Conversion of a *dorsal*-dependent silencer into an enhancer: evidence for *dorsal* corepressors

Jin Jiang¹, Haini Cai, Qing Zhou¹
and Michael Levine

Department of Biology, Center for Molecular Genetics, 9500 Gilman Drive, UCSD, La Jolla, CA 92093-0322, USA

¹Present address: Howard Hughes Medical Institute, Department of Biochemistry and Molecular Biology, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA

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In *Drosophila*, the *dorsal* (*dl*) morphogen gradient initiates the differentiation of the embryonic mesoderm and neuroectoderm by activating the expression of regulatory genes (e.g. *twist* and *snail*) in a concentration-dependent manner. *dl* also functions as a repressor that establishes the dorsal epidermis and amnioserosa by restricting regulatory genes such as *dpp* and *zen* to dorsal regions of the embryo. The ability of *dl* to function as both an activator and repressor distinguishes it from the *bicoid* morphogen, which appears to function solely as an activator. In an effort to determine how *dl* functions as a repressor we have performed a detailed characterization of a *zen* silencer element, called the VRE, which mediates ventral repression in response to the *dl* gradient. A minimal 110 bp VRE sequence is identified, which is able to silence the ventral expression of a heterologous promoter. This sequence contains two *dl* binding sites as well as binding sites for additional nuclear factors present in early embryos. Mutations in the latter binding sites convert the minimal VRE into an enhancer, which mediates transcriptional activation in ventral regions in response to *dl*. These results suggest that *dl* is intrinsically an activator, but is converted into a potent silencer when it interacts with neighboring corepressors.

Key words: corepressors/dorsal/*Drosophila*/morphogen/silencing

Introduction

The *dorsal* (*dl*) morphogen is distributed in a concentration gradient with peak levels in the ventral-most nuclei and progressively lower levels in dorsal nuclei. This gradient is responsible for initiating the differentiation of three embryonic tissues: the mesoderm, neuroectoderm and dorsal ectoderm (reviewed by Govind and Steward, 1991; Ip and Levine, 1992; St Johnston and Nüsslein-Volhard, 1992). *dl* functions as a transcriptional activator that initiates the expression of various target genes in a concentration-dependent manner along the dorsoventral axis of early embryos. High levels of *dl* activate the mesoderm determinants *twist* (*twi*) and *snail* (*sna*) in ventral regions, while low levels of *dl* activate neuroectoderm determinants such as *rhomboid* (*rho*) in lateral regions (Roth *et al.*, 1989; Jiang *et al.*, 1991a; Pan *et al.*, 1991; Thisse *et al.*, 1991;

Ip *et al.*, 1992a,b; Jiang and Levine, 1993). *dl* also functions as a transcriptional repressor that restricts the expression of *decapentaplegic* (*dpp*) and *zerknütt* (*zen*) to dorsal regions, where they are responsible for the differentiation of the dorsal epidermis and amnioserosa, respectively (Rushlow *et al.*, 1987; St Johnston and Gelbart, 1987; Ip *et al.*, 1991).

There are several examples of transcription factors functioning as both activators and repressors. For example, the glucocorticoid receptor (GR) can activate gene expression through glucocorticoid response elements (GRE), and also repress AP-1 mediated activation from other promoters (reviewed by Schule and Evans, 1991). Repression of AP-1 activity by the GR appears to involve specific protein–protein interactions between GR and c-Jun, whereby GR blocks the ability of c-Jun to activate transcription. However, a limitation of these and most other studies in mammalian systems is the use of cultured cells, which often rely on the overexpression of interacting proteins at nonphysiological concentrations. It is conceivable that the GR–c-Jun interaction is not relevant for the regulation of any native promoter *in vivo*. Here we investigate how *dl* functions as both an activator and repressor in the context of transgenic embryos.

Repression by *dl* appears to involve a mechanism that is distinct from the GR–c-Jun ‘quenching’ described above (see review by Levine and Manley, 1989). *dl* represses the ventral expression of *zen* through a distal regulatory sequence (the ‘VRE’) located far from the *zen* transcription start site (at about –1.2 kb). The VRE can act over considerable distances to silence the expression of a number of unrelated heterologous promoters, including *hunchback* (*hb*), *Krüppel* (*Kr*), *fushi tarazu* (*ftz*), *even-skipped* (*eve*) and *twi* (Doyle *et al.*, 1989; Ip *et al.*, 1991; Kraut, 1991; Jiang *et al.*, 1992; Pan *et al.*, 1991). For example, the *zen* VRE represses the ventral expression of the *Kr* promoter when placed >2 kb upstream from its transcription start site. These results suggest a silencing mechanism of repression, whereby *dl* might directly interfere with the assembly or function of the transcription complex (Levine and Manley, 1989; Ip *et al.*, 1991; Jiang *et al.*, 1992).

