Development 129, 4931-4940 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV5051

Anterior repression of a *Drosophila* stripe enhancer requires three position-specific mechanisms

Luiz Paulo Moura Andrioli, Vikram Vasisht, Ekaterina Theodosopoulou, Adam Oberstein and Stephen Small*

Department of Biology, New York University, 1009 Main Building, 100 Washington Square East, New York, NY 10003-6688, USA
*Author for correspondence (e-mail: stephen.small@nyu.edu)

Accepted 17 July 2002

SUMMARY

The striped expression pattern of the pair-rule gene *even skipped* (*eve*) is established by five stripe-specific enhancers, each of which responds in a unique way to gradients of positional information in the early *Drosophila* embryo. The enhancer for *eve* stripe 2 (*eve* 2) is directly activated by the morphogens Bicoid (Bcd) and Hunchback (Hb). As these proteins are distributed throughout the anterior half of the embryo, formation of a single stripe requires that enhancer activation is prevented in all nuclei anterior to the stripe 2 position. The gap gene *giant* (*gt*) is involved in a repression mechanism that sets the anterior stripe border, but genetic removal of *gt* (or deletion of Gt-binding sites) causes stripe expansion only in the anterior subregion that lies adjacent to the stripe border. We identify a well-conserved sequence repeat, $(GTTT)_4$, which is required for repression in a more anterior subregion. This site is bound specifically by Sloppy-paired 1 (Slp1), which is expressed in a gap gene-like anterior domain. Ectopic Slp1 activity is sufficient for repression of stripe 2 of the endogenous *eve* gene, but is not required, suggesting that it is redundant with other anterior factors. Further genetic analysis suggests that the $(GTTT)_4$-mediated mechanism is independent of the Gt-mediated mechanism that sets the anterior stripe border, and suggests that a third mechanism, downregulation of Bcd activity by Torso, prevents activation near the anterior tip. Thus, three distinct mechanisms are required for anterior repression of a single *eve* enhancer, each in a specific position. Ectopic Slp1 also represses *eve* stripes 1 and 3 to varying degrees, and the *eve* 1 and *eve* 3+7 enhancers each contain GTTT repeats similar to the site in the *eve* 2 enhancer. These results suggest a common mechanism for preventing anterior activation of three different *eve* enhancers.

Key words: Repression, *even-skipped*, Enhancer, Embryogenesis, Patterning

INTRODUCTION

In *Drosophila*, a hierarchy of genetic interactions divides the embryo into fourteen segments along the anterior posterior axis (reviewed by Pankratz and Jackle, 1993; Small, 1997). A crucial component of this hierarchy is the pair-rule gene *even skipped* (*eve*), which is expressed in a pattern of seven stripes in early blastoderm embryos (Frasch et al., 1987; Macdonald et al., 1986). This pattern is established by five modular enhancers, each of which controls the expression of a single stripe or a pair of stripes (Fig. 1) (Frasch et al., 1987; Fujioka et al., 1999; Goto et al., 1989; Harding et al., 1989; Macdonald et al., 1986). In general, *eve* stripes are activated by broadly distributed proteins, and stripe borders are set by localized repressors. Each enhancer contains a different combination of activator and repressor binding sites, and consequently a unique patterning activity.

Although all five enhancers are active at the same time in development, they function independently, based in large part on their modularity (Small et al., 1993). Stripe borders are formed by short-range repression mechanisms, which permit different enhancers to be in different states of transcriptional activity within the same nucleus. The best-characterized enhancer directs the expression of *eve* stripe 2 (*eve* 2). This stripe is first activated in a broad anterior domain by the anterior morphogens Bicoid (Bcd) and Hunchback (Hb), and then refined to a stripe by repressive mechanisms involving the gap proteins Giant (Gt) and Kruppel (Kr), which form the anterior and posterior borders of the stripe, respectively (Arnosti et al., 1996; Small et al., 1992; Small et al., 1991; Stanojevic et al., 1991). All four proteins bind in vitro to multiple sites within a 480 bp minimal stripe element (MSE; Fig. 1), which is sufficient for stripe expression in vivo. Mutations in individual sites cause changes in the level of expression or the shape of the stripe, suggesting that the enhancer acts as a switch element that directly measures concentrations of activator and repressor proteins.

The model for *eve* 2 regulation is the result of intensive study, but is incomplete in at least two respects. First, the combined activities of Bcd and Hb do not seem to be sufficient for activation at the position of *eve* stripe 2. For example, reporter genes containing three high affinity Bcd sites and three Hb sites cannot respond to the low levels of these proteins at the position of *eve* stripe 2 (Simpson-Brose et al., 1994). In

addition, several attempts to construct artificial stripe 2 enhancers using up to ten Bcd and/or Hb sites have been unsuccessful (S. S. and M. Levine, unpublished). These results suggest that *cis*-regulatory sequences other than the Bcd and Hb sites are important for activation. Such sequences may contain low-affinity Bcd- and Hb-binding sites, or sites for other activator proteins. Alternatively, they may simply function to provide the correct spacing between known activator or repressor sites.

Second, the mechanism(s) that control repression of *eve* 2 in anterior regions are not well understood. As the activators (Bcd and Hb) are distributed throughout the anterior half of the embryo, repressive mechanisms must exist that prevent activation in all nuclei anterior to the position of the stripe. The gap protein Gt has been suggested as a critical anterior repressor, but there is only a modest expansion of the stripe in embryos lacking *gt* activity (Small et al., 1992). Furthermore, ectopic expression of Gt does not efficiently repress *eve* 2 (Nibu and Levine, 2001; Wu et al., 1998). Thus, Gt activity is required for setting the anterior stripe border, but it probably acts by potentiating the activity of another anterior repressor (X). Deleting the Gt-binding sites causes a more extensive expansion than that seen in loss-of-function *gt* mutants (Small et al., 1992), consistent with the idea that these sites are bound by both Gt and X, which then cooperate in *eve* 2 repression. As Gt contains a b-ZIP dimerization domain (Capovilla et al., 1992), the simplest model is that Gt and X form a heterodimer that mediates effective repression through these sites. However, the expansion of *eve* 2 caused by deleting these sites still does not extend all the way to the anterior tip. This suggests that other unknown mechanism(s) prevent activation in this region.

To identify other activities that regulate *eve* 2, we tested a series of enhancer deletions in the context of a *lacZ* reporter gene. These experiments led to the identification of a binding site $(GTTT)_4$, which is crucial for repression in a more anterior subregion. We show that the forkhead domain (FD) protein Slp1, which is expressed in a broad anterior domain, binds to this site and is sufficient for repression of stripe 2 of the endogenous *eve* gene. Further genetic experiments identify a separate repression activity near the anterior pole that is dependent on the terminal patterning gene *torso*. Thus, three position-specific activities are required for anterior repression of *eve* 2. Two other *eve* stripes (*eve* 1 and *eve* 3) are also repressed by ectopic Slp1 expression. As the enhancers that control these stripes contain sites similar to $(GTTT)_4$, it is possible that repression of all three enhancers in this region is controlled by a similar mechanism.

MATERIALS AND METHODS

In vivo analysis of *eve lacZ* transgenes

For the initial enhancer analysis, four deletions (D1-D4) were generated by oligo-mediated mutagenesis (Muta-Gene, BioRad Laboratories, Richmond, CA) of a pBS-SK+ clone containing the *eve* 2 MSE fused upstream of an *eve* basal promoter fragment. An 800 bp fragment composed of the 500 bp *eve* 3+7 MSE and a 300bp spacer sequence was inserted upstream of the *eve* 2 MSE to control for changes in expression levels (Small et al., 1993). Deletion 1 (D1) removes 78 bp between the Gt 1 and Bcd 2 sites. D2 removes 62 bp between the Bcd 2 and Kr 4 sites. D3 removes 41 bp between the Bcd 3 and Gt 2 sites. D4 removes 38 bp between the Gt 2 and the Gt 3 sites. A fifth deletion (D5) removes 87 bp that lies 5′ of the Bcd 5 site (S. S. and D. Arnosti, unpublished). Precise deletion end points were confirmed by sequence analysis.

The deletion and base pair substitution of the $(GTTT)_4$ site was made in the context of a pBS-SK+ clone containing only the enhancer and the *eve* basal promoter. The following oligos were used in these experiments:

*eve*2delGTTT, 5′ GGCTAATCCCAGCATGCGCCGCAGAAGG-ATG 3′; and

*eve*2mutGTTT, 5′ GCTAATCCCAGCAGGTACCTGCGTGCA-CGCCGCAGAAGG 3′.

