The gypsy insulator can function as a promoter-specific silencer in the *Drosophila* embryo

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The *Drosophila* gypsy retrotransposon disrupts gene activity by blocking the interactions of distal enhancers with target promoters. This enhancer-blocking activity is mediated by a 340 bp insulator DNA within gypsy. The insulator contains a cluster of binding sites for a zinc finger protein, suppressor of Hair wing [su(Hw)]. Recent studies have shown that a second protein, mod(mdg4), is also important for normal insulator function. Mutations in mod(mdg4) exert paradoxical effects on different gypsy-induced phenotypes. For example, it enhances *yellow*² but suppresses *cut*⁶. Here, we employ a stripe expression assay in transgenic embryos to investigate the role of mod(mdg4) in gypsy insulator activity. The insulator was inserted between defined enhancers and placed among divergently transcribed reporter genes (*white* and *lacZ*) containing distinct core promoter sequences. These assays indicate that mod(mdg4) is essential for the enhancer-blocking activity of the insulator DNA. Moreover, reductions in mod(mdg4)⁺ activity cause the insulator to function as a promoter-specific silencer that selectively represses *white*, but not *lacZ*. The repression of *white* does not affect the expression of the closely linked *lacZ* gene, suggesting that the insulator does not propagate changes in chromatin structure. These results provide an explanation for why mod(mdg4) exerts differential effects on different gypsy-induced mutations.

Keywords: *Drosophila*/gypsy/insulator/promoter-specific silencer

Introduction

Insulator DNAs are thought to isolate genetic loci by blocking interactions between *cis* regulatory elements and inappropriate target promoters in neighboring transcription units (Kellum and Schedl, 1991; Chung *et al.*, 1993). The best characterized 'authentic' insulators correspond to *scs* and *scs'*, which flank the *Drosophila hsp70* locus (Kellum and Schedl, 1991). In addition, a variety of studies suggest that a 340 bp DNA element contained within the gypsy retrotransposon also functions as an insulator (Geyer *et al.*, 1986; Geyer and Corces, 1992). Although this insulator has no known cellular counterpart, it behaves in a fashion that is indistinguishable from *scs* and *scs'* in enhancer-blocking assays (see below).

We recently devised a stripe expression assay in transgenic embryos to characterize insulator DNAs (Cai and Levine, 1995). This assay was used to show that the *scs*, *scs'* and gypsy insulators selectively block the expression

of distal, not proximal, *even-skipped* (*eve*) stripe enhancers when positioned between divergently transcribed reporter genes. Enhancers barred from acting on one basal promoter are able to activate the other promoter, suggesting that insulators do not propagate changes in chromatin structure. Insulator DNAs appear to lack an intrinsic polarity, and are equally effective in blocking distal enhancers whether they are positioned in the same or opposite orientation relative to the target transcription unit. Insulators can quantitatively modulate enhancer–promoter interactions, in that a portion of the *scs* element only partially blocks distal stripe enhancers. Finally, insulator DNAs may possess some regulatory specificity. For example, the gypsy insulator selectively blocks the activators, but not silencers, contained within the composite ventral repression element from the *zerknüllt* (*zen*) promoter region (Jiang *et al.*, 1992, 1993; Cai *et al.*, 1996).

It has been proposed that insulators segregate DNA into distinct chromatin loop domains (Galloni *et al.*, 1993; Udvardy and Schedl, 1993; Corces, 1995). Evidence for this type of model stems from the demonstration that *scs*, *scs'* and the gypsy insulator element contain a series of DNase I hypersensitive sites (Udvardy *et al.*, 1985; Udvardy and Schedl, 1993; Vazquez and Schedl, 1994). However, it is equally plausible that insulators function as autonomous, specialized DNA elements which attenuate or block distal enhancers. Insulators might block the tracking of distal enhancers by bending DNA, and mimicking the structural changes mediated by the binding of the TFIID complex to the promoter (Dunaway and Droge, 1989; Shen *et al.*, 1994; Oelgeschlager *et al.*, 1996). Alternatively, insulators might contain binding sites for 'pairing proteins' that are required for the looping of distal enhancers to the transcription complex (Georgiev and Corces, 1995; Zhou *et al.*, 1996).