Comparison of the *zen* and *twi* promoters provides a basis for understanding how *dl* might function as both an activator and a repressor. The *cis* regulatory elements mediating repression (*zen* VRE) and activation (*twi* proximal and distal elements) have been characterized in detail and shown to contain multiple *dl* binding sites (Doyle *et al.*, 1989; Ip *et al.*, 1991; Jiang *et al.*, 1991a; Pan *et al.*, 1991; Thisse *et al.*, 1991). Despite the fact that there are some differences in the core recognition sequences between the *dl* binding sites in the *twi* and *zen* promoters, they are functionally interchangeable (Jiang *et al.*, 1992; Pan and Courey, 1992). For example, a ‘repressor’ site in the *zen* VRE mediates activation when multimerized and placed upstream of a minimal hsp 70 promoter. Conversely, an ‘activator’ site from the *twi* PE mediates repression when placed in the

context of the *zen* VRE (Jiang *et al.*, 1992). These results exclude the possibility that *dl* activation and repression are mediated by qualitatively distinct binding sites. Instead, it appears that *dl* is inherently a transcriptional activator, and in order to function as a repressor it must interact with additional factors, or 'corepressors', bound to neighboring sites in the *zen* VRE. *dl*-corepressor complexes appear to act over long distances to silence transcription.

To identify *dl* corepressors we have conducted a detailed characterization of the *zen* VRE. A 110 bp VRE sequence can mediate repression. This minimal element contains two *dl* binding sites as well as multiple binding sites for additional factors present in whole nuclear extracts from early embryos. Mutations in some of these binding sites abolish *dl*-dependent repression and convert the minimal VRE into an enhancer element that mediates transcriptional activation in ventral regions of early embryos in response to *dl*. These results support a combinatorial mechanism for *dl*-dependent silencing, whereby protein-protein interactions with corepressors convert the *dl* activator into a repressor.

Results

Characterization of the minimal *zen* VRE

The full-length VRE is 600 bp long and mediates both ventral repression and general activation in early embryos (Jiang

et al., 1992; see below). To facilitate the identification of *dl* corepressors we initially sought to uncouple activation and repression by characterizing the minimal VRE sequence sufficient for ventral repression. These experiments involved attaching different regions of the VRE upstream of the 480 bp *eve* stripe 2 enhancer (summarized in Figure 1). Previous studies have shown that the ventral expression of the stripe 2 enhancer is completely repressed by the full-length VRE (Jiang *et al.*, 1992). VRE-stripe 2 fusion promoters were attached to a *lacZ* reporter gene and introduced into the germline via P-element transformation (Rubin and Spradling, 1982). Embryos were collected from transformed lines and reporter gene expression was visualized by whole mount *in situ* hybridization using a *lacZ* antisense RNA probe followed by immunohistochemical staining (Tautz and Pfeifle, 1989; Jiang *et al.*, 1991b).

The stripe 2 enhancer directs *lacZ* expression in a broad band in early cycle 14 embryos, with equally intense staining in dorsal and ventral regions (Figure 2A; Jiang *et al.*, 1992; Small *et al.*, 1992). During the next 20 min this pattern is refined into a sharp stripe, which coincides with the endogenous *eve* stripe 2 (Figure 3A; Small *et al.*, 1992). When the full-length 600 bp *zen* VRE is placed upstream of this stripe 2-*lacZ* fusion gene, expression is completely abolished in ventral regions (Figures 2B and 3B; Jiang *et al.*, 1992). This repression depends on *dl*⁺ gene activity since

Fig. 1. Summary of VRE-stripe 2 fusion genes. The stippled bar represents the 480 bp minimal *eve* stripe 2 enhancer (MSE), and the hatched bar represents the full-length 600 bp *zen* ventral repression element (VRE). The stripe 2 enhancer was placed upstream of an *eve-lacZ* fusion gene that contains the first 44 bp of the *eve* promoter, the 100 bp *eve* leader sequence and the first 22 codons of the protein coding sequence. The arrow indicates the transcription start site, and the unfilled bar corresponds to the *lacZ* coding sequence. The line below the diagram of the VRE-stripe 2 fusion gene represents a restriction map of the 600 bp *zen* VRE. The filled circles above the map represent *dl* binding sites, and the arrows indicate the positions of synthetic *Bam*HI sites which were created by *in vitro* mutagenesis (see Materials and methods). Different regions of the *zen* VRE (represented by lines of different lengths) were inserted upstream of the stripe 2-*lacZ* fusion gene. In some cases multiple copies of fragments were used (e.g. two tandem copies of the 110 bp fragment). The activities of each VRE-stripe 2 fusion gene are indicated to the right of the diagrams. For example, the 350 bp VRE sequence retains nearly full ventral repression activity, but lacks the activator sequences that promote ubiquitous expression in early embryos.

there is uniform expression in both dorsal and ventral regions of mutant embryos derived from *dl*⁻ females (data not shown; see Jiang *et al.*, 1992). In addition, expression is expanded along the dorsal surface due to general activation sequences in the *zen* VRE (Figure 2B; Jiang *et al.*, 1992). Deletion of a 170 bp sequence from the 3' end of the VRE has no effect on general activation or ventral repression in early embryos (the '430' fusion gene, see Figures 1 and 2C), although there is a slight reduction in ventral repression activity in older embryos (Figure 3: compare panel C with B).

