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Conversion of a silencer into an enhancer: evidence for a co-repressor in *dorsal*-mediated repression in *Drosophila*

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The *dorsal* (*dl*) protein gradient determines patterns of gene expression along the dorsal-ventral axis of the *Drosophila* embryo. *dl* protein is at peak levels in ventral nuclei of the embryo where it activates some genes (*twist* and *snail*) and represses others [*zerknullt* (*zen*), *decapentaplegic* and *tolloid*]. It is a member of the rel family of transcription factors and interacts with specific DNA sequences in the regulatory regions of its target genes. These sequences (*dl* binding sites), when taken from the context of either an activated or repressed promoter, mediate transcriptional activation of a heterologous promoter, but not repression. We found that T-rich sequences close to the *dl* binding sites in the silencer region of the *zen* promoter are conserved between three *Drosophila* species. Using this sequence information we defined a minimal element that can mediate repression of a heterologous promoter. This element interacts with at least two factors present in embryonic extracts, one of which is *dl* protein. The other factor binds to the T-rich site. Point mutations in either site abolish ventral repression *in vivo*. In addition, mutations in the T-rich site cause ectopic expression in ventral regions indicating that the minimal silencer was converted into an enhancer.

Key words: co-repressor/*dorsal*/*Drosophila*/silencer/transcriptional regulation

Introduction

The *dorsal* (*dl*) protein is a maternal morphogen that determines cell fates along the dorsal-ventral (DV) axis of the *Drosophila* embryo (reviewed in St Johnston and Nüsslein-Volhard, 1992). During early development, *dl* protein is distributed in a nuclear concentration gradient established by a mechanism of regulated nuclear transport (Roth *et al.*, 1989; Rushlow *et al.*, 1989; Steward, 1989). Ventrally located nuclei receive high levels of *dl* protein while lateral and dorsal nuclei receive progressively lower amounts of protein. In ventral regions, where the *dl* protein is at peak levels, it activates the expression of target genes, such as *twist* (*twi*) and *snail* (*sna*), which are necessary for the formation of ventral cell types (Ip *et al.*, 1992; Jiang *et al.*, 1992; Jiang and Levine, 1993). In ventral-lateral regions where the *dl* protein level is too low to activate *twi* and *sna*, it is able to activate genes such as *rhomboid* (*rho*), which is involved in the differentiation of the neuroectoderm (Ip *et al.*, 1992). In contrast to its role as an activator, *dl* can also function as a repressor. Genes such as *zerknullt* (*zen*), *decapentaplegic* (*dpp*) and *tolloid* (*tld*) are normally expressed only in the dorsal part of the embryo (Rushlow *et al.*, 1987b; St Johnston and Gelbart, 1987; Shimell *et al.*, 1991). In the absence of *dl* protein, these genes are expressed uniformly along the DV axis, indicating that *dl* protein normally represses their transcription (Rushlow *et al.*, 1987a; Ray *et al.*, 1991). Thus, *dl* appears to be able to activate the expression of certain target genes while repressing the expression of others.

Recent studies have addressed the interaction between the *dl* protein and the promoter regions of several target genes. *dl* encodes a sequence-specific DNA binding protein which is a member of the *rel* family of proteins (Steward, 1987). This family includes transcription factors such as the vertebrate oncogene *rel* and the mammalian regulatory protein NF-κB (Ip *et al.*, 1991; reviewed in Rushlow and Warrior, 1992). Multiple *dl* binding sites have been identified in enhancer regions of the *twi*, *sna* and *rho* promoters (Jiang *et al.*, 1991; Pan *et al.*, 1991; Thisse *et al.*, 1991; Ip *et al.*, 1992), and in the negative enhancer, or silencer, regions of the *zen* and *tld* promoters (Ip *et al.*, 1991; N.Kirov, C.Rushlow and M.O'Connor, unpublished results). Point mutations in the *dl* binding sites of the *twi* promoter severely reduce ventral activation, while mutations in the *dl* binding sites in the *zen* silencer abolish ventral repression. These results suggest that *dl* functions as a transcriptional activator or repressor *in vivo* by interacting with the *dl* binding sites within these promoters (Jiang *et al.*, 1991, 1992; Rushlow and Warrior, 1992). Moreover, the *zen* silencer, termed the ventral repression element (VRE), when placed upstream of various heterologous promoters, can act over considerable distances to block expression in ventral regions (Doyle *et al.*, 1989; Ip *et al.*, 1991; Jiang *et al.*, 1992). For example, when the VRE is attached to the *even-skipped* (*eve*) stripe 2 promoter element, only the dorsal half of the stripe is expressed. Point mutations in the *dl* binding sites in the VRE will restore *eve* stripe 2 to a full stripe (Jiang *et al.*, 1992).