As a first step towards determining how insulators block distal enhancers, we have analyzed the expression of synthetic gene complexes containing the gypsy insulator in various mutant embryos. The minimal, 340 bp element contains a cluster of 12 tightly linked binding sites for a *Drosophila* zinc finger protein, suppressor of Hair wing [su(Hw)] (Geyer *et al.*, 1988; Parkhurst *et al.*, 1988; Spana *et al.*, 1988; Harrison *et al.*, 1993; Kim *et al.*, 1996). The binding of the su(Hw) protein to the insulator is required for the blocking of distal enhancers, as well as the mutagenic effects of the gypsy retrotransposon (Harrison *et al.*, 1989; Hoover *et al.*, 1992; Smith and Corces, 1992; Dorsett, 1993).

Recent studies have shown that su(Hw) interacts with a protein encoded by the *mod(mdg4)* locus, which was first identified as an enhancer of position effect variegation (Georgiev and Gerasimova, 1989; Dorn *et al.*, 1993; Gerasimova *et al.*, 1995; Georgiev and Kozycina, 1996). The functional significance of this protein–protein inter-

Fig. 1. Gypsy-induced mutations in *yellow* and *cut*. Wings and notums were dissected from adult flies. The notums (A and B) are oriented with anterior up, while the wings (C and D) are oriented with distal to the left. Schematic diagrams of the *y* and *ct* loci are also shown. The spiral circles in the gypsy elements represent su(Hw) binding sites. The tissue-specific enhancers (hatched circles) in the *y* promoter are: wing (*w*), body cuticle (*c*), mouth part (*m*) and bristles (*b*). The wing margin enhancer in the *ct* promoter is indicated by the hatched box (*w**m*). (A) Notum of an adult fly that is homozygous for the *y*² mutation, which is due to the insertion of a gypsy element as shown in the diagram. Both the microchaete and macrochaete bristles have normal, black pigmentation. (B) Notum of an adult fly that is doubly homozygous for *y*² and the hypomorphic mutation, *mod(mdg4)*^{ul}. There is a loss of pigmentation in the bristles, presumably due to a general repression of *y* gene activity. (C) Wing of an adult fly that is homozygous for *ct*⁶, which results from the insertion of a gypsy element as shown in the diagram. There is a loss of wing margin tissue in distal and posterior regions. (D) Wing of an adult fly that is doubly homozygous for *ct*⁶ and the *mod(mdg4)*^{ul} mutation. The *cut* phenotype is suppressed and the wing margin is restored.

action is unclear. In some cases it appears that *mod(mdg4)* is simply required for gypsy insulator activity. For example, the phenotype caused by a gypsy insertion in the promoter region of the *cut* gene is suppressed by a mutation in *mod(mdg4)* (Georgiev and Gerasimova, 1989; Georgiev and Kozycina, 1996). This observation suggests that the loss of *mod(mdg4)* function permits the distal wing margin enhancer, located upstream of the gypsy insertion, to interact with the *cut* promoter (see Figure 1). In contrast, the same *mod(mdg4)* mutation enhances the phenotype caused by a gypsy insertion in the promoter region of the *yellow* locus (Georgiev and Gerasimova, 1989; Georgiev and Kozycina, 1996). In this case, tissue-specific enhancers located both upstream and downstream of gypsy appear to be repressed (Gerasimova *et al.*, 1995; see Figure 1).

