Individual *dorsal* morphogen binding sites mediate activation and repression in the *Drosophila* embryo

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The *dorsal* (*dl*) morphogen gradient is responsible for initiating the differentiation of the mesoderm, neuroectoderm and dorsal ectoderm in the *Drosophila* embryo. *dl* encodes a sequence-specific DNA binding protein that belongs to the Rel family of transcription factors. Previous studies have shown that *dl* activates the mesoderm determinant *twist* (*twi*); here we use a combination of site-directed mutagenesis and P-transformation assays to demonstrate that it also functions as a direct transcriptional repressor of a second target gene, *zerknüllt* (*zen*). By exchanging *dl* binding sites between the promoters we show that activator sites from *twi* can mediate repression when placed in the context of the *zen* promoter, and that repressor sites from *zen* can mediate activation in the context of the *twi* promoter. This represents the first demonstration that common binding sites for any DNA binding protein can mediate both activation and repression in a developing embryo. Evidence is also presented that the affinities of *dl* binding sites are important for the efficiency of repression, but are not the sole determinants of the threshold response to the *dl* gradient.

Key words: *dorsal*/*Drosophila*/transcription factors/*twist*/*zerknüllt*

Introduction

The *dorsal* (*dl*) morphogen is expressed in a broad gradient in precellular embryos, with peak levels in ventral regions and progressively lower levels in lateral and dorsal regions (reviewed by Govind and Steward, 1991; Ip and Levine, 1992; St Johnston and Nüsslein-Volhard, 1992). This gradient is responsible for initiating three basic tissues, the mesoderm, neuroectoderm and dorsal ectoderm. Peak levels of *dl* activate the expression of two regulatory genes, *twist* (*twi*) and *snail* (*sna*), which initiate the differentiation of the mesoderm (Jiang *et al.*, 1991a; Pan *et al.*, 1991; Thisse *et al.*, 1991). Low levels of *dl* (in lateral regions) coincide with the presumptive neuroectoderm and there is growing evidence that these low levels directly trigger the expression of regulatory genes, such as *rhomboid*, which are required for the differentiation of the tissues that arise from the neuroectoderm (Ip, Y.T., Park, R., Kosman, D., Bier, E. and Levine, M., submitted). *dl* also functions as a repressor and

restricts the activities of certain regulatory genes (i.e. *zen* and *dpp*) to dorsal regions where they are important for the differentiation of the amnioserosa and dorsal epidermis (Rushlow *et al.*, 1987; St Johnston and Gelbart, 1987; Ip *et al.*, 1991).

dl encodes a sequence-specific DNA binding protein that belongs to the Rel family of transcription factors, including the vertebrate oncogene *rel* and the mammalian regulatory factor NF- κ B (Steward, 1987; Ghosh *et al.*, 1990; Kieran *et al.*, 1990; Ip *et al.*, 1991). Evidence that *dl* regulates transcription stems from the identification of multiple *dl* binding sites in the promoter regions of the target genes *twi* and *zen* (Pan *et al.*, 1991; Thisse *et al.*, 1991; Jiang *et al.*, 1991a). Mutations in the sites present in the *twi* promoter severely reduce ventral activation, suggesting that *dl* functions as a transcriptional activator *in vivo* (Jiang *et al.*, 1991a).

dl represses *zen* through a distal region of the *zen* promoter, the VR, which is located between –1.6 and –1 kb upstream of the transcription start site (Doyle *et al.*, 1989). The 600 bp VR element contains four *dl* binding sites (Ip *et al.*, 1991) and, when it is placed upstream of various heterologous promoters, including *hunchback* and *Krüppel*, it can act over considerable distances to block their ventral expression (Doyle *et al.*, 1989; Ip *et al.*, 1991). Using site-directed mutagenesis combined with P-transformation assays, we show that the *dl* binding sites in the *zen* VR directly mediate ventral repression *in vivo*, strengthening the view that *dl* can function both as a transcriptional activator and a repressor.