The removal of 250 bp from the 5' end of the VRE (the

'350' fusion gene, see Figure 1) eliminates general activation, such that expression no longer extends along the dorsal surface (Figures 2D and 3D). However, most of the ventral repression activity is retained by this truncated VRE since there is only slight derepression of the stripe in older embryos (Figure 3D). Thus, this deletion uncouples activation and repression, indicating that general activation sequences are located in the distal 250 bp of the *zen* VRE, while the bulk of the ventral repression sequences are located in the proximal 350 bp. The next set of experiments centered on deleting this 350 bp region to identify the minimal repression element.

Fig. 2. Expression patterns of VRE-stripe 2 fusion genes in early embryos. Embryos are oriented with anterior to the left and dorsal up. Expression of the *lacZ* reporter gene was visualized by *in situ* hybridization. (A) Staining pattern obtained with the stripe 2-*lacZ* fusion gene lacking *zen* VRE sequences. A broad stripe of staining is observed, with equal levels in dorsal and ventral regions. (B) Expression of the same fusion gene containing the full-length VRE (600 bp VRE-stripe 2 fusion gene). Staining is completely repressed in ventral regions. In addition, expression extends along the dorsal surface and posterior pole due to general activation sequences in the VRE. Repression is blocked at the anterior pole by an unknown polar repressor in the stripe 2 enhancer. (C) The 430 bp VRE-stripe 2 fusion gene. The expression pattern is similar to that in panel B. (D) The 350 bp VRE-stripe 2 fusion gene. Expression is repressed in ventral regions, but staining is no longer detected along the dorsal surface. (E) The 180 bp VRE-stripe 2 fusion gene. Ventral expression is only slightly attenuated. (F) Same as (E) except that the fusion gene contains two tandem copies of the 180 bp VRE sequence. Repression is comparable to that obtained with the 350 bp sequence (see D). (G) The 2 × 110 VRE-stripe 2 fusion gene. Ventral repression is more complete than that mediated by one copy of the 180 bp VRE (see E). (H) The 4 × 70 VRE-stripe 2 fusion gene. There is no ventral repression, but instead, weak staining is observed along the ventral surface.

Fig. 3. Expression of VRE–stripe 2 fusion genes in older embryos. Embryos are oriented as in Figure 2. (A) Stripe 2–*lacZ* fusion gene lacking VRE sequences. The stripe 2 enhancer directs the expression of a refined stripe. (B) 600 bp VRE–stripe 2 fusion gene (full-length VRE). There is complete repression in ventral regions. (C) The 430 bp VRE–stripe 2 fusion gene. There is partial derepression of the pattern, even though repression was complete at earlier stages (see Figure 2C). (D) The 350 bp VRE–stripe 2 fusion gene. There is only partial repression in ventral regions. (E) The 180 bp VRE–stripe 2 fusion gene. There is no ventral repression, such that the stripe is expressed at nearly equal levels in dorsal and ventral regions. (F) 2×180 VRE–stripe 2 fusion gene. Two copies of the 180 bp VRE sequence mediates partial repression, comparable to that observed for the 430 and 350 bp VRE sequences (see panels C and D). (G) 2×110 VRE–stripe 2 fusion gene. There is only slight repression in ventral regions. (H) 4×70 fusion gene. Four copies of the 70 bp element fail to mediate ventral repression.

A 180 bp fragment containing three *dl* binding sites (the ‘180’ fusion gene, Figure 1) mediates weak ventral repression in early embryos (Figure 2E). However, there is a nearly complete derepression of the stripe 2 pattern in older embryos (Figure 3E). It is conceivable that this reduced activity is due to the loss of corepressor sites located in 3’ regions of the VRE. However, this explanation seems unlikely since the 430 bp VRE (Figure 1) lacks the same 3’ sequences, yet mediates essentially complete ventral repression (Figures 2C and 3C). Perhaps the loss of repression activity results from placing the 180 bp VRE in reverse orientation relative to the native *zen* promoter (see Materials and methods). Evidence that the 180 bp fragment

contains all of the essential sequences required for ventral repression activity was obtained by placing two tandem copies of this fragment upstream of the stripe 2–*lacZ* fusion gene (2×180 , see Figure 1). This fusion gene directs a pattern similar to that obtained with the 430 bp VRE (compare Figures 2F and 3F with 2C and 3C), indicating a nearly complete restoration of ventral repression activity.