At present, it is not clear how *dl* represses transcription. Although *dl* binding sites in the *twi* and *zen* promoters are not identical, the sequence of the binding site alone does not appear to determine whether *dl* acts as an activator or a repressor. A *dl* binding site from the *twi* enhancer is able to mediate repression when placed in the context of the *zen* silencer, while a site from the *zen* VRE activates when placed in the *twi* promoter (Jiang *et al.*, 1992; Pan and Courey, 1992). Furthermore, a *dl* binding site from the *zen* silencer, when multimerized and attached to a minimal hsp70 basal promoter, can mediate activation (Jiang *et al.*, 1992). This suggests that *dl* functions as an activator by itself and that ventral repression requires the binding of *dl* together with an additional factor(s), or co-repressor, to the *zen* silencer region.

To identify DNA sequences that may interact with the putative co-repressor, we searched for conserved blocks of DNA sequence in the *zen* silencer from different *Drosophila*

species. This search identified several conserved regions which included *dl* binding sites and nearby sites rich in T residues. Here we show that an element that contains a *dl* binding site and a T-rich site, when multimerized, can cause ventral repression. This element binds two different proteins from embryonic nuclear extracts, one of which is *dl*. Point mutations in either of the sites abolished DNA binding and ventral repression. Moreover, in the absence of the T-rich sites, and thus the putative co-repressor interaction, *dl* functions as a transcriptional activator.

Results

dl binding sites and T-rich sequences are highly conserved between Drosophila species

dl mediates repression of *zen* through a distal region of the *zen* promoter, located between −1.6 and −1.0 kb upstream of the transcription start site. This region, termed the ventral repression element (VRE), acts as a silencer on heterologous promoters such as *hunchback* (*hb*), *Krüppel* (*Kr*) and *eve* stripe 2 to induce repression in ventral regions of the embryo (Doyle *et al.*, 1989; Ip *et al.*, 1991; Jiang *et al.*, 1992). The VRE contains four high affinity *dl* binding sites (Ip *et al.*, 1991). In order to identify other important sequences in the VRE, we compared the *zen* promoter sequences of *Drosophila melanogaster* (*D.mel*) with those of *D.virilis* (*D.vir*) and *D.pseudoobscura* (*D.pso*) whose estimated divergence from *D.mel* is >50 million years (Ashburner, 1989). The *zen* regions from *D.vir* and *D.pso* were sequenced (see Materials and methods) and an alignment of DNA sequences is shown in Figure 1. The *dl* binding sites [GGG(A)$_n$CC] in *D.mel* are labelled Z0, Z1, Z2 and Z3 (Z for *dl* binding sites in the *zen* promoter). The Z0 and Z1 sites are conserved in sequence and position among all three species. There is only a single base mismatch in each site—an A residue substitutes for a G residue in the *D.vir* and *D.pso* sequences. The effect of this change would most certainly reduce but not abolish *dl* binding. The Z2 site is conserved between *D.mel* and *D.vir* but not *D.pso*; however, the sequence at this place in *D.pso* could possibly interact with *dl* protein. The Z3 site does not appear to be conserved. It lies in a region of the VRE which overall displays little homology among the three species. Inspection of the *D.pso* sequence in this region reveals a putative *dl* binding site just proximal to the *D.mel* Z3 (Figure 1, italics).

In addition to the *dl* binding sites there are other regions of homology that extend over several nucleotides. In particular, there are T-rich stretches that lie adjacent to the *dl* binding sites (see shaded areas in Figure 1). These regions are labelled AT0, AT1, AT2 and AT3. All four sites are

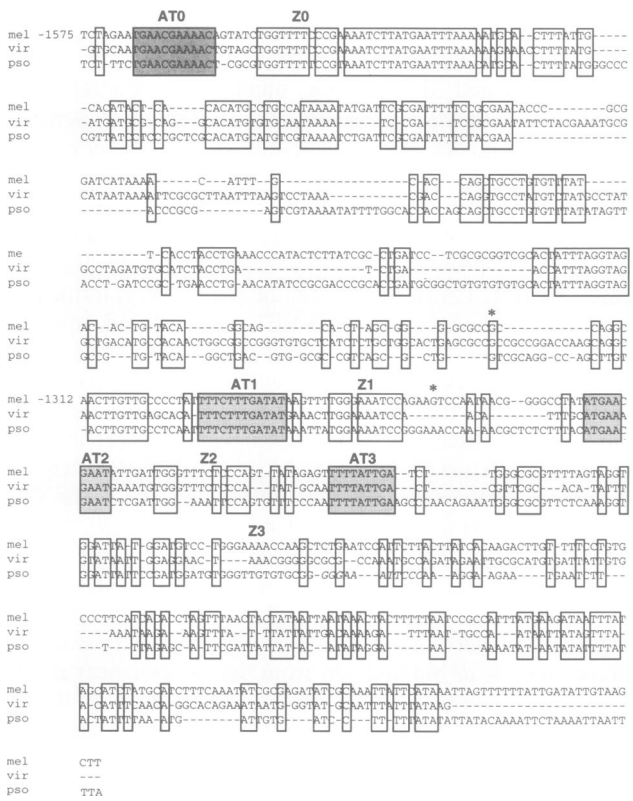

Fig. 1. Comparison of *zen* promoter sequences from *Drosophila melanogaster* (mel), *D.virilis* (vir) and *D.pseudoobscura* (pso). The distal regions of the *zen* promoter from *D.virilis* and *D.pseudoobscura* were sequenced and aligned with the 600 bp *zen* VRE (−1575 to −825 from the transcription start site). Perfect matches between all three promoters are boxed. Four regions of homology that are A-rich (T-rich on the other strand) are shaded and labelled AT0, AT1, AT2 and AT3. The four *dl* binding sites in the *mel* VRE are labelled Z1, Z2, Z3 and Z4. The asterisks mark the limits of the 55 bp oligonucleotide used in this study.