To determine whether *dl* activation versus repression involves functionally distinct binding sites, we have exchanged sites between the *zen* and *twi* promoters. We show that an ‘activator’ site in the *twi* promoter is able to mediate repression when placed in the context of the *zen* promoter, while a ‘repressor’ site from *zen* activates transcription when placed in the *twi* promoter. The repressor site also mediates activation of a minimal hsp70 promoter, suggesting that *dl* is intrinsically an activator and that repression requires additional factor(s) present in the *zen* VR. We also present evidence that the affinities of *dl* binding sites are important, but not sufficient, for determining the threshold response to the *dl* concentration gradient.

Results

dl repression can override activation

It has been proposed that *dl* represses transcription by interfering with the basal transcriptional machinery (Doyle *et al.*, 1989; Ip *et al.*, 1991). A test of this model is to determine whether *dl* repression can block activation from a set of unrelated heterologous promoters. To provide further evidence that *dl* repression can override diverse activators, we took advantage of two well defined promoter elements, one for the *eve* promoter that directs the expression of stripe

2 (Small *et al.*, 1991; Stanojevic *et al.*, 1991; S.Small *et al.*, submitted), and a *twi* proximal element (PE) that directs strong expression in ventral regions (Jiang *et al.*, 1991a; Pan *et al.*, 1991; Thisse *et al.*, 1991). The stripe 2 element is activated by *bicoid*, while the *twi* PE is activated by *dorsal*. The 480 bp stripe 2 element (the minimal stripe element or 'MSE') is located between -1550 and -1070 bp upstream from the *eve* transcription start site (see Figure 1). It initially directs a broad pattern of expression (at nuclear cycle 12–13) in the anterior half of the embryo, with equally intense staining along the dorsal–ventral axis (Figure 2A). During a period of ~20–30 min this pattern is refined into a stripe that coincides with the endogenous *eve* stripe 2 (Figure 2B; S.Small *et al.*, submitted). However, when the

600 bp *zen* VR is placed upstream of the MSE (VR/MSE, see Figure 1), the resulting chimeric promoter is expressed in dorsal but not ventral regions (Figure 2, compare panels C and D with panels A and B). The broad expansion of the stripe 2 pattern along the dorsal surface is due to general activation sequences present in the *zen* VR (Doyle *et al.*, 1989). Evidence that ventral repression is mediated by *dl* was obtained by expressing this fusion in *dl*⁻ embryos. Such mutants exhibit a uniform staining pattern similar to that observed for the MSE without VR sequences (data not shown). Furthermore, mutations in the *dl* binding sites abolish the ventral repression of the stripe 2 pattern (see below).

The *twi* PE is located between -440 and -180 bp

Fig. 1. Summary of promoter fusions. The uppermost horizontal bar represents the 480 bp minimal *eve* stripe 2 element. It was placed upstream of an *eve* basal promoter–*lacZ* fusion gene that contains the first 44 bp of the *eve* promoter. The unfilled horizontal bar represents the *lacZ* coding sequence and the arrow corresponds to the transcription start site. The VR/MSE, mVR/MSE and tVR/MSE (stippled bars) correspond to different forms of the 600 bp *zen* ventral repression element (VR). All three were placed upstream of the MSE–*lacZ* fusion gene. The black dots below the VR element indicate the locations of the *dl* binding sites. The mVR is identical to the VR except that base substitutions (represented by X) were created which abolish the binding activities of all four *dl* sites. The tVR was prepared by converting all four *dl* binding sites (unfilled dots) into the lower affinity TD5 site (from the *twi* promoter). The 2×PE construct contains two copies of the *twi* PE element (striped horizontal bars) attached to a *twi*–*lacZ* fusion gene. The VR/2PE is the same except that a copy of the wild-type *zen* VR was placed upstream of the *twi* sequences. The 1×PE construct contains the first 440 bp of the *twi* promoter fused to the *lacZ* coding sequence. The unfilled dots below the PE element correspond to the TD4 and TD5 *dl* binding sites. The 1×PEm was prepared by disrupting both sites (X). The 3dl/*twi* construct contains three tandem copies of the high affinity oligo B *dl* binding site from the *zen* VR placed upstream of a truncated *twi*–*lacZ* fusion gene, which includes the first 290 bp of the *twi* promoter. The 3dl/hsp70 construct contains three copies of the oligo B sequence placed upstream of a minimal hsp70–*lacZ* fusion gene.

