein) binds nearby DL and blocks its activation function (19).

Silencing can include direct interactions of repressors bound to the silencer element with some components of the transcriptional machinery, thereby locking the machinery in place and preventing transcription initiation (32). Alternatively, repressors can interact with chromatin components to change the chromatin structure, which can interfere with the function of the transcriptional machinery. The repressed state of the silent mating type loci HML and HMR may be an example of this mechanism (15). Repression is mediated by transcription factors (RAP1 and ABF1) bound to specific silencers which interact with numerous chromatin proteins (32) and are believed to organize them into a specific repressed structure spread over large chromatin domains. Whether the *tld* VRE uses a similar mechanism remains to be found.

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