Restriction sites used to screen for mutant clones are indicated by underlined sequences. All mutations were confirmed by DNA sequencing, and *Eco*R1-*Xho*I fragments containing the mutant enhancers were cloned along with a 4.5 kb *Xho*I-*Xba*I *eve* lacZ fragment into the CaSpeR vector (Pirrotta, 1988), which was cut with *Eco*R1 and *Xba*I. Constructs were introduced into the *Drosophila* germline via standard microinjection procedures (Small, 2000; Spradling, 1986). At least three independent lines were generated for each construct, and analyzed by in situ hybridization using an antisense *lacZ* RNA probe as previously described (Jiang et al., 1991b).

Genetic crosses

The mutant alleles used in this study were tor^{PM51}, gt^{YA82}, $croc^{59}$, fkh^{3331}, hb^{14F21} and $slp^{\Delta 34B}$*CyO*/ l(2), which is a small deficiency that makes no *slp1* or *slp2* mRNA (Grossniklaus et al., 1992). The *slp* deficiency is on the CyO balancer chromosome, which facilitated identification of flies carrying this mutation. For analysis of transgenes in zygotic mutants, embryos were collected from inter se crosses among flies carrying a single copy of the transgene and single copies of the mutations to be tested. *slp, gt* double mutant embryos were unambiguously identified by triple staining with anti-sense *slp1*, *eve* and *lacZ* mRNA probes.

Yeast one-hybrid analysis

Four tandem copies of a 30 bp sequence containing the $(GTTT)_4$-binding site were inserted into the pBluescript SK+ vector using standard cloning procedures. As a negative control plasmid, four copies of a mutated version of the same sequence were inserted into a parallel construct. The sequences of the oligos used for these constructs are as follows:

$(GTTT)_4$, 5′ GATCGCGGCGTTTGTTTGTTTGTTTGCTGG 3′; and

$(Gmut)_4$, 5′ GATCGCGGCGTGCACGCAGGTACCTGCTGG 3′.

The integrity of each repeat was assayed by sequence analysis, and fragments containing the intact tetramers were excised as *Sma*I-*Xba*I fragments, blunt ended and cloned into the blunt-ended *Xba*I site of the pHisi-1 vector and into the blunt-ended *Xho*I site of the placZi vector. These vectors were provided in the Matchmaker One-Hybrid kit from Clontech (Palo Alto, CA).

A yeast strain (YM4271) carrying both the *lacZ* and His3 reporter constructs was generated using conditions recommended by the manufacturer (Clontech), and transfected with a λACT library of cDNAs made from 0- to 10-hour-old embryos (Yu et al., 1999).

Slp1 protein expression and DNA-binding assays

An expression clone containing the full length Slp1 coding region, pET3a/slp1c (Cadigan et al., 1994), was transformed into *E. coli* BL21 cells and expression was induced with 1mM IPTG for 3 hours at 37°C. Cells were harvested by centrifuging at 4200 ***g*** for 10 minutes. The bacterial pellet was then resuspended in 2 ml phosphate buffer (20 mM $NaPO_4$, 500 mM NaCl, 1 mM EDTA, 0.1% Tween20, 1×Complete™ Protease Inhibitor Cocktail) followed by sonication three times for 15 seconds on ice. Crude lysates were fractionated by centrifugation at 16,000 ***g*** for 30 minutes. The supernatant was mixed

1:1 with 100% glycerol, flash frozen in liquid nitrogen and stored at –80°C. Fraction content was monitored using SDS-PAGE.

Double stranded probes were created by annealing single stranded oligos containing the $(GTTT)_4$ site and the mutant version of the site. The sequences of the oligos used are as follows:

GTTT top, 5′ GATCGCGGCGTTTGTTTGTTTGTTTGCTGG 3′;

GTTT bottom, 5′ GATCCCAGCAAACAAACAAACAAACG-CCGC 3′;

MutGTTT top, 5′ GATCGCGGCGTGCACGCAGGTACCTG-CTGG 3′; and

MutGTTT bottom, 5′ GATCCCAGCAGGTACCTGCGTGCACG-CCGC 3′.

Annealed oligos were labeled using the Klenow fragment of *E. coli* DNA polymerase I and ^{32}P-dGTP, and purified on a 15% polyacrylamide gel. Gel retardation assays were performed by incubating nuclear extracts from 0-12 hours wild-type embryos (Han et al., 1993) or bacterial extracts with $(GTTT)_4$ or a mutant version of the probe in 20 μl binding cocktail (50% glycerol, 12.5 mM Hepes pH 7.5, 62.5 mM NaCl, 7 mM β-mercaptoethanol, 3.125 mM $MgCl_2$, 1.25 mg/ml bovine serum albumin and 0.1 mg/ml poly dIdC) at 25°C for 30 minutes and resolving DNA-Protein complexes on a 4% acrylamide, 2.5% glycerol gel containing 1×TBE. Labeled complexes were visualized by autoradiography.

Ventral misexpression of Slp1 and Gt

A general transformation vector for ventral misexpression was constructed using a 2.8 kb *Nsi*I-*Asp*718 *snail* (*sna*) promoter fragment (Ip et al., 1992), which includes the native basal promoter and 100 bp of the 5′ UTR. This was fused upstream of a 2.5 kb *Asp*718 fragment containing an FRT-STOP-FRT cassette, and a double-stranded *Kpn*I-*Not*I linker that contains a *Pme*I site for the insertion of any cDNA sequence. These fragments were ligated into the CaSpeR CGB3 vector 'PBKN' (a gift from Miki Fujioka), which was cut with *Pst*I and *Not*I. The *slp1* cDNA used here is a 1.6 kb *Eco*RV-*Xho*I fragment isolated from the one-hybrid clone. The *Eco*RV site is a native site that lies 17 bp upstream of the translation initiation site (Grossniklaus et al., 1992). This fragment includes the entire *slp1* ORF and 370 bp of the 3′ UTR. The *gt* cDNA used here is a 1.6 kb fragment that extends from an artificial *Nde*I site at the translation initiation codon to an *Eco*RI site that lies ~400 bp downstream of the termination codon (Kraut and Levine, 1991). These fragments were blunt-ended, and cloned into the *Pme*I site of the ventral expression vector described above. Transgenic flies carrying these constructs were then generated using standard microinjection techniques (Small, 2000; Spradling, 1986).

For these experiments, three independent *sna-slp1* and five *sna-gt* transgenic lines were analyzed. To activate mis-expression, we generated males containing a given misexpression transgene along with a *β2-tubulin-FLP* transgene, which activates expression of the yeast FLP recombinase and recombination between the FRTs during spermatogenesis (Struhl et al., 1993). These males were then mated with w^{1118} females or with females carrying various *lacZ* reporter genes. The reporter genes used here are *eve*2-*lacZ* (Small et al., 1992), *eve*3+7-*lacZ* (Small et al., 1996), *–7.8eve-lacZ* (D. Kosman and S. S., unpublished) and *eve*1+5-*lacZ* (Fujioka et al., 1999). All reporter genes contain the proximal eve basal promoter and 5′ untranslated region. Embryos were collected and analyzed by in situ hybridization (Kosman and Small, 1997). All misexpression lines gave similar results in the assays described here.

RESULTS

Identification of a novel repressor site in the *eve* 2 MSE

Previous DNA-binding experiments identified twelve sites

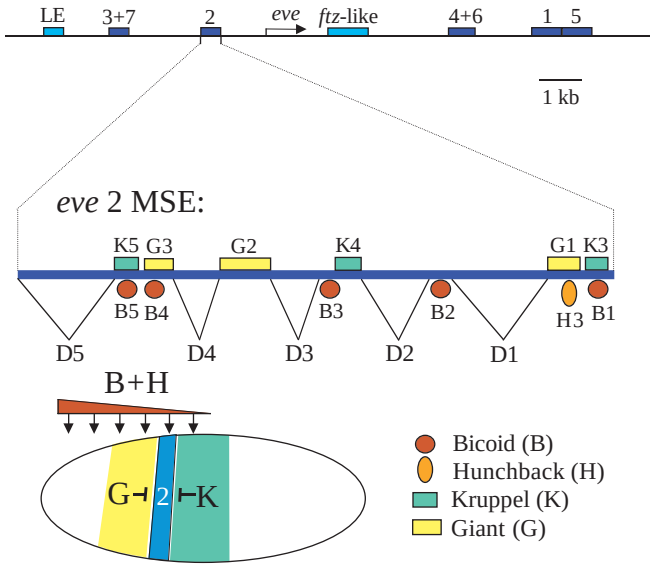

Fig. 1. A map of the *eve* locus is shown at the top. The positions of five enhancers that control early stripe formation (1-7) are shown. Two other enhancers that control the refinement of the initial stripes (LE), and later expression in inter-stripe regions (*ftz*-like) are also indicated. A map of the *eve* 2 minimal stripe element (MSE) is shown in the middle with positions of defined binding sites for transcription factors. Activator and repressor sites are closely linked, especially in two clusters, each of which contains two pairs of overlapping sites. Regions tested by deletion analyses are marked (D1-D5). A model for *eve* 2 regulation is presented at the bottom. Activation is mediated by Bcd and Hb, while Gt and Kr are involved in repression mechanisms that form the stripe borders.

for the genetically defined regulators within the *eve* 2 MSE (Small et al., 1991; Stanojevic et al., 1989). To identify other sequences important for *eve* 2 regulation, we constructed a series of mutant enhancers that contain deletions (D1-D5) of the regions between the known binding sites (Fig. 1). Each deletion was tested separately in the context of an *eve* 2 *lacZ* fusion gene that also contains the *eve* 3+7 MSE as an internal control for levels of expression. Several independent lines for each construct were obtained by P-element mediated transformation. Embryos were collected from these lines, and examined by in situ hybridization for expression of *lacZ* mRNA.