The 180 bp VRE was split into two fragments of 110 and 70 bp (see Figure 1). Two tandem copies of the 110 bp fragment (2×110) provide intermediate levels of ventral repression (Figures 2G and 3G), which is more complete than that mediated by one copy of the 180 bp VRE (Figure 2E). The 110 bp fragment is the minimal sequence

Fig. 4. Nuclear factor binding in the *zen* VRE. (A) DNase I protection in the 180 bp VRE using a nuclear extract from 0–4 h embryos. The 180 bp VRE fragment was labeled either at the 5' end of the noncoding strand (lanes 1–5), or at the 5' end of the coding strand (lanes 6–10). The labeled fragments were incubated with increasing amounts of nuclear extract. Lanes 1 and 6: G+A sequence; lanes 2, 5, 7 and 10: DNase I digestion patterns of naked DNA templates (without added extract); lanes 3 and 8: DNase I patterns obtained after addition of 25 μ g of nuclear extract; lanes 4 and 9: 50 μ g of nuclear extract. Protected regions are indicated to the left of the autoradiograms. The unfilled boxes indicate the *dl* binding sites, the filled boxes bracket the AT sites and the hatched boxes represent the GC sites. (B) DNA sequence of the 180 bp VRE. The three *dl* binding sites (ZD1, ZD2 and ZD3) are boxed, and regions protected by addition of nuclear extract are shaded. Nuclear factor binding sites were mutagenized by substituting the underlined sequences. The arrows at –1.24 and –1.29 kb indicate the locations of two synthetic *Bam*HI sites created by *in vitro* mutagenesis. (C) Alignment of the core AT recognition sequences. Two additional sequences, AT0 and AT4, match the AT consensus sequence and are located elsewhere in the full-length VRE (the AT0 site is located at –1.6 kb and the AT4 site at –1.0 kb). The core sequences of the three GC binding sites are also shown. (D) Summary of *dl*, AT and GC binding sites in minimal VRE sequences. The 180 bp VRE contains three of each class of binding site, while the 110 bp VRE contains two of each.

that retains ventral repression activity. For example, even four tandem copies of the 70 bp fragment (4×70 , see Figure 1) fail to mediate ventral repression (Figures 2H and 3H); the weak ventral activation that is observed might be due to the single *dl* binding site contained within this fragment (see below). Smaller regions of the 110 bp VRE, the 4×50 and 7×37 fusion genes (Figure 1), completely fail to repress stripe 2 expression (data not shown). Interestingly, a similar study by Kirov *et al.* (1993) identified a minimal 55 bp VRE that retains weak repression activity. This sequence encompasses the 37 bp fragment that fails to give repression activity in our assays. The spacing between

dl and a putative corepressor site (AT1, see below) is different in these sequences, such that there is virtually no spacer between the two binding sites when the 37 bp sequence is multimerized.

Nuclear factor binding sites in the *zen* VRE

In an effort to identify *dl* corepressors, DNA binding assays were done with the minimal 110 bp repression element (mVRE) and whole nuclear extracts from early embryos. The initial experiments involved DNase I footprint analyses with the 180 bp VRE. Multiple regions of protection were identified using extracts from 0–4 h embryos (Figure 4A),

Fig. 5. Conversion of the VRE into a ventral activator. Early to mid cleavage cycle 14 embryos are presented as in Figures 2 and 3. (A) Pattern obtained with the 180 bp VRE–stripe 2 fusion gene (same as Figure 2E). There is reduced expression in ventral regions. (B) Same as (A) except that the AT1, AT2 and AT3 binding sites were mutagenized. The mutagenized promoter not only lacks ventral repression activity, but also shows weak activation along the entire ventral surface. (C) 180 bp VRE–stripe 2 fusion with the three GC sites mutated. There is normal repression in ventral regions, comparable to that obtained with the wild-type 180 bp VRE (see A). (D) An embryo expressing the 2×110 VRE–stripe 2 fusion lacking the AT1 site. There is only a slight reduction in ventral expression. (E) Same as (D) except that the AT2 site was mutagenized. Ventral repression activity is lost and there is weak staining along the ventral surface. (F) 2×110 VRE–stripe 2 fusion lacking both the AT1 and AT2 sites. There is intense staining along the ventral surface.

which corresponds to the time when *dl* represses the ventral expression of *zen*. The three known *dl* binding sites were identified among these regions of protection (ZD1, ZD2 and ZD3; Figure 4A). Additional factor binding sites were also identified, as summarized in Figure 4B. These can be divided into two classes based on the core sequences within the protected regions. One class of binding sites, called the AT sites, are closely related to one another and contain an AT-rich core with the consensus sequence TTT(C/T)NTT-(G/C)AT (Figure 4C). There are three copies of this sequence (AT1, AT2 and AT3) in the 180 bp VRE; two additional sequences in the 600 bp VRE contain a perfect match to this consensus (AT0 and AT4; see Figure 4C). The other class of binding sites, called the GC sites, are related in that they all contain a GC-rich half site and an AT-rich half site (Figure 4C). There are additional sequences in the 600 bp VRE that are related to the GC class of sites. The 110 bp mVRE contains two *dl* binding sites and two of each class of nuclear factor binding sites, as summarized in Figure 4D.