upstream from the transcription start site and has been shown to mediate activation by *dl* (Jiang *et al.*, 1991a; Pan *et al.*, 1991; Thisse *et al.*, 1991). Strong ventral expression is obtained with a *twi-lacZ* fusion gene containing two tandem copies of the PE (2×PE; see Figure 1). However, it is silenced when the 600 bp *zen* VR is placed upstream (compare Figure 2 panel F with E). The dorsal on/ventral off pattern that is observed with this VR-*twi* chimeric promoter (VR/2PE; Figure 1) is due to general activator sequences present in the VR, which also contributes to the polar expression observed with the VR/MSE promoter. Repression of the *twi* PE involves a distance of at least 300 bp, which corresponds to the minimal spacing between the proximal-most *dl* site in the VR and the distal-most *dl* site in the PE. The minimal spacing between a *dl* repressor site and a *bicoid* activator site in the VR/MSE promoter is 200 bp.

The *dl* binding sites in the *zen* VR mediate ventral repression

To determine whether the *dl* binding sites present in the *zen* VR directly mediate repression, base substitutions were

introduced in the core recognition sequences of all four *dl* binding sites (Figure 1; Materials and methods). These point mutations abolished binding, as judged by mobility shift assays (data not shown). This mutagenized VR (mVR, Figure 1) failed to block the ventral expression directed by the MSE (Figure 3A and B; compare with Figure 2C and D). This result provides strong evidence that the *dl* binding sites in the VR are responsible for repression, and indicates that *dl* functions as both a transcriptional repressor and activator in early embryos.

An activator site from the *twi* promoter mediates repression when placed in the *zen* VR

To determine whether activator sites from the *twi* promoter mediate activation or repression when placed in the *zen* VR, we prepared the tVR/MSE chimeric promoter summarized in Figure 1. It is identical to the VR/MSE promoter, except that all four *dl* sites were converted into the lower affinity TD5 binding site present in the *twi* PE promoter sequence (see Jiang *et al.*, 1991a; Materials and methods). Previous studies have shown that when TD5 and the neighboring TD4 binding sites are disrupted there is a substantial reduction

Fig. 2. *dl* repression overrides activation mediated by the *eve* MSE and *twi* PE. P-transformed embryos are oriented with anterior to the left and dorsal up. The staining patterns were detected in whole mount preparations using a digoxigenin-11-UTP labeled *lacZ* antisense RNA probe. (A) Early and (B) mid-nuclear cycle 14 embryos containing the MSE *eve-lacZ* fusion gene. Early expression includes a broad band in the anterior half of the embryo (A), but during a 20–30 min period this pattern is refined into a stripe (B). (C) Early and (D) late cycle 14 embryos containing the VR/MSE chimeric promoter, which is identical to those shown in (A) and (B), except that the 600 bp *zen* VR was placed upstream of the stripe 2 element. The VR represses staining in ventral regions. (E) Early cycle 14 embryo containing the VR/2PE chimeric promoter, which contains the *zen* VR placed upstream of two tandem copies of the *twi* PE element. Staining is restricted to dorsal and polar regions, with little or no expression in ventral regions. The expression that is observed is due to general activator sequences present in the *zen* VR (Doyle *et al.*, 1989). (F) Mid-cycle 14 embryo containing the 2×PE gene fusion, which is identical to that shown in (E) except that it lacks the *zen* VR element.

Fig. 3. *dl* binding sites in the *zen* VR mediate repression. P-transformed embryos are oriented with anterior to the left and dorsal up. (A) Early cycle 14 and (B) cellularized embryo containing the mVR/MSE chimeric promoter. This is identical to the VR/MSE shown in Figure 2C and D, except that the four *dl* binding sites were inactivated by site-directed mutagenesis. This results in uniform staining along the dorsal–ventral axis; expression is no longer excluded from ventral regions. (C) Early and (D) mid-cycle 14 embryos containing the tVR/MSE chimeric promoter. This is identical to the VR/MSE except that all four *dl* binding sites were substituted with the lower affinity TD5 site from the *twi* promoter. The lateral limits of expression are comparable to that observed for the VR/MSE promoter, although weak staining is observed in ventral regions of older embryos (D).

in *twi* expression. The tVR/MSE shows a dorsal on/ventral off pattern of expression comparable to that observed for the normal VR/MSE fusion promoter (Figure 3C and D), indicating that the TD5 site can mediate transcriptional repression in the context of the *zen* VR. However, repression is not quite as efficient since older embryos show weak staining in ventral regions (Figure 3D). In contrast, the normal VR mediates complete ventral repression.