All five deletions disrupt the normal function of the *eve* 2 enhancer. Four (D1, D3, D4, and D5) cause a significant reduction in the level of stripe activation (Fig. 2; data not shown). It is not clear whether these reductions are caused by removing discrete activator sites or by changing the spacing between the known sites. By contrast, the D2 deletion, which removes a 62 bp sequence between the Bcd 2 and the Kr 4 sites (Fig. 1), causes an apparent strengthening of stripe 2 activation, and ectopic expression in more anterior regions (Fig. 2E,F). The derepression is quite broad early in nuclear cycle 14 (Fig. 2E), but refines later to an ectopic anterior stripe (Fig. 2F). These results suggest that the D2 region contains sequences required for repression in the region of the ectopic stripe.

Because important functional binding sites are likely to be evolutionarily conserved, we compared the *eve* 2 sequence from *D. melanogaster* with published sequences from four

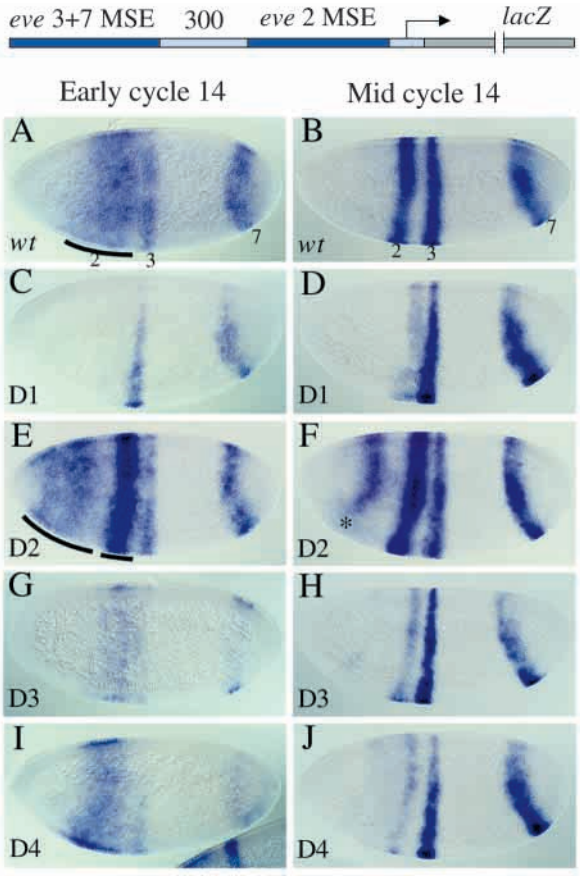

Fig. 2. Deletion analysis of the *eve* 2 MSE. The *lacZ* reporter construct used in these assays is shown schematically at the top, and contains the *eve* 2 MSE and the *eve* 3+7 MSE to control for levels of expression. Whole-mount *lacZ* mRNA expression patterns directed by this construct are shown for the wild-type *eve* 2 MSE (A,B), and for *eve* 2 MSEs containing deletions (D1-4; C-J) as shown in Fig. 1. Unless otherwise indicated, all embryos in this paper are oriented with anterior towards the left and dorsal upwards. Expression patterns are shown for embryos early (left column) or mid-way through (right column) cleavage cycle 14. The wild-type *eve* 2 enhancer is first activated in a broad anterior domain (marked by the broad line, A), which is then refined to a stripe (B). Deletions 1, 3 and 4 each cause a failure to activate or maintain wild-type expression levels of *eve* 2 (C,D,G-J). D2 causes a premature strengthening of *eve* 2 with a more extensive anterior expansion (E). This expansion refines to form an ectopic stripe (*) in mid-cycle 14 (F).

other *Drosophila* species (Fig. 3A) (Ludwig et al., 1998; Sackerson, 1995). The Kr4 and Bcd2 sites are well-conserved among all five species, and thus represent excellent anchors for the careful analysis of the intervening region. The best-conserved sequence block in this region is a 16 bp sequence that consists of four repeats of the sequence GTTT. Although this type of repeat is unusual for a functional binding site, we tested it by deletion or mutagenesis in the context of an *eve* 2-*lacZ* reporter gene. Both disruptions cause severe anterior derepressions (Fig. 4C-F). By contrast, a deletion that removes the rest of the D2 sequence (46 bp), but leaves the $(GTTT)_4$ sequence intact, does not cause any detectable change in enhancer activity (data not shown). Thus, the $(GTTT)_4$ sequence is the major binding site for a repressive activity that prevents expression of the *eve* 2 enhancer in a specific anterior region.

Anterior repression of *eve* 2 requires three independent activities

Previous experiments suggested that the gap gene *gt* and the Gt-binding sites are required for the correct positioning of the anterior *eve* 2 border (Small et al., 1992). To test the relationship between the $(GTTT)_4$-binding activity and Gt-mediated repression, the *eve*2Δ$(GTTT)_4$-*lacZ* construct was crossed into a *gt* mutant background. If the two repression mechanisms are independent, we expect an additive effect from combining these two perturbations. If, however, Gt-mediated repression is partially redundant with the $(GTTT)_4$-binding activity, removing both might cause a more severe derepression. The result of this cross is shown in Fig. 5D. There is an anterior shift and slight expansion of stripe 2 that is similar to the effects on the wild-type *eve* 2 transgene in *gt* mutants (Fig. 5C). No new effect is detected on the band of

A

```
          ----Kr4-----
D. mel    ACC--GGGTTGCGAAG-----TCAGGG------------------CATTCCGCCGATCT----A--------GCCATCG
D. yak    ACC--GGGTTGCAAAG-----TCAGGG------------------GATTCCGACGATCTCGCCATATCCATCGCCATCG
D. ere    ACC--GGGTTGCAAAG-----TCAGGG------------------GATTCCATCGCCGTCGCCATCGCCATCGCCACCG
D. pse    ACCAAGGGTTGTCTCCTGGCCTCAGGA------------------GTTTCCACA------------------GTCAACG
D. pic    ACC--GGGTTACCCTCAACCTACGAGTTTAACTTTCAACTTTGACGTTACCAAAACGACT-------------TCAACT

                                                                                  --Bcd2---
D. mel    CCATCTTCT--------GCGGGC----------------GTTTGTTTGTTTGTTT-------------GCTGGGATTAGC
D. yak    CCATCTTCT--------GCGGGC----------------GTTTGTTTGTTTGTTT-------------GCTGGGATTAGC
D. ere    CCATCTTCT--------GCGGGC----------------GTTTGTTTGTTTGTTT-------------GCTGGGATTAGC
D. pse    C----------------------TTTCG-C-TG-----GTTTGTTTATTTGTTTGTTT-GT---TTTAGCCAGGATTAGC
D. pic    CCATTTTCGACTTTACTGGCGGAGTTTCCACATGCCTCTGTTTGTTTATTTGTTTATTTTGTGGATTTA---GAGATTAGG
```

B

```
eve 2          GTTTGTTT  GTTTGTTT
eve 3+7        GTTTGTTTGTGTTTGTTT
eve 1          GTTTGTTTTCGTTTGTTT
```

Fig. 3. Evolutionary conservation of the *eve* 2 D2 region. (A) Sequences of the *eve* 2 enhancer region between the Bcd2 and Kr4 sites are shown for *D. melanogaster* (*mel*), *D. erecta* (*ere*), *D. yakuba* (*yak*), *D. pseudoobscura* (*pse*) and *D. picticornis* (*pic*). Blocks of identical sequences at least three bases long are shaded in blue. (B) Sequences of similar repeats of the GTTT motif in three different *eve* enhancers.