Converting the *zen* VRE into an activation element

To determine whether the AT or GC class of binding sites function as *dl* corepressors, they were mutagenized in the context of otherwise normal VRE–stripe 2 fusion genes. These experiments involved the use of the 180 bp VRE, which contains three *dl* binding sites, three AT sites and three GC sites (see Figure 4D). The initial experiments involved disrupting all three AT sites by substituting 6–9 nucleotides within the core consensus sequence (see Materials and methods). Gel shift assays indicate that these mutations have no effect on the binding of *dl* protein to the neighboring *dl* sites (data not shown). When this mutagenized VRE was placed upstream of the stripe 2–*lacZ* fusion gene, it not only failed to repress the ventral expression of the stripe 2 enhancer, but activated expression along the entire ventral surface (Figure 5; compare panel B with A). Particularly intense staining is observed in ventral regions where stripe 2 is expressed, suggesting additive or cooperative interactions between the stripe 2 enhancer and the mutagenized VRE. Mutations in the three GC sites (see Figure 4B) had no effect

on the ability of the 180 bp VRE to repress the ventral expression of the stripe 2 enhancer (Figure 5C).

These results strongly suggest that the AT sites bind one or more corepressors to convert the *dl* activator into a repressor. Individual AT binding sites were mutagenized in the context of the minimal 110 bp VRE, which contains two *dl* sites and two AT sites (see Figure 4D). Nucleotide substitutions were introduced into either the AT1 site or AT2 site alone, or both sites simultaneously. Mutagenized VREs were dimerized and placed upstream of the stripe 2 enhancer. Mutations in the AT1 site cause a severe reduction in ventral repression activity (compare Figures 5D and 2G), such that stripe 2 expression is only slightly reduced in ventral regions. Occasionally, embryos show weak expression along the ventral surface, in addition to the slight ventral repression of the stripe 2 pattern (data not shown). Mutations in the AT2 site cause both a complete loss in ventral repression activity and the conversion of the VRE into a ventral activation element (Figure 5E). Even more potent ventral activation is observed when both the AT1 and AT2 sites are mutagenized (Figure 5F). These results strongly suggest that the AT1 and AT2 sites bind one or more corepressors that convert the *dl* activator into a repressor.

The AT1 and AT2 sites may be recognized by distinct corepressors

The core sequences of the AT1 and AT2 binding sites differ by only three nucleotides (Figure 4C). Gel retardation assays were performed to determine whether they are recognized by the same or distinct nuclear factor(s). Short DNA fragments containing either the AT1 or AT2 sequence were mixed with crude nuclear extracts prepared from 0–4 h embryos, and fractionated on native polyacrylamide gels (Figure 6). AT1 and AT2 complexes migrate at slightly different positions (lanes 1 and 4), suggesting that they might be recognized by different proteins. Further evidence for distinct proteins was obtained by performing competition assays. Formation of the AT1–protein complex is specifically inhibited by an excess of unlabeled AT1 DNA (lane 2), but not by a similar excess of the AT2 sequence (lane 3). Similarly, the AT2 complex is competed by the AT2 sequence (lane 6), but not by AT1 (lane 5). A further check on the specificity of the AT1 and AT2 complexes involved the use of mutagenized AT1 and AT2 DNA fragments for competition assays. These mutant DNAs contain four nucleotide substitutions in the core AT1 and AT2 binding sites (see Materials and methods), and completely fail to inhibit the formation of either AT1 or AT2 complexes (data not shown). These results suggest that *dl* may interact with two different corepressors in the *zen* VRE; perhaps closely related isoforms of the same protein recognize the AT1 and AT2 sites.

Discussion

We have identified a 110 bp repression element (mVRE) that can silence the ventral expression of the *eve* stripe 2 enhancer. The mVRE contains two *dl* binding sites and two closely linked corepressor sites. Mutations in the corepressor sites convert the mVRE into an enhancer. Although these sites (AT1 and AT2) contain similar core sequences, several lines of evidence suggest they bind distinct, but possibly related, nuclear factors. A model for how *dl* might function as both an activator and repressor is summarized in Figure 7.