The affinity of the *dl* binding sites determines the efficiency of repression

Previous DNA binding assays have shown that the TD5 *dl* binding site in the *twi* promoter is considerably weaker than the four *dl* sites present in the *zen* VR (Jiang *et al.*, 1991a). The results presented above suggest that the TD5 site might not mediate repression as efficiently as the native *dl* binding sites normally present in the *zen* VR. We have further investigated this issue by examining the expression of the various VR/MSE hybrid promoters in embryos containing low, uniform levels of the *dl* morphogen. *Toll* ‘lateralizing’ mutations cause partial constitutive activation of the *Toll* receptor (Schneider *et al.*, 1991). As a result, low levels of total cytoplasmic *dl* (~5–10% of the total) enter nuclei in both dorsal and ventral regions. The low levels of nuclear *dl* protein present in *Toll* lateralizing mutants are not sufficient to activate *twist*, but are able to repress *zen*. This observation was taken as evidence that the *twist* and *zen* promoters respond to distinct thresholds of the morphogen (Roth *et al.*, 1989). Perhaps this differential response results from different affinities of the *dl* binding sites, with *zen* being more sensitive to low levels of the morphogen since it contains higher affinity sites.

When the native VR/MSE *zen*–*eve* chimeric promoter is crossed into a *Toll* lateralizing mutant, there is a marked

reduction in the overall levels of expression in early embryos. In addition, stripe 2 fails to form in older embryos (Figure 4A and B). The staining seen in the posterior pole is due to activation sequences located in the *zen* VR, and involves the masking of the *dl* repressor by the *torso* terminal system (Rushlow *et al.*, 1987; J.Jiang, unpublished results). The tVR/MSE chimeric promoter, containing the lower affinity TD5 site from the *twi* promoter, drives weak, but detectable expression from the MSE when crossed into the *Toll* lateralizing mutant (Figure 4C and D). By cellularization, a fully refined stripe 2 is observed, and staining is also detected at the posterior pole (Figure 4D). As a control experiment, the mutagenized VR/MSE fusion promoter (mVR/MSE), lacking all four *dl* sites, was expressed in the lateralizing mutant (Figure 4E and F). As expected, the chimeric promoter is fully derepressed, and directs high levels of expression that are comparable to those observed in wild-type embryos (compare with Figure 3B). These results suggest that the affinities of the *dl* binding sites are not the sole determinant of the threshold response to the *dl* gradient. Perhaps interactions with neighboring factors in the *zen* VR permit the efficient filling of even low affinity *dl* binding sites (see Discussion).

A repressor site from the *zen* VR can mediate activation

To determine whether high affinity repressor sites from the *zen* VR can activate transcription, we attached multiple copies of one of the sites (oligo B; see Materials and methods) to a truncated *twi* promoter lacking its own *dl* sites. As shown previously, the first 440 bp of *twi* 5' flanking sequences is sufficient to drive strong expression of the *lacZ* reporter gene in ventral regions of early embryos (Jiang *et al.*, 1991a; Pan *et al.*, 1991; Thisse *et al.*, 1991; see