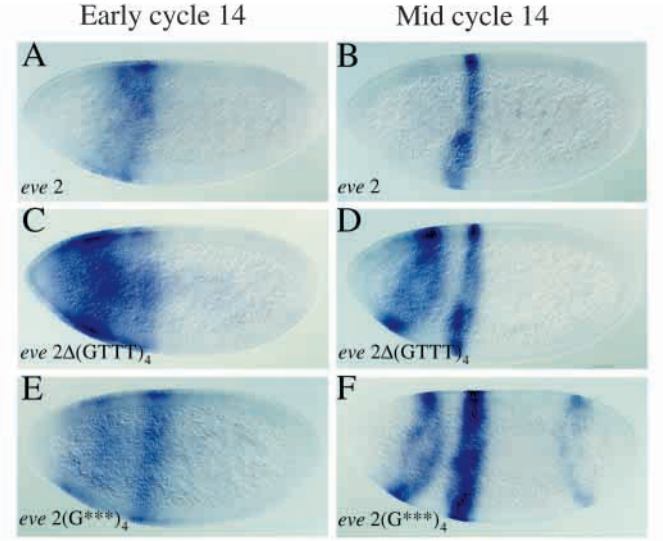

Fig. 4. The $(GTTT)_4$ repeat is critical for anterior repression of *eve* 2. *lacZ* mRNA expression patterns are shown for early (left) and mid cycle 14 (right) embryos containing the wild-type *eve 2-lacZ* transgene (A,B) or an identical construct in which the $(GTTT)_4$ sequence was either deleted (C,D) or mutated (E,F).

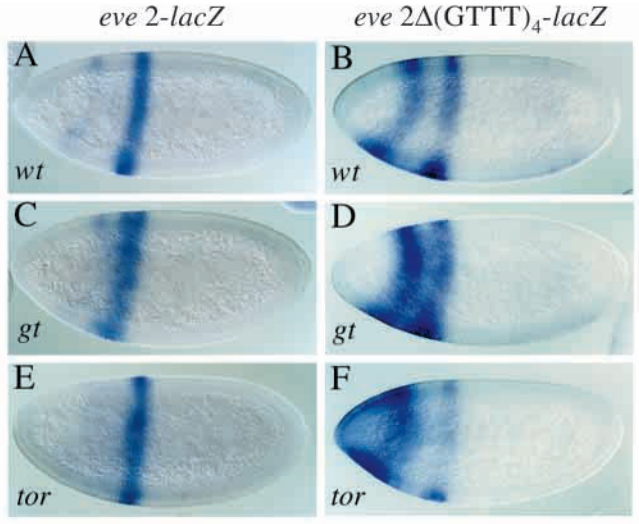

Fig. 5. Genetic analysis of anterior *eve* 2 repression. *lacZ* expression patterns are shown for embryos containing the *eve* 2-*lacZ* and *eve*2Δ$(GTTT)_4$-*lacZ* transgenes in a wild-type embryo background (A,B), and in mutant embryos lacking *gt* (C,D) or *tor* activity (E,F). Both genetic mutants expand the derepression caused by the deletion of the $(GTTT)_4$ site.

derepression created by deleting the $(GTTT)_4$ site, and a small repressed area is still maintained between the two parts of the pattern (Fig. 5D). This result is consistent with an additive effect, and suggests that the $(GTTT)_4$-binding activity functions independently of Gt-mediated repression. The failure to derepress in the region between the two parts of the pattern probably reflects the activity of the unknown protein X, which normally participates with Gt in repression.

The *eve*2Δ$(GTTT)_4$-*lacZ* transgene is also repressed at the anterior tip (Fig. 5B), even in *gt* mutants (Fig. 5D), suggesting that yet another mechanism prevents activation in this region. This mechanism could work through another localized repressor activity, or by modifying Bcd, the major activator of *eve* 2. It has been previously shown that Bcd-dependent activation of *hb* and *orthodenticle* (*otd*) is downregulated by the Tor phosphorylation cascade at the anterior tip (Ronchi et al., 1993), consistent with the latter possibility. To test whether *tor* controls the ability of Bcd to activate *eve* 2, the *eve*2Δ$(GTTT)_4$-lacZ transgene was crossed into embryos lacking *tor* activity. This causes a significant derepression at the anterior tip (Fig. 5F), suggesting that *tor*-mediated modification of *bcd* activity is important for preventing activation in this region. A similar derepression is not detected with the wild-type *eve*2-lacZ transgene in *tor* mutants (Fig. 5E), suggesting that Tor-mediated repression is dependent on the $(GTTT)_4$-binding activity (see Discussion). In summary, these results suggest that multiple activities are required for anterior repression of *eve* 2, and that three different mechanisms prevent activation in different anterior regions.

A role for Slp1 in anterior repression of the *eve* stripe 2 response

Because of the peculiar sequence of the $(GTTT)_4$ site, we performed gel shift experiments using nuclear extracts from 0- to 12-hour-old wild-type embryos (Fig. 6A). These experiments showed the formation of several specific protein-DNA complexes. To identify specific proteins that bind the $(GTTT)_4$ sequence, we conducted a yeast one-hybrid assay with constructs containing four tandem copies of the intact site. From an initial screen of ~500,000 clones, we obtained 66 true positives (based on survival on his^- medium and increased *lacZ* production). These clones were then transformed into a yeast strain containing identical reporters except for base pair substitutions in the $(GTTT)_4$ sequence. Forty-nine clones also activated one of the mutant constructs, leaving only 16 that activated the $(GTTT)_4$ constructs, but not the negative controls (Table 1). Among these 16 were two clones that encode histone H1 and a single clone that encodes the forkhead domain (FD) protein Slp1. *slp* was originally classified as a pair-rule mutation (Nusslein-Volhard et al., 1985), but the *slp* locus contains two tightly linked genes, *slp1* and *slp2* (Grossniklaus et al., 1992). These genes are related in their primary structure, and their expression patterns overlap significantly. However, *slp1* is expressed much earlier in an anterior 'gap gene-like' domain, which first appears as an anterior cap, and then evolves into a broad stripe at approximately 80% egg length (Grossniklaus et al., 1992). Double staining experiments with *gt* show that both genes are expressed at the same time, and

Table 1. Clones isolated in the yeast one hybrid experiment with the $(GTTT)_4$ site

Isolate number	Gene name	Putative function
8, 10, 29, 30, 31,33	PK61C (CG1210)	Ser Thr kinase
15, 66	Histone H1	Linker histone
11, 16	CG12288	RNA-binding protein
18, 56	CG3838	Unknown
58	CG11533	Kinase
40	CG5454	RNA-binding protein
27	CG2201	Enzyme
28	Slp1	FD transcription factor

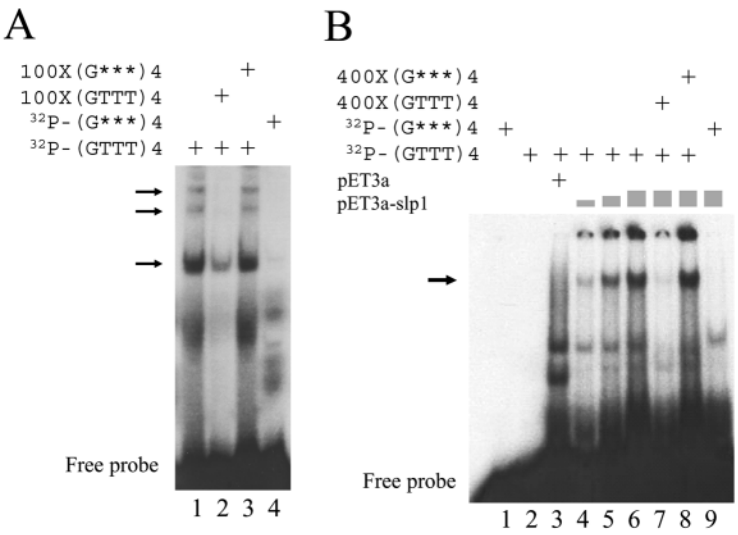

Fig. 6. Gel shift analysis of the $(GTTT)_4$ sequence. (A) Incubation of an ^{32}P-labeled oligo containing the $(GTTT)_4$ sequence with *Drosophila* embryonic extracts causes the formation of several complexes (arrows). Most of these complexes can be competed by excess cold probe (lane 2), but not by the mutant probe (lane 3). Complexes are not formed with the mutant probe (lane 4). (B) Slp1 protein binds specifically to the $(GTTT)_4$ repeat. Increasing concentrations of a bacterial extract containing full-length Slp1 protein (lanes 4-6) causes formation of a specific complex (arrow). Binding can be competed by excess cold probe (lane 7), but not by the mutant probe (lane 8).

that *slp1* expression overlaps the anterior part of the *gt* expression domain (Fig. 7A). Thus, the temporal and spatial expression patterns of *slp1* are consistent with a role in anterior repression of *eve* 2.