To investigate the seemingly paradoxical effects of *mod(mdg4)* mutants on gypsy insulator function, we examined transgenic embryos expressing fusion promoters with defined enhancers and divergently transcribed test promoters. Mutations in *mod(mdg4)* cause distal enhancers to be derepressed on both the *white* and *eve/lacZ* test promoters, suggesting that *mod(mdg4)* is essential for gypsy insulator function. Moreover, there is a general repression of *white*, suggesting that the gypsy insulator

functions as a promoter-specific silencer in *mod(mdg4)* mutants. This selective repression is observed even when the gypsy insulator is located far from the *white* promoter. In contrast, the closely linked *eve/lacZ* gene, which contains a distinct basal promoter sequence, is not repressed in *mod(mdg4)* mutants, suggesting that silencing of the *white* gene does not involve long-range changes in chromatin structure. These studies provide the first evidence for promoter-specific silencing, and offer an explanation for why *mod(mdg4)* mutants suppress some gypsy-induced mutations, but enhance others.

Results

A hypomorphic mutation in *mod(mdg4)*, *mod(mdg4)*^{ul} (Georgiev and Gerasimova, 1989; Gerasimova *et al.*, 1995; Georgiev and Kozycina, 1996), exerts paradoxical effects on two different gypsy-induced mutations (Figure 1). It enhances the *y*² phenotype, so that there is a loss of pigmentation in mesothoracic bristles (Figure 1B; compare with A). Conversely, *mod(mdg4)*^{ul} suppresses the *ct*⁶ phenotype, resulting in the restoration of the wing margin (Figure 1D; compare with C).

Both the *yellow* and the *cut* promoter regions contain a series of tissue-specific enhancers (Jack, 1985; Geyer

Fig. 2. The *mod(mdg4)* protein is required for gypsy insulator activity. Transgenic embryos are undergoing cellularization and are oriented with anterior to the left and dorsal up. These embryos express one of two fusion genes, indicated by the diagrams below (A–D) and (E–H). The fusion gene in the upper panels (A–D) contains the *eve* stripe 3 (E3) and stripe 2 (E2) enhancers separated by the 340 bp gypsy insulator DNA (SU). The fusion gene in the lower panels (E–H) contains a 400 bp spacer sequence (T0.4) in place of the insulator. Embryos were hybridized either with a digoxigenin-labeled *white* antisense RNA probe or *lacZ* probe. (A) Pattern of *white* expression obtained with the E3–SU–E2 fusion gene in embryos derived from *mod(mdg4)^{u1}* homozygous females. Only residual stripes are observed (compare with the control embryo in E, which was stained in parallel). Note that the distal E2 directs weak expression, suggesting both a breakdown in insulator function and general repression of the *white* promoter. (B) Same as (A), except that the fusion gene was expressed in embryos derived from wild-type flies. The proximal E3 directs *white* expression within the limits of *eve* stripe 3. The distal E2 is blocked. (C) Pattern of *lacZ* expression in a wild-type embryo. The proximal E2 directs a stripe 2 pattern. The distal E3 is severely attenuated so that there is only a residual stripe 3 pattern. The anterior-most stripe in the head is due to vector sequences. (D) Same as (C), except that the fusion gene was expressed in an embryo derived from a *mod(mdg4)^{u1}* homozygous female. This results in a substantial derepression of the distal E3 so that *lacZ* is expressed within the limits of both stripes 2 and 3. The weak stripe 7 pattern is also due to the derepression of E3. (E) *white* expression pattern of the E3–T0.4–E2 fusion gene in a *mod(mdg4)^{u1}* mutant embryo. Both E2 and E3 direct a composite staining pattern consisting of stripes 2 and 3. Staining is substantially stronger than that observed with a fusion gene containing the gypsy insulator (see A). This result suggests that the general repression of *white* is due to the presence of the insulator. (F) Same as (E), except that the fusion gene is expressed in a wild-type embryo. (G) *lacZ* staining pattern in a wild-type embryo. Both E2 and E3 direct *lacZ* expression in a composite stripe 2–stripe 3 pattern. The weak, posterior stripe 7 is due to E3. (H) Same as (G), except that the fusion gene was crossed into a *mod(mdg4)^{u1}* mutant. There is no substantial change in the staining pattern as compared with wild-type embryos. Augmented expression in stripe 7 (compare with G) might be due to slight differences in embryonic age.