Fig. 4. *dl* repression in lateralized embryos. Male transformants containing the VR/MSE, tVR/MSE and mVR/MSE chimeric promoters were mated with mutant females that produce lateralized embryos (*Toll^{rm9}/Toll^{rm10}*). Embryos are oriented with anterior to the left. (A) Mid-cycle 14 and (B) cellularizing lateralized embryo containing the VR/MSE promoter. There is complete repression, except at the posterior pole. The faint stripe of staining seen in older embryos (B, arrowhead) is anterior to *eve* stripe 2 and is due to vector sequences present in the P-transposon (Jiang *et al.*, 1991a). (C) Mid-cycle 14 and (D) cellularized embryo containing the tVR/MSE promoter. The substitution of the *dl* binding sites in the VR with the lower affinity TD5 site results in less efficient repression of stripe 2. A weak stripe 2 is detected in both embryos, in addition to the intense staining at the posterior pole. The arrowhead in (D) indicates the weak head stripe due to the vector. (E) Mid-cycle 14 and (F) cellularized embryo containing the mVR/MSE chimeric promoter. The inactivation of the *dl* binding sites in the *zen* VR allows the *eve* stripe 2 element to direct intense expression. The levels of staining are comparable to that observed for the mVR/MSE fusion gene in wild-type embryos (see Figure 3A and B).

Figure 5A). This 440 bp region contains a cluster of *dl* binding sites, located between -330 and -286 bp. Mutations in two of the sites (called TD4 and TD5) cause a marked reduction in expression, suggesting that these sites are important for activation (Figure 5B). A truncated *twi* promoter containing the first 290 bp of 5' flanking sequence is essentially inactive (data not shown). However, when three tandem copies of the *zen* oligo B site are placed upstream to create the 3dl/*twi* fusion (see Figure 1), strong ventral expression is restored (Figure 5C). The limits of expression are quite broad, and encompass the entire presumptive mesoderm (~18 cells in width; see Figure 5E). This is a broader pattern than that obtained with the native TD4 and TD5 sites, which suggests that the dorsal-ventral limits of expression depend on the affinity of the *dl* binding sites (see Discussion). The oligo B sites also mediate activation when placed upstream of the minimal *hsp70* promoter (Figure 5D and F), although staining is not nearly as intense as that obtained when the sites are placed upstream of the *twi* promoter sequences.

Discussion

Previous studies have established that *dl* binding sites present in the *twi* promoter function as activator sites (Jiang *et al.*, 1991a; Pan *et al.*, 1991; Thisse *et al.*, 1991), and here we have demonstrated that *dl* binding sites present in the *zen* VR mediate repression. We have also shown that *dl* activator sites can repress transcription, while repressor sites can activate, depending on promoter context. This represents the first demonstration that common binding sites for any DNA binding protein can mediate both transcriptional activation and repression in a developing embryo. Repression might involve one or more short range mechanisms, such as competitive binding to DNA (reviewed by Levine and Manley, 1989), since the *zen* VR element appears to contain general activator sites (Doyle *et al.*, 1989). The *zen* VR-*eve* stripe 2 fusion gene (VR/MSE) is expressed at overall higher levels than the *eve* stripe 2 element alone, and the binding of *dl* may interfere with general activators that enhance expression in dorsal regions (see Figure 2C). However, short

Fig. 5. *dl* repressor sites can mediate activation. Embryos are oriented with anterior to the left. (A) Late cycle 14 embryo that contains the 1×PE *twi-lacZ* fusion gene. This truncated *twi* promoter sequence directs strong expression in the ventral-most 12–14 cells. The arrowhead indicates the head stripe due to vector sequences. (B) Late cycle 14 embryo that contains the same fusion gene as in (A) except that the TD4 and TD5 *dl* binding sites were inactivated by site-directed mutagenesis. There is a substantial reduction in the levels of expression, indicating that the TD4 and TD5 are *bona fide* activator sites *in vivo*. (C) Lateral and (E) ventral–lateral views of embryos containing the 3dl/*twi* chimeric promoters, which contain three tandem copies of the high affinity oligo B binding site from the *zen* VR placed upstream of a truncated *twi-lacZ* fusion gene. Intense staining is observed in the ventral-most 18–20 cells. This pattern is broader than that obtained with the PE fusion gene containing the native TD4 and TD5 binding sites. (D) Lateral and (F) ventral–lateral views of embryos containing the 3dl/*hsp70* promoter, which contains three copies of the oligo B site placed upstream of a minimal *hsp70* promoter. Weak, non-uniform staining is observed in the ventral-most 18–20 cells.

range repression is probably not sufficient to account for the ability of the VR to silence the ventral expression of various heterologous promoters over distances of 1–2 kb. Thus, the interaction of *dl* with factors bound to neighbouring sites within the VR might convert the weak *dl* activator into a potent repressor that may directly block the basal transcription machinery (see below). The identities of such neighboring ‘corepressors’ are currently unknown, and must await a detailed biochemical dissection of the VR element.