The $(GTTT)_4$ sequence bears little resemblance to the only previously defined Slp1-binding site (TCTTCGATGTCAA-CACACC) (Yu et al., 1999). Thus, we tested whether bacterially expressed Slp1 can bind directly to the $(GTTT)_4$ sequence in vitro (Fig. 6B). These experiments show that Slp1 binds specifically to this sequence, suggesting that it may directly interact with this sequence in vivo. Similar results were obtained using a fragment of the Slp1 protein that contains only the forkhead domain (data not shown).

If Slp1 acts as an anterior repressor of *eve* 2, genetically removing it might cause an anterior derepression of the *eve*2-*lacZ* expression pattern. To test this, we crossed the reporter into a *slp* deletion mutant that completely removes the *slp1*-coding region and disrupts *slp2* (Grossniklaus et al., 1992). No anterior expansion was detected in this experiment (data not shown). We also analyzed endogenous *eve* expression in this mutant background, and did detect slight anterior shifts of stripes 1 and 2 (data not shown), but no significant derepression in anterior regions. To test whether Slp1-mediated repression requires *gt*, we examined *eve* and the *eve*2-*lacZ* reporter gene in *gt; slp* double mutant embryos. The double mutant shows no increase in the anterior derepression over that caused by removal of *gt* alone (data not shown). These results argue against a role for Slp1 in anterior repression of *eve*, but do not rule out the possibility that Slp1 is one of several redundant proteins that repress through the $(GTTT)_4$ site. Two other FD proteins, Fkh and Crocodile (Croc), are expressed in anterior regions of the embryo (Hacker et al., 1995; Weigel et al., 1989). However, the expression domains of both proteins are located very near the anterior pole, making it unlikely that either gene is involved in this repression mechanism. To make sure, we examined *eve* and *eve* 2-*lacZ* expression in each mutant; neither shows an anterior derepression. Thus these two genes are unlikely to play important roles in this repression mechanism.

Ectopic Slp1 is sufficient for repression of *eve* stripes 1, 2 and 3

To further test the roles of Slp1 and Gt in *eve* patterning, we used a fragment of the *snail* (*sna*) promoter (Ip et al., 1992) and the yeast FLP-FRT system (Struhl et al., 1993) to drive ectopic domains of each gene along the ventral surface of the embryo (Fig. 7B,C). This method is an efficient way to test whether any gene is sufficient for repression of individual stripes because the ventral expression domain intersects all seven *eve* stripes. In this assay, Slp1 expression alone distorts

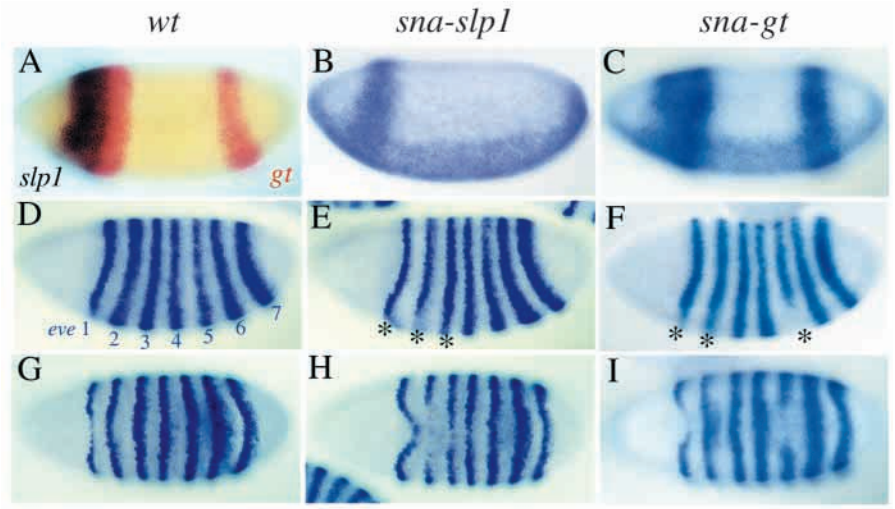

Fig. 7. Effects of ectopic *slp1* or *gt* expression on the *eve* striped pattern. (A) Wild-type expression patterns of *slp1* (black) and *gt* mRNA (red) in an early cleavage cycle 14 embryo. Embryos carrying activated *sna-slp1* (B) or *sna-gt* (C) transgenes express low levels of ectopic mRNA along the entire ventral surface in addition to their endogenous patterns. (D-I) *eve* RNA expression patterns in wild-type embryos (D,G) and those containing either ectopic *slp1* (E,H) or ectopic *gt* (F,I). Images in A-F are lateral views with anterior towards the left and dorsal upwards. Those in G-I are ventral views.

the expression of *eve* 1 in ventral regions by shifting it posteriorly, and causes a strong repression of *eve* 2 and a weaker repression of *eve* 3 (Fig. 7E,H). By contrast, there is no detectable effect on the posterior stripes. Thus, Slp1 activity is sufficient for repression of specific anterior stripes including *eve* 2. By contrast, ventrally misexpressed Gt causes only a weak repression of *eve* 1 and 2, but strongly affects *eve* 5, a repression target of the posterior *gt* expression domain (Fig. 7F) (Nibu and Levine, 2001). The minor effect of Gt on *eve* 2 is transient, and the stripe recovers and expands posteriorly later in cycle 14 (Fig. 7I). This expansion is probably caused by repression of *Kr*, which forms the posterior border of *eve* 2 (Wu et al., 1998). These results confirm that Gt is not sufficient for effective repression of *eve* 2, and that its effect is much weaker than Slp1-mediated repression. We also generated embryos that contain ventral expression domains of both Slp1 and Gt. While effects of both genes were detected within the same embryos, there was no evidence of synergistic repression activity in these experiments (data not shown). This is consistent with the demonstration that the $(GTTT)_4$ site is independent of Gt-mediated repression (Fig. 5).

The repressive effects of ectopic Slp1 on the three anterior *eve* stripes suggest a common mechanism for repression in anterior regions of the embryo. To test this, we searched the *eve* locus for binding sites similar to the $(GTTT)_4$ site in the *eve* 2 enhancer (Adams et al., 2000). Interestingly, there are only two other such sites in the *eve* locus, which are located within the boundaries of the stripe 1 and stripe 3+7 enhancers (Fig. 3B). We next tested whether repression by ectopic Slp1 is mediated through these enhancers and the *eve* 2 MSE. In these experiments, ectopic Slp1 caused a ventral repression of stripe 1 in the context of an *eve*1+5-*lacZ* transgene (Fig. 8B). A similar repression of stripe 3 was observed in the context of an *eve*3+7-*lacZ* transgene (Fig. 8D,F). These results are consistent with the idea of a common mechanism. By contrast, no ventral repression of the *eve*2-lacZ transgene was detected (data not shown), which is surprising in light of the fact that *eve* 2 is the most strongly affected stripe in the context of the endogenous gene.

The reason for the discrepancy between the reporter and the endogenous gene is not clear. It is possible that Slp1-mediated repression of *eve 2* requires *eve* sequences outside the minimal enhancer. For example, the late element (LE) (Fig. 1) mediates the refinement of all seven *eve* stripes after they are initially positioned (Fujioka et al., 1996; Jiang et al., 1991a). Perhaps interactions between the LE and/or other *cis* sequences are required for effective repression by Slp1. To test this, we used a larger reporter gene (*–7.8 eve-lacZ*) that contains all native sequences from the 5′ border of the locus to the transcription start site. This transgene contains the LE, the *eve* 2 and *eve* 3+7 enhancers, and all native sequences that lie between these elements, and drives expression of stripes 2, 3 and 7, and a single line of nuclei located within the normal position of stripe 1 (Fig. 8G). Expression from this reporter is effectively repressed at the position of stripes 1 and 3 in embryos containing ventrally expressed Slp1, but there is still no effect on the stripe 2 response (Fig. 8H). Thus, the addition of these extra sequences does not restore the sensitivity of *eve* 2 to Slp1-mediated repression. This suggests that undefined properties of the endogenous *eve* locus are required for Slp1-mediated repression of *eve* 2.