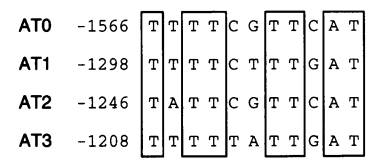

Fig. 2. Alignment of the AT-rich sequences from the *D.melanogaster zen* promoter. The T-rich strands of the four AT sites from the *mel* sequence were aligned for the most significant homology. The number indicates the nucleotide position of the 5′-most residue from the transcription start site. Note that most of the residues that align are T residues.

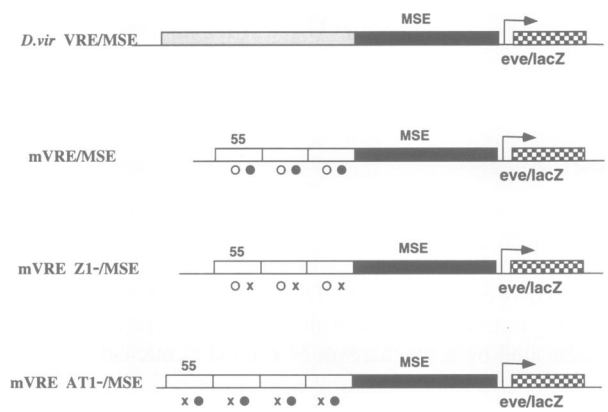

Fig. 3. Summary of promoter fusions. The arrow corresponds to the transcription start site in the *eve* basal promoter. The hatched bar represents 3 kb (~75%) of the *lacZ* coding sequence. The filled black bar represents the 480 bp minimal *eve* stripe 2 element. The shaded bar represents 980 bp of the *D.vir* promoter. The unfilled boxes correspond to different forms of the 55 bp minimal VRE. Multiple copies (three or four) of the minimal VRE (mVRE) were fused to the MSE. The black dots underneath the boxes refer to the *dl* binding site, Z1, while the unfilled dots refer to the AT1 site. Point mutations within each of the sites are represented by an X. The orientation of each 55 bp element is not denoted in the schematic diagram. In each case the order is 3′ to 5′ except for the third copy of the wild-type 55 bp element which is 5′ to 3′.

very well conserved between the three species. Comparison of the four sites in *D.mel* revealed that the T residues align (see Figure 2). In fact, if all 12 sites (from *D.mel*, *D.vir* and *D.pso*) are compared, the same alignment results (see sequences in Figure 1).

D.virilis zen promoter sequences function as a VRE in D.melanogaster

The observed DNA sequence homology between species could be said to be significant if the *zen* promoter sequences of *D.vir*, for example, was able to direct a *zen*-like expression pattern when transformed into *D.mel*. We tested a 900 bp fragment (which includes the 600 bp shown in Figure 1) for its ability to function as a VRE. The assay involves a well defined heterologous promoter element from the *eve* promoter that directs the expression of *eve* stripe 2 (Small *et al.*, 1992). The 480 bp stripe 2 element (the minimal stripe 2 element or MSE) was fused to the *eve* basal promoter and a *lacZ* reporter gene (schematized in Figure 3), and inserted into flies by P-element mediated transformation. It directs a broad pattern of *lacZ* expression in the anterior half of the embryo at cell cycle 12−13. This pattern refines into a stripe (*eve* stripe 2) by mid cycle 14 (Jiang *et al.*, 1992). An example of a cycle 14 transformant embryo carrying the MSE is shown in Figure 4A. The *lacZ* expression is equally intense in dorsal and ventral regions. However, if the 900 bp *D.vir* DNA sequence is placed upstream of the MSE (see Figure 3), stripe 2 is expressed in dorsal but not ventral regions of the embryo. Figure 4B shows a mid-cycle 14 embryo carrying the *D.vir* VRE/MSE transgene. The stripe is slightly obscured by the presence along the dorsal half of the embryo of broad *lacZ* expression, which is probably due to general activation sequences present in the *D.vir* VRE. A similar expression pattern was observed when the 600 bp *D.mel* VRE was used in previous studies (Jiang *et al.*, 1992). Thus *D.vir* sequences can function in *D.mel* to mediate ventral repression. To show that the ventral repression of stripe 2 is mediated by *dl*, males carrying the *D.vir* VRE/MSE transgene were mated to dl^- females. Embryos that inherit the transgene exhibit a full stripe 2 pattern (Figure 4C).