In contrast, the identities of some of the factors that help *dl* function as an activator are known. The demonstration that *dl* binding sites from the *zen* VR can mediate activation when placed upstream of the minimal *hsp70* promoter suggests that *dl* is inherently a weak transcriptional activator (see Figure 5). Interactions between *dl* and other factors bound to the *twi* promoter might enhance activation. For example, a general transcriptional activator called *zeste* (Benson and Pirrotta, 1988; Biggin *et al.*, 1988; Wu and Goldberg, 1989) has been shown to bind to proximal regions of the *twi* promoter, and augments activation by *dl* (Pan *et al.*, 1991). In addition, genetic studies suggest that *dl* can

interact with the helix–loop–helix protein encoded by *twi* to direct optimal expression of target genes such as *snail* (Kosman *et al.*, 1991).

Studies on the interaction between the gradient morphogen *bicoid* (*bcd*) and the gap gene *hunchback* led to the suggestion that the limits of target gene expression depend on the affinities of the *bcd* binding sites (Driever *et al.*, 1989; Struhl *et al.*, 1989). According to this view, promoters containing high affinity sites can be activated in posterior regions where there are low levels of *bcd*, while those containing low affinity sites are restricted to anterior regions where there are high levels. The results obtained by replacing the low affinity TD4 and TD5 *dl* binding sites in the *twi* PE with the high affinity oligo B site from the *zen* VR (see Figure 5E) lend some support to this model. The normal PE element directs expression in the ventral-most 12–14 cells containing peak levels of the *dl* morphogen. Perhaps expression is restricted to this region by the low affinity TD4 and TD5 sites, which can be filled only where there are high concentrations of *dl*. In contrast, the chimeric PE element containing three copies of the oligo B sequence is expressed

in a broader domain that includes 18–20 cells. Expression extends into ventral-lateral regions containing moderate levels of the morphogen, which may be sufficient to fill the high affinity oligo B sites.

However, several observations suggest that the affinities of binding sites are not the sole determinant of the differential regulation of the *twi* and *zen* target promoters by the *dl* gradient. *twi* is activated only in the 18–20 ventral-most cells where there are peak levels of *dl* (Thisse *et al.*, 1988). In contrast, *zen* is repressed in the entire ventral half of the embryo, spanning a total of ~36 cells (Doyle *et al.*, 1986). Considered in cross-section, there are at least six to eight cells in lateral regions on both sides of the ventral midline where *dl* is able to repress *zen* but fails to activate *twi*. Nonetheless, the low affinity TD5 site can repress the transcription of the *eve* stripe 2 element, and direct lateral limits comparable to those obtained with the native high affinity sites in the *zen* VR. Thus, when placed in the context of the *zen* VR, the TD5 site can repress transcription in lateral cells containing low levels of *dl* that are not sufficient for activation. Conversely, the high affinity oligo B binding site fails to activate a truncated *twi* promoter in lateral cells where it is able to mediate repression when in the context of the *zen* VR.

We propose that the binding of *dl* to DNA is facilitated by protein–protein interactions with neighboring co-repressors in the *zen* VR. These interactions may permit the efficient filling of the binding sites even in lateral regions containing low levels of *dl*. Perhaps interactions between *dl* and coactivators bound to the *twi* promoter do not mediate cooperative binding to DNA. In this case, occupancy may depend solely on the inherent affinities of the sites. *In vivo* footprint assays using chimeric *zen* VR and *twi* PE sequences should provide a critical test of this proposal.