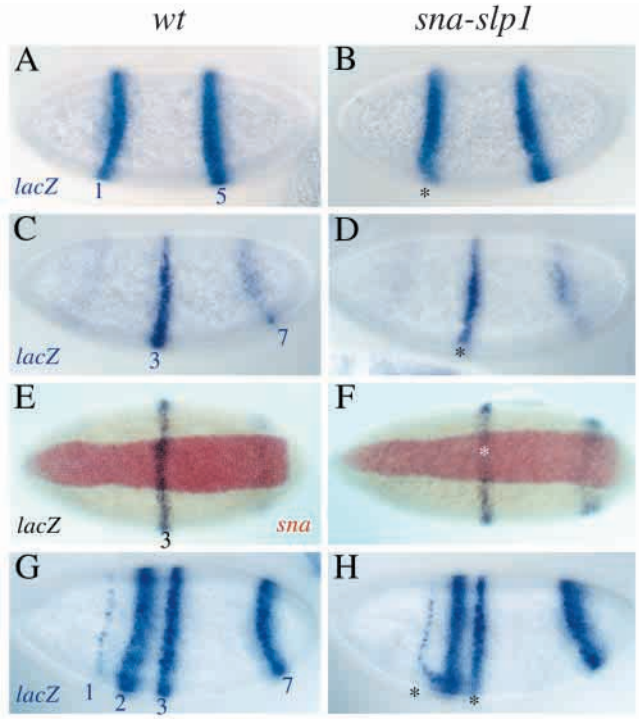

Fig. 8. Ectopic Slp1 represses reporter gene expression driven by the *eve* 1+5 and the *eve* 3+7 enhancers, but not the *eve* 2 enhancer. *lacZ* mRNA expression patterns (blue or black) driven by the *eve* 1+5 enhancer (A,B), the *eve* 3+7 enhancer (C-F) or a 7.8 kb 5′ regulatory fragment that contains the *eve* 2 and 3+7 enhancers (G,H) are shown in wild-type embryos (left column) or embryos that ventrally misexpress Slp1 (right column). Stripe numbers are indicated on each panel, and repression events are marked with asterisks. (E,F) Ventral views of embryos double stained to detect *lacZ* (black) and *sna* mRNA (red), which is expressed in ventral-most regions.

DISCUSSION

Region-specific repression of *eve* 2

The results presented here indicate that three distinct mechanisms are required for anterior repression of *eve* 2, with each activity functioning within a specific subregion (Fig. 9A).

In subregion III, the Gt-binding sites (Fig. 1) are crucial for repression – deletion of these sites leads to an anterior expansion of the stripe. However, it is clear that Gt does not act alone, and that at least one other factor (X) must be involved in repressing through these sites (Small et al., 1992). The identity of X is not clear, but genetic studies have localized a Gt-like patterning activity to the left arm of chromosome II (Vavra and Carroll, 1989). Segmental aneuploids that remove this arm show an expansion similar to that seen in *gt* mutants.

In subregion II, repression of *eve 2* is mediated by the $(GTTT)_4$ site described in this paper. We have further identified a candidate protein, Slp1, which is expressed at the right time and place for the repression activity and binds specifically to this site in the yeast 1-hybrid experiment and in vitro. The $(GTTT)_4$ site shows little similarity to the other known Slp1-binding site (Yu et al., 1999), but is quite similar to sites bound by other members of the FD protein family. For example, a 115 amino acid FD fragment of Fkh binds specifically to the site

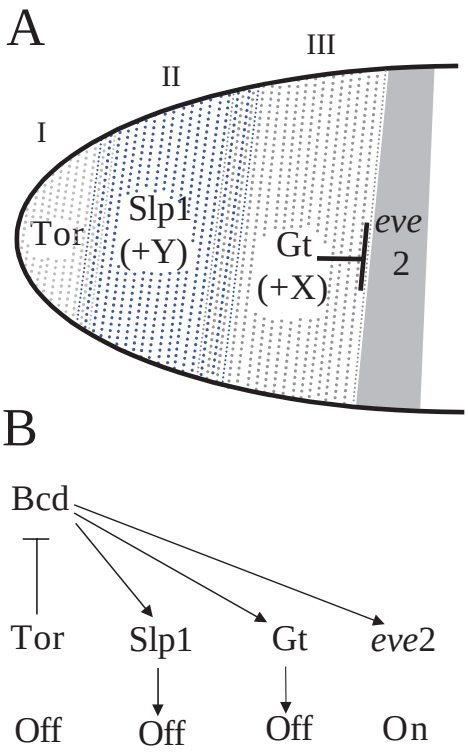

Fig. 9. (A) Three different mechanisms control repression of *eve* 2 in anterior regions of the embryo. The activity of Gt and at least one other factor (X) is required for repression very near the anterior border. Anterior to this domain, Slp1 and at least one other factor (Y) mediate *eve* 2 repression. At the anterior pole, Tor activity may downregulate the activity of Bcd, the primary activator of *eve* 2. (B) A model for Bcd coordination of *eve* 2 regulation. Bcd activates transcription of the *eve* 2 enhancer even at quite low concentrations. Bcd also is required for activation of the repressors of *eve* 2 (Gt and Slp1). We propose that these genes are positioned based on their decreased sensitivity to the Bcd gradient. At the anterior tip of the embryo, the activity of Bcd may be disrupted by the Tor phosphorylation cascade, which prevents activation of several Bcd target genes in this region.

CTTTGTAAA (Kaufmann et al., 1994), which bears some resemblance to the $(GTTT)_4$ site. Also, the hepatocyte FD protein HNF-3 binds to a site (TGTTTGTTTTAGTT) that contains two perfect GTTT repeats (Pani et al., 1992). Ventrally expressed Slp1 specifically represses *eve* 2, strongly supporting a role in regulation of the endogenous *eve* gene. However, there is no effect on *eve* 2 in *slp* mutants, suggesting that Slp1 is redundant with at least one other protein (Y), which also mediates repression through the $(GTTT)_4$ site. The existence of multiple complexes in gel shifts with embryo extracts is consistent with this, but the identity of Y is still unknown.

In subregion I, *eve* 2 repression is controlled by Tor, which may act by downregulating Bcd-dependent activation. This is consistent with the previous demonstration that Tor interferes with Bcd-dependent activation of *hb*, *otd* and *slp1* (Grossniklaus et al., 1994; Ronchi et al., 1993).

In summary, at least five different protein activities are involved in three distinct mechanisms that repress *eve* 2 in anterior regions. Interestingly, it seems that all aspects of *eve* 2 regulation are controlled, directly or indirectly, by the Bcd morphogen gradient (Fig. 9B). The *eve* 2 enhancer is directly activated by Bcd, but activation is prevented near the anterior pole by Tor. The anterior expression patterns of the defined repressors of *eve* 2 (Slp1 and Gt) are also activated by Bcd. We propose that the relative positions of these domains, and the ultimate position of *eve* 2, are controlled by differential sensitivity to the Bcd concentration gradient. Future experiments on the cis-elements that regulate *slp1* and *gt* transcription will be required to test this.

Shared mechanisms of *eve* repression in anterior regions

The study of *eve* regulation is an excellent paradigm for how complex promoters integrate the activities of multiple enhancers. Previous studies suggest that all five enhancers function independently in the segmented part of the embryo. The autonomy of each enhancer depends on short-range repression mechanisms and sufficient linear spacing between enhancers along the DNA sequence (Gray et al., 1994; Small et al., 1993). These factors are crucial for creating the pattern of seven *eve* stripes because they permit different enhancers to be in different transcriptional states within the same nuclei. For example, in nuclei at the position of stripe 1, the *eve* 1 enhancer will activate transcription even though the *eve* 2 enhancer is in a repressed state.

A different scenario exists in regions anterior to the striped pattern, where none of the *eve* enhancers are activated. The genetic removal of various repression activities suggests that at least two *eve* enhancers (*eve* 2 and *eve* 3+7) can be activated in this region. The mechanism of *eve* 1 activation is still unknown, but it is reasonable to suggest that its activators are also distributed in this region. Thus, mechanisms must be in place to prevent activation by each enhancer. Alternatively, repression could occur by an anterior repression activity that directly contacts the basal transcription complex. We have shown that the $(GTTT)_4$ site in the *eve* 2 enhancer mediates anterior repression, and that there are similar binding sites in the *eve* 1 and *eve* 3+7 enhancers. Furthermore, ectopic Slp1 expression represses all three stripes. These results argue against the mechanism of direct contact with the basal machinery, and suggest that these three enhancers share a common mechanism for repression in a specific anterior region of the embryo. Genetic experiments also show that the Tor phosphorylation cascade participates in polar repression of the *eve* 2 and *eve* 3+7 enhancers, which suggests another common repression mechanism that is shared by at least two of these enhancers.

By contrast, the *eve* 5 and *eve* 4+6 enhancers do not appear to contain sites similar to the $(GTTT)_4$ sites (Adams et al., 2000), and they are immune to repression by ectopic Slp1. These enhancers are expressed in posterior regions of the embryo, and thus may be activated by factors localized there, making anterior repression unnecessary for their function.