A minimal VRE contains one dl binding site and one AT site

In order to facilitate biochemical studies, it was necessary to determine a minimal segment of DNA from the *zen*

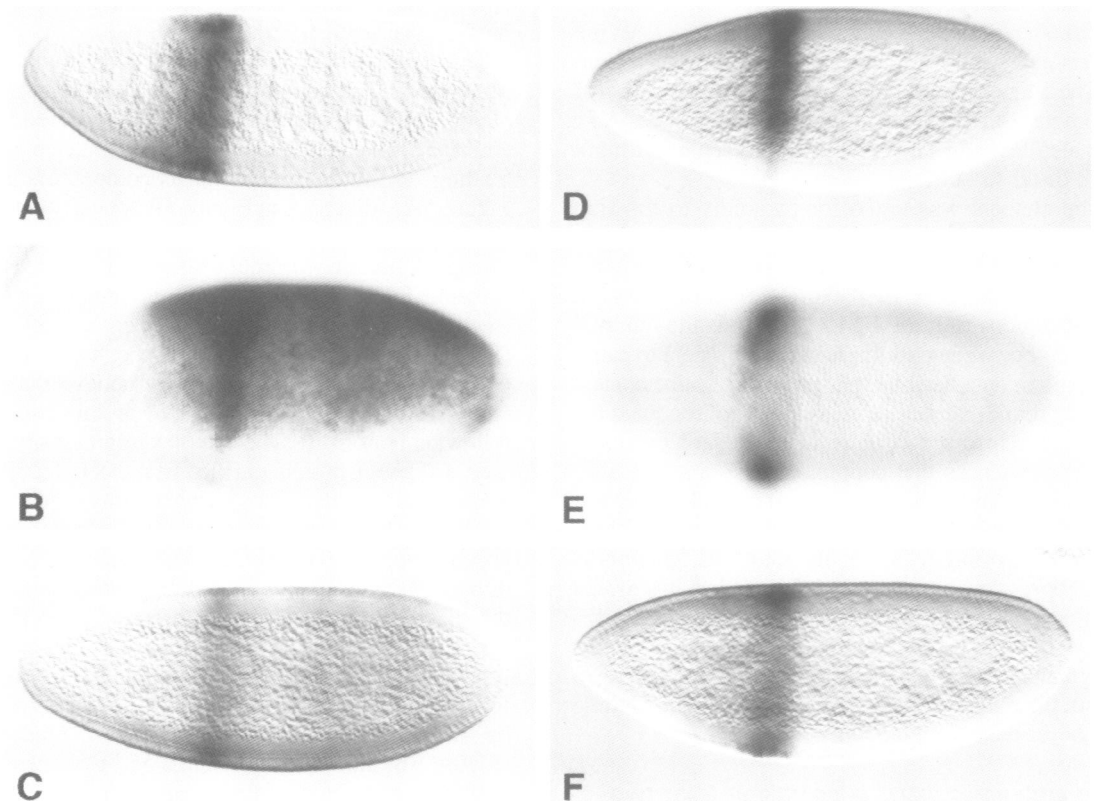

Fig. 4. The *vir* VRE and minimal *zen* VRE function as ventral repression elements. Whole mount preparations of P-transformed embryos were hybridized to digoxigenin-11-UTP *lacZ* antisense probes. All embryos are in mid-nuclear cycle 14 and oriented with anterior to the left and dorsal up, except for that in panel E which shows the ventral side. (**A**) Embryos containing the MSE−*eve*−*lacZ* fusion gene. The expression pattern has refined into a stripe with equal levels of staining in dorsal and ventral regions. (**B**) Embryo containing the *D.vir* VRE/MSE chimeric promoter. The *D.vir* VRE represses expression in ventral regions. Arrowheads mark the extent of stripe 2 expression which is obscured by the broad *zen*-like staining pattern due to activation sequences in the 980 bp *D.vir* promoter. Note that the extent of stripe 2 is similar to the extent of the dorsally localized *zen*-like staining. (**C**) Embryo derived from a dl^- female and a transformant male carrying the *D.vir* VRE/MSE construct. Stripe 2 expression is not repressed. Moreover the *zen* pattern is no longer restricted and *lacZ* transcripts are now found in ventral as well as dorsal regions. (**D** and **E**) Embryos containing the minimal 55 bp (×3) VRE/MSE chimeric promoter. The minimal VRE represses stripe 2 expression in ventral regions (arrowheads). (**F**) Embryo derived from a dl^- female and a transgenic male carrying the minimal VRE/MSE construct. Stripe 2 expression is restored to a full stripe.