Materials and methods

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. The MSE is located between –1.55 and –1.07 kb upstream of the *eve* transcription initiation site (Small *et al.*, 1991; Stanojevic *et al.*, 1991; S.Small *et al.*, submitted). The basal *eve*–*lacZ* fusion gene contains the proximal-most 44 bp from the *eve* promoter, the entire *eve* leader sequence and the first 22 codons fused in-frame with the *lacZ* coding sequence. The MSE–*lacZ* fusion gene was inserted into the CaspeR injection vector (Thummel *et al.*, 1988), which contains the *white* gene as a selection marker. The orientation of the *lacZ* fusion gene is opposite to that of *white*. The VR/MSE chimeric promoter was prepared by inserting a 600 bp *Xba*I–*Hind*III fragment containing the *zen* repression element (VR) at a unique *Eco*RI site upstream of the MSE–*lacZ* fusion gene. The VR maps between –1.6 and –1 kb upstream of the *zen* transcription start site. The orientation of the VR in the VR/MSE construct is the same as in the native *zen* promoter. The mVR/MSE and tVR/MSE constructs were made in the same way as the VR/MSE, except that mutagenized VR sequences were used in place of the wild-type VR. The mVR/MSE construct was made by inactivating all four *dl* binding sites via site-direct mutagenesis. The tVR/MSE construct involved replacing the four binding sites with the low affinity TD5 *dl* binding site from the *twi* promoter (see below). The 1×PE P-transposon has been described previously (Jiang *et al.*, 1991a). The 2×PE fusion gene was prepared by attaching a copy of the 260 bp *twi* PE (located between –440 and –180 bp in the *twi* promoter) upstream of a *twi*–*lacZ* fusion gene containing *twi* promoter sequences from –440 to +160 bp fused in-frame to the *lacZ* coding sequence. The VR/2×PE construct was made by inserting the 600 bp *zen* VR into an *Eco*RI site located just upstream of the 2×PE fusion gene. The 3dl/hsp70 construct contains three copies of the oligo B *dl* binding site from the *zen* VR fused to the hsp70–*lacZ* fusion gene in the PWHL vector. Two complementary oligonucleotides, each containing 24 nucleotides that span the oligo B binding site (AATTGATTGGGTTTCTCCCATG and

AATTACTGGGAGAAACCAATCA), were annealed and ligated at high concentrations into the *Eco*RI site of the pGem+7 vector (Promega) in order to obtain multiple copy insertions. pG3dl, a pGem+7 plasmid containing three tandem copies of the oligo B sequence, was obtained. An *Xho*I–*Bam*HI fragment containing the three *dl* binding sites was excised from the pG3dl and cloned between the *Xho*I and *Sph*I sites of the PWHL injection vector, which are located 55 bp upstream of the hsp70–*lacZ* fusion gene. The 3dl/*twi* construct contains the same three *dl* binding sites attached to a *twi*–*lacZ* fusion gene that contains *twi* sequences from –290 to +160 bp fused in-frame with the *lacZ* coding region.

In vitro mutagenesis

Base substitutions were made with mutagenic oligonucleotides using the Muta-gene kit (Bio-Rad) as described previously (Jiang *et al.*, 1991b). The mVR mutagenized sequence, containing base substitutions in all four *dl* binding sites, was prepared with the following oligonucleotides: AAACAG-TATCTAGAATTCACGAAAATCTTCT, ATATAAGTTTTCTGAATTCTAGAAGTCCAAT, ATATTGATTGGCTTTCTAGAAGTTATAGAGT and TGGATGTCCTGTGAATTCTAAGCTCTGAAT. All four mutagenic oligonucleotides were annealed simultaneously with a single-stranded DNA template prepared from a pBluescript SK[–] recombinant phagemid containing the 600 bp *zen* VR. The base substitutions targeted the core recognition sequence, GGG(A/T)–CC, of each binding site. In each case, at least one G and one C were substituted, and these changes virtually abolished *dl* binding based on mobility shift assays (data not shown). The tVR mutagenized sequence, which replaces all four *dl* binding sites with the low affinity TD5 sequence, was prepared with four oligonucleotides containing 10–11 bases flanking either side of the TD5 core recognition sequence, TGGGTTTTCTCT. DNA binding studies indicate that the underlined nucleotide is the basis for the low affinity of the TD5 binding site. When this is substituted with a C residue, there is a substantial increase in binding affinity (J.Jiang, unpublished results). The TD5 *dl* binding site is located between –310 and –298 bp upstream from the *twi* transcription start site, and has ~5-fold lower affinity than the oligo B *dl* binding site in the *zen* VR (Jiang *et al.*, 1991a).