The mechanism of Slp1-mediated repression

slp was originally classified as a pair-rule gene based on its cuticular phenotype, and has been shown to function at both the level of the pair-rule genes and the segment polarity genes (Grossniklaus et al., 1992). Specific patterning functions for the early anterior Slp1 expression domain, however, have remained

unclear, although strong alleles of *slp1* exhibit severe defects in the mandibular lobe. Our results suggest that Slp1 acts at the level of the gap genes by repressing enhancer elements that control the initial *eve* stripes. Thus, Slp1 function is required at three different levels of the segmentation hierarchy.

The mechanism involved in Slp1-mediated repression of *eve* is unknown, but may involve an interaction with the co-repressor Groucho (Gro) (Paroush et al., 1994; Paroush et al., 1997). The Slp1 protein sequence contains a motif (FSIDAIL) (Grossniklaus et al., 1992), which is very similar to the EH1 Gro-binding consensus (FSIDNIL) (Jimenez et al., 1997) and Slp1 has been shown to bind Gro in vitro (Kobayashi et al., 2001).

It has been proposed that Gro mediates repression by creating a direct physical link between DNA-bound proteins and components of the basal transcription machinery (Courey and Jia, 2001; Nibu et al., 2001). As such, repressors that act through Gro can function over very long distances, and have been classified as long-range repressors. The ability of Slp1 to interact with Gro suggests such a long-range mechanism, but several considerations are not consistent with this model. For example, ventral expression of a long-range repressor that 'locks' the basal transcription machinery should repress all seven *eve* stripes, not just the anterior three. Also, the three $(GTTT)_4$ sites described here are all located within minimal enhancer elements that control specific stripes. In a long-range mechanism, these sites could be located anywhere in the promoter, and need not be associated with specific enhancers.

One of the most intriguing findings of this study is that ectopic Slp1 represses *eve* 2 in the endogenous gene, but not in the context of several *lacZ* reporter genes. Despite much effort, we have not resolved this discrepancy, but it is informative to compare the structural differences between the endogenous gene and the tested transgenes. One obvious difference between the *lacZ* reporter genes tested in these experiments and the endogenous *eve* gene is copy number. Perhaps Slp1-mediated repression requires two copies of the enhancer in a homozygous situation. A pairing-sensitive element (PSE) that reduces marker gene expression in homozygotes has been identified in the far 3′ region of the *eve* gene (Fujioka et al., 1999), which is consistent with this hypothesis. Two experiments argue against this hypothesis. First, ectopic Slp1 still represses endogenous *eve* in *Df (eve)/+* heterozygotes (data not shown). Also, Slp1 fails to repress *eve* 2-containing transgenes when they are homozygosed (data not shown).

Another difference between the endogenous gene and the reporters is that the endogenous gene contains genomic regions outside those tested in the reporter genes, and is located in a different genomic position. Perhaps control sequences in the 3′ region of the gene, or further 5′ are required for this repression mechanism. As mentioned above, the *eve* 1 enhancer, which is located in the 3′ region, contains a $(GTTT)_4$-binding site. Perhaps effective repression of *eve* 2 requires all three sites contained in the three different enhancers. This will be tested in future experiments.

Finally, it is possible that the native *eve* locus is organized in a specific chromatin conformation that permits repression by Slp1, and this configuration is not maintained when *eve* 2 transgenes are inserted into ectopic genomic locations. The fact that Slp1 protein contains an FD DNA-binding domain is interesting in this regard. Structural studies suggest that FD domains form a 'winged-helix' very similar to the globular DNA-binding domain of the linker histone H1 (Clark et al., 1993). Furthermore, it has been shown that the mammalian FD protein hepatic nuclear factor 3 (HNF3) competes with H1 for binding to specific sites, and that this competition is critical for the in vivo regulation of the albumin liver-specific enhancer (Cirillo et al., 1998). Such a mechanism may be involved in Slp1-mediated repression of *eve* 2. Consistent with this, we isolated two clones that encode histone H1 in the one-hybrid experiment with the $(GTTT)_4$ site (Table 1). This suggests that both proteins can bind to this site, and supports the idea that regulation of chromatin structure may be an important part of Slp1-mediated repression of *eve* 2. More experiments will be required to test this hypothesis.

The complexities of enhancer regulation

The *eve* 2 enhancer is one of the best-characterized patterning elements in *Drosophila* development. Proteins involved in activation and repression have been identified, and a simple model has emerged that explains the basic activity of the enhancer (Fig. 1). We have shown that anterior repression of this element requires at least three position-specific mechanisms, which significantly extends our understanding of this aspect of enhancer function. Our results also suggest that the current model for activation of this enhancer is also incomplete. The deletion analysis (Fig. 2) identified four regions that are required for efficient activation of the enhancer. These effects of these deletions may be caused by changing the spacing between known activator and/or repressor sites within the enhancer. However, it possible that these regions contain specific binding sites required for activation. Consistent with this, there are several well-conserved sequence blocks that might represent specific sites required for activation. Base-pair substitutions that disrupt the conserved sequences without changing site spacing will be used to initially test this.

We thank Gary Struhl for the FRT cassette and the β-tub-FLP flies; Miki Fujioka for the modified CaSpeR vector with glass-binding sites and flies containing the *eve* 1+5 reporter gene; Leslie Pick for the Gal4AD library used in the one-hybrid experiment, the pET-Slp1 expression vector and the embryonic extracts used for gel shift analysis; Tony Ip for the *sna* promoter fragment; Alain Vincent for the *croc* flies; and Manfred Frasch for the $slp^{\Delta 34B}$ flies. We thank members of Eric Chang's laboratory for advice on the yeast one-hybrid experiment, and members of the Small and Desplan laboratory for support. We thank Claude Desplan, Dot Clyde and Mary Anne Pultz for comments on the manuscript. L. P. A. was supported by a fellowship from the FAPESP organization in Brazil. This work was funded by NIH grant GM 51946, NSF grant 9982535 and the Margaret and Herman Sokol Foundation.

REFERENCES

Adams, M. D. Celniker, S. E. Holt, R. A. Evans, C. A. Gocayne, J. D. Amanatides, P. G. Scherer, S. E. Li, P. W. Hoskins, R. A. Galle, R. F. et al. (2000). The genome sequence of Drosophila melanogaster. *Science* **287**, 2185-2195.

Arnosti, D. N., Barolo, S., Levine, M. and Small, S. (1996). The eve stripe 2 enhancer employs multiple modes of transcriptional synergy. *Development* **122**, 205-214.

Cadigan, K. M., Grossniklaus, U. and Gehring, W. J. (1994). Localized expression of sloppy paired protein maintains the polarity of Drosophila parasegments. *Genes Dev.* **8**, 899-913.

Capovilla, M., Eldon, E. D. and Pirrotta, V. (1992). The giant gene of Drosophila encodes a b-ZIP DNA-binding protein that regulates the expression of other segmentation gap genes. *Development* **114**, 99-112.

Cirillo, L. A., Mcpherson, C. E., Bossard, P., Stevens, K., Cherian, S., Shim, E.-Y., Clark, E. A., Burley, S. K. and Zaret, K. S. (1998). Binding of the winged-helix transcription factor HNF3 to a linker histone site on the nucleosome. *EMBO J.* **17**, 244-254.

Clark, K. L., Haley, E. D., Lai, E. and Burley, S. (1993). Co-crystal structure of the HNF3/forkhead DNA recognition motif resembles histone H5. *Nature* **364**, 412-420.

Courey, A. J. and Jia, S. (2001). Transcriptional repression: the long and the short of it. *Genes Dev.* **15**, 2786-2796.

Frasch, M., Hoey, T., Rushlow, C., Doyle, H. and Levine, M. (1987). Characterization and localization of the even-skipped protein of Drosophila. *EMBO J.* **6**, 749-759.

Fujioka, M., Emi-Sarker, Y., Yusibova, G. L., Goto, T. and Jaynes, J. B. (1999). Analysis of an even-skipped rescue transgene reveals both composite and discrete neuronal and early blastoderm enhancers, and multi-stripe positioning by gap gene repressor gradients. *Development* **126**, 2527-2538.

Fujioka, M., Miskiewicz, P., Raj, L., Gulledge, A. A., Weir, M. and Goto, T. (1996). Drosophila Paired regulates late even-skipped expression through a composite binding site for the paired domain and the homeodomain. *Development* **122**, 2697-2707.

Goto, T., Macdonald, P. and Maniatis, T. (1989). Early and late periodic patterns of even skipped expression are controlled by distinct regulatory elements that respond to different spatial cues. *Cell* **57**, 413-422.