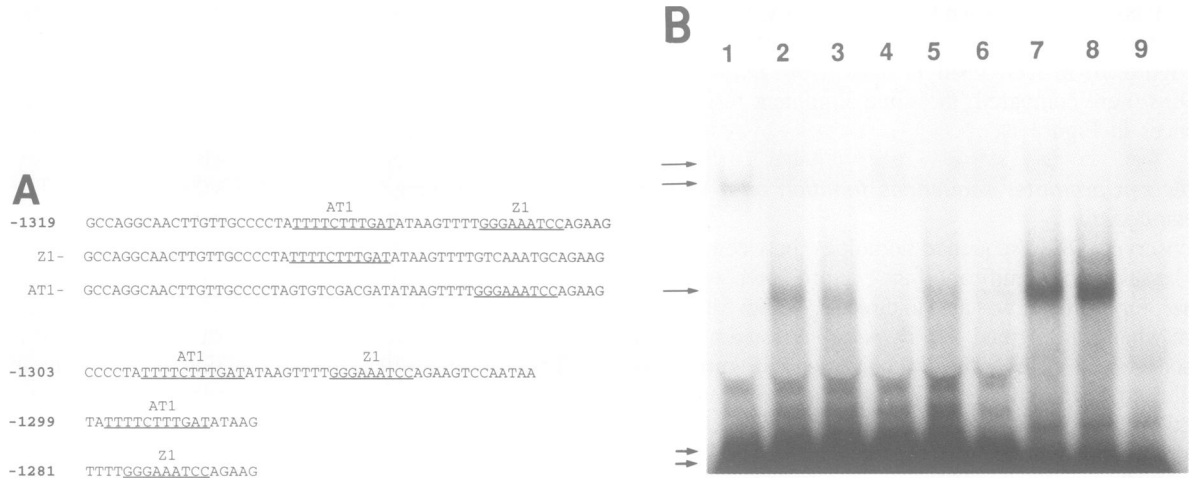

Fig. 5. At least two proteins, *dl* and an additional factor, bind to the minimal VRE. (**A**) DNA sequence of oligos containing Z1 and/or AT1 that were used in DNA binding assays. The position of the 5′-most nucleotide is to the left of each oligo sequence. Wildtype and base substituted mutant sequences of the 55 bp minimal VRE (Z1$^-$ and AT1$^-$) are listed for comparison. Shorter oligos used as probes (−1303) and unlabelled competitor DNAs (−1299 and −1281) are also listed. (**B**) Gel mobility shift assays were performed using *Drosophila* nuclear extracts from 0−4 hour old embryos (except for lane 1 which contains *dl* protein made in *E.coli*). The arrows point out the free probes (shorter arrows) and the DNA−protein complexes (longer arrows). The probe and specific unlabelled competitors which are all listed in (A), vary across the gel. Lanes 1 and 2: −1319, no competitor. Lane 3: −1319, Z1 competitor. Lane 4: −1319, AT1 competitor. Lane 5: −1319 Z1$^-$, no competitor. Lane 6: −1319 AT1$^-$, no competitor. Lane 7: −1303, no competitor. Lane 8: −1303, Z1 competitor. Lane 9: −1303, AT1 competitor.

promoter that could function as a VRE. We chose a 55 bp sequence from a region that is highly conserved between the three species and which contained a *dl* binding site and an AT site. The sequence extends from −1319 to −1264 of the *zen* promoter (see Figure 1, asterisks), and includes AT1 and Z1. It also includes another conserved region of eight nucleotides distal to AT1 (see Figure 1).

The 55 bp element was tested for its ability to function as a VRE in the stripe 2 repression assay. When multiple copies of the 55 bp element are placed upstream of the MSE (VRE/MSE, see Figure 3), *lacZ* expression is repressed in ventral-most regions (Figure 4D and E). Thus the minimal sequence of 55 bp, when multimerized, is sufficient to cause ventral repression on a heterologous promoter. In the context of a *dl*$^-$ background, ventral repression is abolished and the full stripe 2 is expressed (Figure 4F). We are presently testing DNA sequences that contain other Z and AT sites in the repression assay. Jiang *et al.* (1993) have also analyzed the Z and AT sites. They tested a shorter sequence (37 bp), which includes Z1 and AT1, for its ability to function as a minimal repression element, and found that it was not sufficient for ventral repression. We do not understand this discrepancy; it might be due to the difference in the lengths of the oligonucleotides and how the proteins are able to interact with them *in vivo*. The spacing between the binding sites may be critical for function.

Drosophila embryonic extracts contain at least two factors that bind to the minimal VRE

A simple model for the mechanism by which the *zen* VRE mediates ventral repression is that the VRE interacts with the *dl* protein and another factor, or co-repressor, both of which bind to VRE sequences. *dl* and the co-repressor are then able to interact and are both required to interfere with transcriptional activity. Since *dl* protein is present in ventral nuclei, repression is seen only in ventral regions. The co-repressor need not be specifically localized, and may be a general factor. The model predicts that at least two proteins bind to the minimal VRE, and that mutations in either binding site abolish repression.