et al., 1986; Geyer and Corces, 1987; Jack and DeLotto, 1995). In the y^2 mutant, gypsy is inserted between an upstream body cuticle enhancer and a downstream bristle enhancer. In the y^2 mutant (Figure 1A), the pigmentation of the body cuticle is yellow but the bristles are dark, indicating that the enhancers located upstream of gypsy are blocked while the proximal bristle-specific enhancer is active. The mutant phenotype is enhanced in flies doubly mutant for y^2 and *mod(mdg4)^{u1}*, in that both the cuticle and the bristles are yellow (Figure 1B, compare with A), indicating that the proximal bristle-specific enhancer is inactivated. It has been suggested that this loss of function of the proximal enhancer is caused by the conversion of the gypsy insulator into a bidirectional repressor (Gerasimova *et al.*, 1995). The *ct^f* mutant contains a gypsy insertion between the *cut* promoter and a distal wing-margin enhancer that maps nearly 90 kb upstream of the promoter. This enhancer is blocked in the *ct^f* mutant, resulting in the loss of wing margin tissue (Figure 1C). In contrast, the enhancer appears to be reactivated in the double mutant, *ct^f* and *mod(mdg4)^{u1}*, resulting in the restoration of the wing margin (Figure 1D, compare with C).

The enhancer-blocking assay (Cai and Levine, 1995) was used to investigate how *mod(mdg4)* mutants exert these opposing effects on *yellow* and *cut* gene activity. The 340 bp gypsy insulator was placed between divergently transcribed *white* and *lacZ* reporter genes. The promoter-proximal sequences associated with these reporters are distinct. The leftward *white* gene lacks a canonical TATA (O'Hare *et al.*, 1984), while the rightward *lacZ* gene is driven by a small segment of the *eve* promoter, which contains an optimal TATA element (Frasch and Levine, 1987). Transgenic embryos were hybridized with either digoxigenin-labeled *white* or *lacZ* antisense RNA probes to visualize the expression of the reporter genes.

***eve* stripe assay**

The insulator was placed between the 500 bp *eve* stripe 3 enhancer ('E3') and the 480 bp *eve* stripe 2 enhancer ('E2'). As shown previously, the insulator selectively blocks interactions between the distal enhancer and target promoter (Cai and Levine, 1995). The proximal E3 directs the expression of the leftward *white* reporter gene within the limits of stripe 3 (Figure 2B), but the distal E2 is

Fig. 3. Promoter-specific activities of the gypsy silencer. Transgenic embryos express one of two fusion genes (see diagrams below A–D and E–H), and are oriented with anterior to the left and dorsal up. Embryos were hybridized with *white* or *lacZ* antisense RNA probes as described in Figure 2. (A) *white* expression pattern obtained with the 2PE–SU–E3 fusion gene in a *mod(mdg4)^{u1}* mutant. Both the mesoderm-specific 2PE and E3 enhancers are attenuated due to a general repression of the *white* promoter. There is residual expression mediated by the proximal 2PE and distal E3, suggesting both a breakdown in insulator activity as well as a general repression of *white*. (B) Same as (A), except that the fusion gene is expressed in a wild-type embryo. The distal E3 is blocked, but the proximal 2PE directs strong staining in ventral regions. (C) *lacZ* staining pattern mediated by the 2PE–SU–E3 fusion gene in a wild-type embryo. The distal 2PE is blocked, while the proximal E3 directs a strong stripe 3 pattern. (D) Same as (C), except that the fusion gene is expressed in a *mod(mdg4)^{u1}* mutant. The distal 2PE is derepressed, resulting in a composite staining pattern consisting of a central segmentation stripe and a band of staining in the ventral mesoderm. (E) *white* expression pattern obtained with the 2PE–λ1.4–E3 fusion gene in a *mod(mdg4)^{u1}* mutant. The proximal 2PE directs essentially normal levels of expression in the presumptive mesoderm, but the distal E3 is somewhat attenuated by the large spacer sequence used in this experiment. However, this reduction in stripe 3 staining is not due to the *mod(mdg4)^{u1}* mutation, since a weak stripe is also observed in wild-type embryos (e.g. F). (F) Same as (E), except that the fusion gene is expressed in a wild-type embryo. The 2PE and E3 direct a composite pattern of *white* expression although, as indicated above, the large spacer DNA used in this experiment reduces the distal E3. (G) *lacZ* expression pattern obtained with the 2PE–λ1.4–E3 fusion gene in a wild-type embryo. Both the proximal E3 and distal 2PE interact with the *eve/lacZ* promoter, resulting in an additive staining pattern. (H) Same as (G), except that the fusion gene is expressed in embryos derived from *mod(mdg4)^{u1}* homozygous females. There is no change in the staining pattern.