Gray, S., Szymanski, P. and Levine, M. (1994). Short-range repression permits multiple enhancers to function autonomously within a complex promoter. *Genes Dev.* **8**, 1829-1838.

Grossniklaus, U., Cadigan, K. M. and Gehring, W. J. (1994). Three maternal coordinate systems cooperate in the patterning of the Drosophila head. *Development* **120**, 3155-3171.

Grossniklaus, U., Pearson, R. K. and Gehring, W. J. (1992). The Drosophila sloppy paired locus encodes two proteins involved in segmentation that show homology to mammalian transcription factors. *Genes Dev.* **6**, 1030-1051.

Hacker, U., Kaufmann, E., Hartmann, C., Jurgens, G., Knochel, W. and Jackle, H. (1995). The Drosophila fork head domain protein crocodile is required for the establishment of head structures. *EMBO J.* **14**, 5306-5317.

Han, W., Yu, Y., Altan, N. and Pick, L. (1993). Multiple proteins interact with the fushi tarazu proximal enhancer. *Mol. Cell. Biol.* **13**, 5549-5559.

Harding, K., Hoey, T., Warrior, R. and Levine, M. (1989). Autoregulatory and gap gene response elements of the even-skipped promoter of Drosophila. *EMBO J.* **8**, 1205-1212.

Ip, Y. T., Park, R. E., Kosman, D., Yazdanbakhsh, K. and Levine, M. (1992). dorsal-twist interactions establish snail expression in the presumptive mesoderm of the Drosophila embryo. *Genes Dev.* **6**, 1518-1530.

Jiang, J., Hoey, T. and Levine, M. (1991a). Autoregulation of a segmentation gene in Drosophila: combinatorial interaction of the even-skipped homeo box protein with a distal enhancer element. *Genes Dev.* **5**, 265-277.

Jiang, J., Kosman, D., Ip, Y. T. and Levine, M. (1991b). The dorsal morphogen gradient regulates the mesoderm determinant twist in early Drosophila embryos. *Genes Dev.* **5**, 1881-1891.

Jimenez, G., Paroush, Z. and Ish-Horowicz, D. (1997). Groucho acts as a corepressor for a subset of negative regulators, including Hairy and Engrailed. *Genes Dev.* **11**, 3072-3082.

Kaufmann, E., Hoch, M. and Jackle, H. (1994). The interaction of DNA with the DNA-binding domain encoded by the Drosophila gene fork head. *Eur. J. Biochem.* **223**, 329-337.

Kobayashi, M., Goldstein, R. E., Fujioka, M., Paroush, Z. and Jaynes, J. B. (2001). Groucho augments the repression of multiple Even skipped target genes in establishing parasegment boundaries. *Development* **128**, 1805-1815.

Kosman, D. and Small, S. (1997). Concentration-dependent patterning by an ectopic expression domain of the Drosophila gap gene knirps. *Development* **124**, 1343-1354.

Kraut, R. and Levine, M. (1991). Mutually repressive interactions between the gap genes giant and Kruppel define middle body regions of the Drosophila embryo. *Development* **111**, 611-621.

Ludwig, M. Z., Patel, N. H. and Kreitman, M. (1998). Functional analysis of eve stripe 2 enhancer evolution in Drosophila: rules governing conservation and change. *Development* **125**, 949-958.

Macdonald, P. M., Ingham, P. and Struhl, G. (1986). Isolation, structure, and expression of even-skipped: a second pair- rule gene of Drosophila containing a homeo box. *Cell* **47**, 721-734.

Nibu, Y. and Levine, M. S. (2001). CtBP-dependent activities of the short-range Giant repressor in the Drosophila embryo. *Proc. Natl. Acad. Sci. USA* **98**, 6204-6208.

Nibu, Y., Zhang, H. and Levine, M. (2001). Local action of long-range repressors in the Drosophila embryo. *EMBO J.* **20**, 2246-2253.

Nusslein-Volhard, C., Kluding, H. and Jurgens, G. (1985). Genes affecting the segmental subdivision of the Drosophila embryo. *Cold Spring Harb. Symp. Quant. Biol.* **50**, 145-154.

Pani, L., Quian, X. B., Clevidence, D. and Costa, R. H. (1992). The restricted promoter activity of the liver transcription factor hepatocyte nuclear factor 3 beta involves a cell-specific factor and positive autoactivation. *Mol. Cell. Biol.* **12**, 552-562.

Pankratz, M. and Jackle, H. (1993). Blastoderm Segmentation. In *The Development of* Drosophila melanogaster (ed. M. Bate and A. Martinez-Arias), pp. 467-516. New York: Cold Spring Harbor Laboratory Press.

Paroush, Z., Finley, R. L., Jr, Kidd, T., Wainwright, S. M., Ingham, P. W., Brent, R. and Ish-Horowicz, D. (1994). Groucho is required for Drosophila neurogenesis, segmentation, and sex determination and interacts directly with hairy-related bHLH proteins. *Cell* **79**, 805-815.

Paroush, Z., Wainwright, S. M. and Ish-Horowicz, D. (1997). Torso signalling regulates terminal patterning in Drosophila by antagonising Groucho-mediated repression. *Development* **124**, 3827-3834.

Pirrotta, V. (1988). Vectors for P-mediated transformation in Drosophila. *Biotechnology* **10**, 437-456.

Ronchi, E., Treisman, J., Dostatni, N., Struhl, G. and Desplan, C. (1993). Down-regulation of the Drosophila morphogen bicoid by the torso receptor-mediated signal transduction cascade. *Cell* **74**, 347-355.

Sackerson, C. (1995). Patterns of conservation and divergence at the even-skipped locus of Drosophila. *Mech. Dev.* **51**, 199-215.

Simpson-Brose, M., Treisman, J. and Desplan, C. (1994). Synergy between the hunchback and bicoid morphogens is required for anterior patterning in Drosophila. *Cell* **78**, 855-865.

Small, S. (1997). Mechanisms of segmental pattern formation in Drosophila melanogaster. In *Progress in Developmental Biology*, Vol. VII (ed. J. Collier), pp. 137-178. New Delhi: Oxford and IBH Publishing.

Small, S. (2000). In vivo analysis of lacZ fusion genes in transgenic Drosophila melanogaster. *Methods Enzymol.* **326**, 146-159.

Small, S., Kraut, R., Hoey, T., Warrior, R. and Levine, M. (1991). Transcriptional regulation of a pair-rule stripe in Drosophila. *Genes Dev.* **5**, 827-839.

Small, S., Blair, A. and Levine, M. (1992). Regulation of even-skipped stripe 2 in the Drosophila embryo. *EMBO J.* **11**, 4047-4057.

Small, S., Arnosti, D. N. and Levine, M. (1993). Spacing ensures autonomous expression of different stripe enhancers in the even-skipped promoter. *Development* **119**, 762-772.

Small, S., Blair, A. and Levine, M. (1996). Regulation of two pair-rule stripes by a single enhancer in the Drosophila embryo. *Dev. Biol.* **175**, 314-324.

Spradling, A. (1986). P-element mediated transformation. In *Drosophila: A Practical Approach* (ed. D. B. Roberts): Oxford: IRS Press.

Stanojevic, D., Hoey, T. and Levine, M. (1989). Sequence-specific DNA-binding activities of the gap proteins encoded by hunchback and Kruppel in Drosophila. *Nature* **341**, 331-335.

Stanojevic, D., Small, S. and Levine, M. (1991). Regulation of a segmentation stripe by overlapping activators and repressors in the Drosophila embryo. *Science* **254**, 1385-1387.

Struhl, G., Fitzgerald, K. and Greenwald, I. (1993). Intrinsic activity of the Lin-12 and Notch intracellular domains in vivo. *Cell* **74**, 331-345.

Vavra, S. H. and Carroll, S. B. (1989). The zygotic control of Drosophila pair-rule gene expression. I. A search for new pair-rule regulatory loci. *Development* **107**, 663-672.

Weigel, D., Jurgens, G., Kuttner, F., Seifert, E. and Jackle, H. (1989). The homeotic gene fork head encodes a nuclear protein and is expressed in the terminal regions of the Drosophila embryo. *Cell* **57**, 645-658.

Wu, X., Vakani, R. and Small, S. (1998). Two distinct mechanisms for differential positioning of gene expression borders involving the Drosophila gap protein giant. *Development* **125**, 3765-3774.

Yu, Y., Yussa, M., Song, J., Hirsch, J. and Pick, L. (1999). A double interaction screen identifies positive and negative ftz gene regulators and ftz-interacting proteins. *Mech. Dev.* **83**, 95-105.