To visualize factors that bind to the minimal VRE, we performed DNA gel mobility shift assays using ^{32}P-labelled oligonucleotides and nuclear extracts prepared from staged embryos. The sequences of the oligonucleotides used as binding substrates (probes) or as competitors are listed in Figure 5A. All assays in Figure 5B contain a mixture of two detergents, sodium deoxycholate and Nonidet P40. This disrupted protein−protein interactions to reveal only the protein−DNA interactions with the oligonucleotide probes (see Materials and methods). When the 55 bp element (−1319 to −1264) is used as probe, two band shifts are observed (Figure 5B, lane 2). We believe the upper band (slower complex) represents the *dl* protein complex for the following reasons. (i) The complex migrates similarly to that seen with the *dl* protein purified from bacterial cells (lane 1). It migrates a little more slowly but this is probably due to modifications of the *dl* protein in the embryo. The complex appears as a poorly resolved doublet, and the same is true of bacterially expressed *dl* (compare lanes 1 and 2). Perhaps the added detergents disrupt *dl* protein dimers leading to the formation of complexes containing single *dl* molecules which run more quickly in the gel shift assay. (ii) Complex formation is strongly prevented by competition with an 18 bp oligonucleotide that spans the *dl* binding site Z1 (Figure 5B, lane 3). (iii) When a 55 bp element containing point mutations in Z1 (see sequence in Figure 5A), is used as labelled substrate in this assay the slower complex is not observed (lane 5). Thus the slower complex in lane 2 contains *dl* protein. The faster complex (lower band shift) is of particular interest and could correspond to the binding of a putative co-repressor to the AT site. It appears as a broad band which is strongly reduced, but not abolished, by competition with an 18 bp oligonucleotide that spans AT1 but does not include Z1 (Figure 5B, lane 4). Moreover, point mutations that change five T residues severely reduced complex formation (lane 6).

We have also performed DNA binding assays with a 46 bp probe (−1303 to −1255). It contains AT1 and Z1, but not the 8 bp conserved sequence distal to AT1. The two probes behave similarly, but not identically. *dl* complexes are present (compare lanes 2 and 7), and are reduced by competition with specific unlabelled oligonucleotides that contain Z1 (lanes 3 and 8). The faster complex is also present in both cases. Its formation is mostly prevented, but as with the 55 bp oligonucleotide, not totally prevented with unlabelled AT1 oligonucleotides (lanes 4 and 9). The faster complex is broader and stronger with the 46 bp probe. The reason for the residual binding in the region of the faster complex after competition with AT1 oligonucleotide is not clear. It could be an artifact of the gel shift assay or most probably reflects the inability of the short AT1 oligonucleotide to compete efficiently with the 55 bp labelled probe. We repeated the binding experiments with different preparations of embryonic extracts and did not find any additional binding activity with the 55 bp oligonucleotide probe as compared with the 46 bp probe. Thus it appears that the conserved 8 bp sequence is not involved in the formation of any distinctive complex in our binding reactions.

The AT site is required for repression; dl becomes an activator in its absence

The minimal VRE interacts *in vitro* with *dl* protein and another undefined factor, the putative co-repressor. To show that these interactions are necessary for ventral repression, we tested the binding site mutants, described above for the DNA binding studies, in the stripe 2 repression assay. Schematic representations of the transformation constructs are shown in Figure 3. The *dl* binding site mutations have previously been tested in the context of the 600 bp VRE and were shown to abolish ventral repression (Jiang *et al.*, 1992). Here we test a Z1 mutant site in the context of the 55 bp minimal VRE. A transgenic embryo carrying mutant Z1 sites is shown in Figure 6A. Stripe 2-directed expression is no longer repressed in ventral regions because *dl* protein cannot interact with the mutant VRE. This 'derepression' of stripe 2 was also observed when mutations in the AT1 site were tested (Figure 6B and C). However, in addition to the derepression of stripe 2, many embryos exhibited *lacZ* transcripts along the ventral side (Figure 6B and C). Without the AT site (and therefore, co-repressor) interaction, *dl* protein can no longer repress transcription, and appears instead to function as an activator. To test this possibility, transgenic males from one line were mated with dl^- virgins. Figure 6D shows an embryo derived from this cross. Expression along the ventral midline is absent, indicating that *dl* is indeed responsible for the ventral activation seen in the AT^- mutant embryo. Thus *dl* activity is influenced by the AT site and presumably the factor that occupies that site.

Fig. 6. *dl* is a repressor in the presence of the AT site and an activator in its absence. Whole mount preparations of mid-nuclear cycle 14 embryos were hybridized with digoxigenin-11-UTP-labelled *lacZ* RNA probes. All embryos are oriented anterior to the left and dorsal up. (**A**) Embryo containing the VRE Z1-/MSE promoter. The VRE is no longer able to mediate ventral repression. (**B** and **C**) Embryos from two different transgenic lines (nos 1 and 4, respectively) containing the VRE AT1-/MSE promoter. The mutant VRE can no longer mediate ventral repression of stripe 2 expression, and in fact mediates ventral activation. The transgenic embryo in C showed stronger ventral activation (relative to the stripe) than any of the other five lines. This is not due to a fortuitous position effect because not only is there variation in strength of activation between lines, but also within each line there were some embryos (10−50%) that showed a full stripe 2 (derepressed) but little or no ventral activation. It is unclear why this variation occurs. (**D**) Embryo derived from a transgenic male from line no. 4 (VRE $AT1^-$/MSE) and a dl^- female. Neither ventral repression nor ventral activation is seen in the absence of *dl* protein.