Fig. 6. The AT1 and AT2 sites bind distinct factors. Gel retardation assays were done with nuclear extracts from 0–5 h embryos and oligonucleotides containing either the AT1 or AT2 binding site. Lanes 1–3, labeled AT1 oligonucleotide; lanes 4–6, labeled AT2 oligonucleotide. Lane 1, labeled AT1 without added competitor. A single protein–DNA complex is observed. Lane 2, same as lane 1 except that an 800-fold molar excess of unlabeled AT1 was added; there is a severe reduction in the amount of shifted complex formed. Lane 3: same as lane 1 except that an 800-fold molar excess of unlabeled AT2 was added. There is little or no reduction in the levels of the protein–DNA complex. Lane 4: labeled AT2 without added competitor. A single protein–DNA complex is observed. It has a slightly higher mobility than the AT1 complex. Lane 5: same as lane 4 except that a 400-fold molar excess of unlabeled AT1 was added. There is only a slight reduction in the amount of AT2–protein complex formed. Lane 6: same as lane 4 except that a 400-fold excess of unlabeled AT2 was added. There is a severe reduction in the levels of the protein-labeled AT2 complex.

The *dl* protein is intrinsically a weak transcriptional activator; tandem copies of an optimal *dl* binding site can activate a naive promoter in response to *dl* protein (Jiang *et al.*, 1992; Pan and Courey, 1992). Efficient activation requires synergistic interactions with other regulatory proteins, such as *zeste* or basic helix–loop–helix (bHLH) proteins (Jiang *et al.*, 1991a; Pan *et al.*, 1991; Ip *et al.*, 1992a,b; Jiang and Levine, 1993). Repression is achieved when *dl* interacts with a different set of cofactors, such as those that bind to the AT1 and AT2 sites in the VRE.

Modes of repression

There are several distinct modes of transcriptional repression (reviewed by Levine and Manley, 1989). Competition has been implicated in the regulation of *eve* stripes along the anteroposterior axis of early *Drosophila* embryos; activators and repressors appear to compete for overlapping or closely linked binding sites within individual stripe enhancers (Small *et al.*, 1991, 1992; Stanojevic *et al.*, 1989, 1991). This form of repression has been invoked for the regulation of various target genes by the steroid hormone receptor superfamily; a vast array of these regulatory proteins can interact with common binding sites (e.g. Mietus-Snyder *et al.*, 1992). The regulation of neuroectoderm stripes of gene expression in *Drosophila* appear to involve a ‘quenching’ mechanism,

Fig. 7. Model for dual functions of *dl*. The *dl* protein is an inherent activator which can stimulate the transcription of a naive promoter containing multiple *dl* binding sites (Jiang *et al.*, 1992; Pan *et al.*, 1992). In order to function as a strong activator, it must interact with additional 'coactivators' bound to nearby sites within a ventral activation element (VAE). Several coactivators have been identified, including *zeste* and bHLH proteins (Pan *et al.*, 1991; Jiang and Levine, 1993). The *dl* protein can mediate repression when it associates with corepressors in the VRE. It is possible that the *dl*-corepressor complex silences the activities of transcriptional activators bound upstream of the transcription start site (a). Alternatively, perhaps repression involves direct interference with the assembly of the transcription complex (b). In either case, disruption of the corepressor binding site allows *dl* to stimulate transcription.

whereby activators and repressors co-occupy neighboring sites, but the repressor masks the ability of the activator to contact the transcription complex (Ip *et al.*, 1992b). In particular, evidence was presented that the *sna* repressor can act over distances of 7–25 bp to block activation by *dl* (Jiang and Levine, 1993). This mechanism has also been proposed for the repression of c-Jun-mediated activation by GR, although repression can be obtained in the absence of GR binding sites if the protein is overexpressed at high concentrations in cultured cells (Schule and Evans, 1991).

Repression over long distances has been observed in a number of different systems. For example, the *zen* VRE can act over distances of 2–3 kb to repress the ventral expression of a number of different heterologous promoters (Doyle *et al.*, 1989; Ip *et al.*, 1991; Kraut, 1991). Long range repression has also been implicated in the regulation of the homeotic selector gene *Ultrabithorax*, whereby the *hb* protein can silence the BXD regulatory element in anterior regions of the embryo (Zhang and Bienz, 1992).

Mechanism of repression

The conversion of the *dl* activator into an 'active' repressor is not without precedent. For example, it has recently been

shown that the mammalian E2F activator can repress heterologous promoters when it forms a complex with the retinoblastoma (Rb) protein (Weintraub *et al.*, 1992). The most thoroughly characterized example of this phenomenon involves interactions between MCM1 and $\alpha 2$ in yeast. MCM1 activates the expression of a number of different target genes, including various *a*-specific genes. These latter genes are repressed in α cells due to interactions with the $\alpha 2$ homeodomain protein, which binds to sites that flank MCM1 (Keleher *et al.*, 1989). This interaction not only blocks the ability of MCM1 to function as an activator, but converts it into a repressor. Recent studies suggest that the $\alpha 2$ -MCM1 complex mediates repression by recruiting general repressors, including Ssn6 and Tup1 (Keleher *et al.*, 1992).