P-transformation and in situ hybridization

P-transposons were introduced into the *Drosophila* germ-line by injecting *white*[–] embryos (w56/w56 homozygotes), as described previously (Jiang *et al.*, 1991a). At least three independent lines were obtained for each of the fusions summarized in Figure 1. The expression patterns directed by the various fusion promoters were examined in P-transformed embryos by whole mount *in situ* hybridization using an antisense *lacZ* RNA probe (Jiang *et al.*, 1991a).

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References

- Benson, M. and Pirrotta, V. (1988) *EMBO J.*, **7**, 3907–3915.
- Biggin, M.D., Bickel, S., Benson, M., Pirrotta, V. and Tjian, R. (1988) *Cell*, **53**, 713–722.
- Doyle, H.J., Kraut, R. and Levine, M. (1989) *Genes Dev.*, **3**, 1518–1533.
- Driever, W., Thoma, G. and Nüsslein-Volhard, C. (1989) *Nature*, **337**, 138–143.
- Ghosh, S., Gifford, A.M., Rivière, L.R., Tempst, P., Nolan, G.P. and Baltimore, D. (1990) *Cell*, **62**, 1019–1029.
- Govind, S. and Steward, R. (1991) *Trends Genet.*, **7**, 119–125.
- Ip, Y.T. and Levine, M. (1992) *Sem. Dev. Biol.*, **3**, in press.
- Ip, Y.T., Kraut, R., Levine, M. and Rushlow, C. (1991) *Cell*, **64**, 439–446.
- Jiang, J., Kosman, D., Ip, Y.T. and Levine, M. (1991a) *Genes Dev.*, **5**, 1881–1891.
- Jiang, J., Hoey, T. and Levine, M. (1991b) *Genes Dev.*, **5**, 265–277.
- Kieran, M., Blank, V., Logeat, F., Vandekerckhove, J., Lotspeich, F., Le Bail, O., Urban, M.B., Kourilsky, P., Baeuerle, P.A. and Israel, A. (1990) *Cell*, **62**, 1007–1018.
- Kosman, D., Ip, Y.T., Levine, M. and Arora, K. (1991) *Science*, **254**, 118–122.
- Levine, M. and Manley, J.L. (1989) *Cell*, **59**, 405–408.
- Pan, D., Huang, J.-D. and Courey, A.J. (1991) *Genes Dev.*, **5**, 1892–1901.

- Roth,S., Stein,D. and Nüsslein-Volhard,C. (1989) *Cell*, **59**, 1189–1202.
- Rushlow,C., Frasch,M., Doyle,H. and Levine,M. (1987) *Nature*, **330**, 583–586.
- Schneider,D.S., Hudson,K.L., Lin,T.-Y. and Anderson,K.V. (1991) *Genes Dev.*, **5**, 797–807.
- Small,S., Kraut,R., Hoey,T., Warrior,R. and Levine,M. (1991) *Genes Dev.*, **5**, 827–839.
- St Johnston,R.D. and Gelbart,W.M. (1987) *Embo J.*, **6**, 2785–2791.
- St Johnston,R.D. and Nüsslein-Volhard,C. (1992) *Cell*, **68**, 201–209.
- Stanojevic,D., Small,S. and Levine,M. (1991) *Science*, **254**, 1385–1387.
- Steward,R. (1987) *Science*, **238**, 692–694.
- Struhl,G., Struhl,K. and Macdonald,P. (1989) *Cell*, **57**, 1259–1273.
- Thisse,B., Stoetzel,C., Gorostiza-Thisse,C. and Perrin-Schmitt,F. (1988) *EMBO J.*, **7**, 2175–2183.
- Thisse,C., Perrin-Schmitt,F., Stoetzel,C. and Thisse,B. (1991) *Cell*, **65**, 1191–1201.
- Thummel,C.S., Boulet,A.M. and Lipshitz,H.D. (1988) *Gene*, **74**, 445–456.
- Wu,C. and Goldberg,M.L. (1989) *Trends Genet.*, **5**, 189–194.

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