Discussion

The *dl* protein is able either to activate or to repress target gene expression. *dl* repression is mediated by silencer sequences which function in both orientations and can act over considerable distances to suppress transcription from heterologous promoters. Thus, the mechanism of *dl* repression is different from that of short-range repressors such as *Kr*, which involves competition with activators for overlapping binding sites (Small *et al.*, 1991; for review on mechanisms of repression, see Jackson, 1991). We have begun to address the mechanism of *dl*-dependent repression. This report establishes three important points. First, *dl* repression is mediated by two distinct kinds of DNA binding site in the *zen* silencer: a *dl* binding site and a T-rich site,

both of which are required for repression. Second, a factor present in embryonic nuclear extracts binds to the AT site. This factor is a candidate for a co-repressor that could interact with *dl* protein (also present in extracts) to repress transcription. Third, in the absence of the AT site, and presumably co-repressor binding, *dl* no longer acts as a repressor but instead now functions as an activator.

Little is known about the putative co-repressor. We are presently purifying a DNA binding activity from nuclear extracts in order to identify and characterize the co-repressor. Our gel shift assay does not rule out the possibility that more than one protein binds to the AT sites. It is also unclear whether the same factor binds to the other AT sites. Oligonucleotides that contain AT0, AT2 or AT3 form complexes that migrate to similar positions but with weaker affinities compared with AT1 (data not shown). In addition, AT2 was a weak competitor for AT1 complex formation (data not shown). However, we have shown that AT1 and Z1, when multimerized, can mediate repression. Perhaps any pair of AT and *dl* binding sites can act as a minimal VRE.

The DNA sequence of the AT sites provides no clues to the nature of the co-repressor or mechanism of binding. Comparison of the AT sites reveals an alignment of several residues (Ts in Figure 2). However, it is not clear whether any consensus sequence that can be derived from the alignment is meaningful. Examination of a compilation of motifs for known DNA binding proteins (Faisst and Meyer, 1992) failed to identify a motif corresponding to the AT sites. The binding sites that most closely resembled AT1 are simply pyrimidine rich. Thus, it may be significant that the sequence of one strand is pyrimidine rich. Further point mutation analysis of the AT sites should determine which residues are important for DNA binding.

There are examples of long range repression from a variety of systems (reviewed in Jackson, 1991). In *Drosophila* one other well defined silencer is located in the *Ultrabithorax* (*Ubx*) gene, and is involved in the negative spatial regulation of *Ubx*. It mediates long-range repression by interacting with the DNA binding domain of the *hb* protein in anterior regions of the embryo (Zhang and Bienz, 1992). Most of what is known about transcriptional silencing comes from studies of the yeast mating type loci. The speculated *dl*−co-repressor interaction is reminiscent of that between the yeast mating type factors α2 and MCM1 (Keheler *et al.*, 1988). α2 is an α-cell specific homeodomain protein which represses transcription of **a**-specific genes. MCM1 is a non-cell-specific DNA binding protein that binds cooperatively with α2 and is also required for repression. As in the case of the *dl*−co-repressor complex, it is not yet known why both proteins are required for repression. Keheler *et al.* (1988) proposed that the α2−MCM1 complex contacts a component(s) of the transcription machinery and locks it in place to prevent transcription initiation or elongation.

Other proposed mechanisms for silencing invoke alterations in chromatin structure (reviewed in Jackson, 1991). These alterations are mediated by general (or global) factors and result in negative effects on the transcription of certain genes. For example, in the yeast *Saccharomyces cerevisiae*, negative regulators of the HO mating type switching endonuclease gene were identified genetically and include SIN1, [a high mobility group (HMG)-like protein], SIN2, which encodes histone H3, and SIN4 whose amino acid sequence provides little information about its function (Wang and Stillman, 1990; Kruger and Herskowitz, 1991; Jiang and Stillman, 1992). A *sin4* mutation causes a decrease in nucleosome density which in turn changes chromatin structure, and is accompanied by alterations in transcriptional regulation (Jiang and Stillman, 1992).

Nuclear matrix proteins are another type of global protein that are thought to interact with silencers. It has been proposed that attachment of silencer DNA to the nuclear scaffold forms a domain within which changes in chromatin structure occur, thereby rendering a locus transcriptionally inactive (Hofmann *et al.*, 1989). In the yeast mating type HML locus, the silencer E site binds RAP-1, a protein which appears to mediate DNA loop formation and attachment to the nuclear matrix in reconstitution experiments (Hofmann *et al.*, 1989). The HML loop correlates well with transcriptionally inactive DNA.

General factors like those mentioned above cannot account for the temporal and spatial specificity of silencer action. Tissue specific transcription factors such as the *dl* and α2 repressors direct the specificity. Thus any mechanism that employs general factors must also involve interactions between the general and specific transcriptional regulators. Such an interaction has been proposed for α2 and SIN4. In a *sin4*$^-$ strain, repression of an α2 target promoter fused to a *lacZ* reporter gene was relieved by 70% (Chen *et al.*, 1993). Thus, the gene-specific repression by α2 seems to be mediated by the general factor SIN4 which influences chromatin structure.