A similar mechanism might govern repression by the *zen* VRE. Direct physical associations between the AT1/AT2 corepressors and *dl* is suggested by the ability of both high and low affinity *dl* binding sites to mediate efficient repression in the context of the *zen* VRE (Jiang *et al.*, 1992). The *dl* sites present in the VRE have high binding affinities, and can mediate repression in lateral regions where there are low levels of the morphogen (Ip *et al.*, 1991). In contrast, the *twi* promoter is activated only by high concentrations of *dl* in ventral regions because its promoter contains low affinity binding sites (Jiang *et al.*, 1991; Pan *et al.*, 1991; Thisse *et al.*, 1991). When the high affinity *dl* sites in the *zen* VRE are replaced by the low affinity sites from the *twi* promoter, the modified VRE mediates repression by low levels of *dl* that are insufficient for activating *twi*. This observation was taken as evidence that corepressors can facilitate the binding of *dl* to adjacent sites within the VRE (Jiang *et al.*, 1992). A prediction of this model is that purified AT1/AT2 factors and *dl* will cooperatively bind DNA.

Materials and methods

Preparation of VRE-stripe 2 fusion genes

The stripe 2-*lacZ* fusion gene and the 600 bp VRE-stripe 2 fusion genes have been described previously (Jiang *et al.*, 1992; Small *et al.*, 1992). The 430 and 350 bp VRE-stripe 2 fusion genes were prepared by inserting either a 430 bp *Xba*I-*Xmn*I or a 350 bp *Nar*I-*Hind*III fragment from the 600 bp *zen* VRE into a unique *Eco*RI site upstream of the stripe 2-*lacZ* fusion gene. The 430 and 350 bp VREs were inserted in the same orientation as in the native *zen* promoter. The 180 bp VRE-stripe 2 and 2 × 180 VRE-stripe 2 fusion genes were prepared with a 180 bp *Nar*I-*Xmn*I fragment from the 600 bp VRE. Either one copy or two tandem copies of this fragment were inserted in the *Sma*I site of the pBluescriptSK+ vector (Promega). A *Bam*HI-*Eco*RV fragment containing either one or two tandem copies of the 180 bp VRE was excised from these pBluescript plasmids and inserted at the unique *Eco*RI site upstream of the stripe 2-*lacZ* fusion gene. The orientation of the 1 × 180 and 2 × 180 VRE sequences is opposite to that in the native *zen* promoter. The 2 × 110 and 4 × 70 VRE-stripe 2 fusion genes were prepared by creating a *Bam*HI site in the 180 bp VRE at -1.24 kb (see Figure 4B). The 70 bp VRE sequence was obtained by digestion with *Bam*HI (see Figure 1); four tandem copies of this sequence were inserted upstream of the stripe 2-*lacZ* fusion gene at the unique *Eco*RI site. After the 70 bp *Bam*HI-*Bam*HI sequence was removed from the Bluescript plasmid containing a copy of the 180 bp VRE, the remaining vector was religated to create the 110 bp VRE sequence. This was removed by digestion with *Xba*I and *Eco*RV, and inserted between the *Xba*I site and *Bam*HI site of the religated plasmid to obtain the 2 × 110 VRE sequence. This fragment was obtained after digestion with *Xba*I and *Eco*RV, and inserted into the unique *Eco*RI site of the stripe 2-*lacZ* fusion gene. 180 bp VRE-stripe 2 fusion genes and 2 × 110 VRE-stripe 2 fusions containing mutations in various nuclear factor binding sites were prepared in the same way as their wild-type counterparts. To create the 4 × 50 VRE-stripe 2 fusion gene, another *Bam*HI site was introduced

at -1.29 kb by site directed mutagenesis (see Figure 4B). This 50 bp sequence, contained within two synthetic *Bam*HI sites, was excised by *Bam*HI digestion, multimerized and inserted into the *Bam*HI site of the pBluescript SK+ vector. A *Xba*I-*Eco*RV fragment containing four copies of the 50 bp sequence was obtained from the pBluescript plasmid and inserted into the unique *Eco*RI site of the stripe 2-*lacZ* fusion gene. The 7 × 37 VRE-stripe 2 fusion gene was obtained by annealing and then multimerizing the following two oligonucleotides: GATCCATTTTCTTTGATATAAG-TTTTGGGAAATCCAG and GATCCTGGATTTCCTAAACTTATA-TCAAGAAAATG.

Multiple copies were inserted into the *Bam*HI site of the pBluescript SK+ vector, and an *Xba*I-*Eco*RV fragment containing seven tandem copies of the 37 bp sequence was placed upstream of the stripe 2-*lacZ* fusion gene in the unique *Eco*RI site.