Proposed mechanisms for *dl*-mediated regulation must account for how *dl* acts as an activator versus a repressor. One model proposes that the *dl*−co-repressor complex interacts with general factors involved in chromatin structure. The *dl*−co-repressor complex (but not *dl* alone) could act as a specific platform for changes in nucleosome positioning to prevent transcriptional activity. The co-repressor itself could be a general factor. On the other hand regulation by *dl* could be more direct. *dl* might interact directly with the basal transcription machinery to activate transcription (of genes such as *twi* and *sna*). In the presence of the co-repressor, the interaction of *dl* with the transcription machinery might change such that certain components of the machinery are excluded or locked in place, preventing transcription initiation and/or elongation. DNA binding of *dl* and the co-repressor to nearby sites would ensure the required protein−protein interaction between them. Our results from the repression assays indicate that neither *dl* nor the co-repressor can mediate repression on their own. In the absence of the co-repressor, *dl* changes its mode of action to activate transcription from the *zen* silencer. Further biochemical studies will reveal whether the co-repressor is a protein specific to *dl*-mediated repression or a general factor involved in the transcriptional regulation of many genes.

Materials and methods

DNA sequencing

Recombinant phage clones of the *zen* region from the Antennapedia complex of *D.vir* and *D.pso* were kindly provided by M.Seeger and T.Kaufman. *Eco*RI fragments from the phage DNAs were subcloned into BlueScript SK− vectors (Stratagene, La Jolla, CA) and sequenced by standard dideoxy sequencing methods using Sequenase kits (US Biochemicals, Cleveland, OH). The DNA sequences were aligned in the Gene Works Program (IntelliGenetics, Inc., Mountain View, CA).

Electrophoretic mobility shift assay

Oligonucleotides used as probes and nonlabelled competitors are shown in Figure 5A. The probes were end-labelled with [γ-^{32}P]ATP (ICN, 7000 Ci/mmol) to a specific activity of 10^7 c.p.m./μg. Nuclear extracts were prepared from 0–4 hour old embryos as described by Biggin and Tjian (1988) except that the nuclei were extracted with 0.35 M NaCl and the extracts were clarified by centrifugation at 30 000 r.p.m. for 1 h in a Beckman SW41 rotor. 10 μg of embryo nuclear extract were preincubated for 10 min at 20°C in 20 μl of 10 mM HEPES pH 7.9, 50 mM NaCl, 1 mg/ml BSA, 3 mM $MgCl_2$, 10 mM EDTA, 6 mM 2-mercaptoethanol, 10% glycerol, 2 μg dI-dC in the presence of 50 ng of Z1 oligonucleotide, 350 ng of AT1 oligonucleotide or without competitor as specified in the legend to Figure 5. 700 pg of labelled probe were then added and incubation was continued for 10 min at 20°C. Sodium deoxycholate (DOC) and Nonidet P40 were added to final concentrations 0.4 and 0.8% respectively, and after 5 min the mixtures were loaded on to a 4% acrylamide gel in 25 mM Tris base, 190 mM glycine. The electrophoresis was run for 50 min at 4°C at 15 V/cm and the gel was dried and autoradiographed. It is known that DOC disrupts protein–protein interactions, but at low concentration does not interfere with protein binding to DNA (Baeuerle and Baltimore, 1988). Thus the band shift assay in the presence of detergents shows only DNA–protein complexes free of intermolecular protein–protein interactions. This reduces the complexity of the binding pattern obtained with the crude nuclear extracts, especially with long oligonucleotide probes. *dl* protein used in DNA-binding assay was expressed and purified from *Escherichia coli* as described by Ip *et al.* (1991).

Preparation of P-transposons

The MSE P-transposon contains the 480 bp stripe 2 element from the *eve* promoter attached to a basal *eve–lacZ* fusion gene (Small *et al.*, 1991). The MSE–*lacZ* fusion gene was inserted into the CaSpeR injection vector which contains the *white* gene as a selectable marker (Thummel *et al.*, 1988). The chimeric promoters were obtained by inserting various multimerized oligonucleotides into the *Eco*RI site at the 5′ end of the MSE (Jiang *et al.*, 1992), and are summarized in Figure 3. The DNA sequences of the 55 bp oligonucleotides (oligos) used in this study are listed in Figure 5A. The oligos contain an additional four base pairs, AATT (*Eco*RI ends), at their 5′ ends. Oligos were purified by gel electrophoresis and handled using standard methods (Sambrook *et al.*, 1989). Annealed oligos were kinased and ligated, and multimers isolated by gel electrophoresis. After re-kinasing, they were inserted by ligation into the *Eco*RI site of MSE P-transposon vector. All inserts of recombinant clones were sequenced by standard dideoxy sequencing methods.

P-transformation and in situ hybridization

P-transposons were introduced into the *Drosophila* germ-line by injecting *white*$^-$ [Df(1) w^{67c23}] embryos using standard methods (Ashburner, 1989). At least three lines were studied for each construct. In the case of the VRE AT$^-$/MSE construct, seven lines were examined due to the variation observed within and between the lines (see text). *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 using an antisense *lacZ* RNA probe (Boehringer Mannheim kits, Indianapolis, IN). Photography was done using Nomarski optics on a Nikon FXA microscope. Composite figures were prepared using the computer program Adobe Photoshop (Macintosh), scanned on a Kodak (Rochester, NY) scanner RFS2035 and printed from the Kodak printer XL7700.

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