In vitro mutagenesis

Nucleotide substitutions were made with mutagenic oligonucleotides using the Muta-gene kit (Bio-Rad) as described previously (Jiang *et al.*, 1991b). Single-stranded DNA templates containing the 180 bp VRE sequence or two copies of the 110 bp VRE were prepared from appropriate pBluescript plasmids described above. The following mutagenic oligonucleotides were used to disrupt each nuclear factor binding site. In all cases a *Sal*I site was created in the mutagenized sites. AT1 site, TATAGAGTTTGTGCGACT-CTTGGGCGC; AT2 site, GGGCCTATATGAGTGTGCGACGTGTGG-GTTTCTCC; AT3 site, TTGCCCTATGTGTGCGACGTATAAGTTT-TGG site; GC1 site, TTTTATTGATCGTGTGCGACGTGTTTTAGTAGGT; GC2 site, CCAGAGTCCGTGTGTGAATCTATATGAACG; GC3 site, CGCCAGGCAACGTGTGCGACGTATTTCCTTTGA.

For double or triple mutations, the corresponding mutagenic oligonucleotides were simultaneously mixed with the single strand DNA template. The mutated recombinant plasmids were screened by *Sal*I digestion, and then confirmed by DNA sequencing. The following two oligonucleotides were used to introduce *Bam*HI sites in the 180 bp VRE sequence: GTTT-CTCCAGGATCCGAGTTTATTG and GGAAATCCAGGGATCC-AATAACGG.

Preparation of nuclear extracts

Embryos were collected at 25°C from population cages of the Oregon R strain. In order to obtain large amounts of material, staged embryos were kept at 4°C and then pooled. Embryos were dechorionated in sodium hypochlorite and then rinsed in NaCl-Triton X-100 and H₂O. Nuclear extracts were prepared exactly as described by Soeller *et al.* (1988) and Biggin and Tjian (1988).

DNase I footprinting

DNA templates containing the 180 bp VRE were labeled by PCR at either end using a pair of primers, T3 and KS, which flank the 180 bp VRE in the pBluescript vector. The KS primer was end-labeled with ³²P using polynucleotide kinase and [α -³²P]ATP. The coding strand of the 180 bp VRE fragment was labeled with the KS primer, which was end-labeled with ³²P using polynucleotide kinase and [α -³²P]ATP. 3 pmol of labeled KS primer and 20 pmol of cold T3 primer were mixed with 100 ng of 180 bp VRE/pBluescript plasmid DNA. The PCR reaction was done with ampliTaQ DNA polymerase (purchased from Perkin Elmer Cetus) as described by the manufacturer. The noncoding strand of the 180 bp VRE sequence was labeled with a combination of hot T3 primer and cold KS primer. The amplified DNA templates were purified from a 5% nondenaturing polyacrylamide gel.

DNase I footprint assays were done essentially as described by Jiang *et al.*, (1991a). An aliquot containing 10 000 c.p.m. of labeled DNA template was incubated with up to 50 μ g of nuclear extracts from 0-4 h embryos, and 0.5 μ g of poly(dI-dC) competitor. Because the crude extract contains considerable exonuclease activity, MgCl₂ was omitted from the normal binding buffer. Control DNase I digestions were done at room temperature for 1 min with 20 ng freshly diluted DNase I (purchased from Worthington). Ten-fold more DNase I was used when nuclear extract was added.

Gel mobility shift assays

Oligonucleotides containing AT binding sites were end-labeled with [γ -³²P]ATP and filled. DNA binding reactions were done in 5 mM Tris-HCl, pH 7.9; 12 mM HEPES; 50 mM KCl; 3 mM EDTA; 1 mM DTT; 5 mg/ml BSA; 10% glycerol; 0.1 mg/ml poly(dI-dC). 7 μ g of 0-5 h embryo nuclear extract were preincubated in this buffer in the presence or absence of 40 ng unlabeled competitor DNA for 10 min at 25°C. Afterwards, 50-100 pg of the labeled oligonucleotide was added to the reaction mix, which was then incubated for another 15 min at 30°C. The reactions were fractionated on a 4% native polyacrylamide gel in low ionic

buffer (7 mM Tris-HCl, pH 7.9; 3.3 mM sodium acetate; 1.25 mM EDTA).

The oligonucleotides used in the gel mobility shift assays were AT1 (GATCCATTTTCTTTGATAGGATC) and AT2 (GATCAATATTCGT-TCATAGGATC).

P-transformation and in situ hybridization

P-transposons containing various VRE-MSE (minimal stripe 2 element) fusion genes were introduced into the *Drosophila* germ-line by injecting white-embryos from yw56/yw56 homozygotes (Rubin and Spradling, 1982). Expression patterns directed by each fusion promoter were visualized by whole mount *in situ* hybridization using an antisense *lacZ* RNA probe followed by immunochemical staining (Jiang *et al.*, 1991a,b).

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