blocked. Conversely, the proximal E2 directs the expression of the rightward *lacZ* reporter within the limits of stripe 2, while the distal E3 is severely attenuated (Figure 2C). The head stripe observed with the *lacZ* probe is due to cryptic regulatory sequences in the P-element transformation vector (Small *et al.*, 1992).

Distinct patterns of expression are observed when this fusion promoter is expressed in embryos derived from *mod(mdg4)^{u1}* homozygous females (Figure 2A and D). There is a severe reduction in *white* expression (Figure 2A), suggesting that both the distal E2 and proximal E3 are ineffective in activating the *white* promoter (compare with Figure 2B). Moreover, there is residual expression of the *white* reporter gene within the limits of both stripes 2 and 3, suggesting that the distal E2 is derepressed. These results suggest both a loss of gypsy insulator function, and a general repression of *white*.

Embryos derived from *mod(mdg4)^{u1}* homozygous mothers exhibit intense *lacZ* expression (Figure 2D), even though the closely linked *white* gene is repressed. Staining is detected within the limits of stripes 2, 3 and 7, although stripes 3 and 7 are somewhat weaker than stripe 2 (stripe 7 is regulated by sequences within E3; Small *et al.*, 1996). This result indicates that the distal E3 is severely derepressed (Figure 2D; compare with Figure 2C). A

similar derepression of the *lacZ* reporter gene is observed in hypomorphic mutations in *su(Hw)* (Cai and Levine, 1995; data not shown). Thus, both *mod(mdg4)* and *su(Hw)* are required for gypsy insulator function.

Control experiments were done to determine whether the repression of the *white* promoter seen in *mod(mdg4)^{u1}* mutants (Figure 2A) depends on the presence of the gypsy insulator DNA. For this purpose, we examined a fusion promoter that contains E3 and E2 separated by a 400 bp spacer sequence ('T0.4'; see diagram below Figure 2E–H). This spacer permits both enhancers to interact with both the leftward *white* promoter (Figure 2F) and the rightward *eve/lacZ* gene (Figure 2G) in wild-type embryos. These staining patterns are not altered in embryos derived from females homozygous for the *mod(mdg4)^{u1}* mutation (Figure 2E and H). In particular, the *white* reporter is expressed within the limits of stripes 2 and 3, indicating that the *mod(mdg4)^{u1}* mutation does not cause a general silencing of *white* when the gypsy insulator is replaced by a random spacer sequence (Figure 2E).

Analysis of additional embryonic enhancers

The gypsy insulator was placed between additional combinations of embryonic enhancers. The results obtained with a fusion promoter containing a mesoderm-specific

Fig. 4. Analysis of *rhomboid* and *hairy* enhancers. Transgenic embryos express one of two fusion genes indicated below (A–D) and (E–H). The embryos were hybridized and oriented as described in Figures 2 and 3. (A) Pattern of *white* expression mediated by the NEE–SU–H1 fusion gene in a *mod(mdg4)^{u1}* mutant. Weak lateral stripes are observed, suggesting that both the proximal NEE and distal H1 are blocked due to a general repression of the *white* promoter. (B) Same as (A), except that the fusion gene is expressed in a wild-type embryo. The proximal NEE directs strong lateral stripes in the presumptive neuroectoderm, while the distal H1 enhancer is blocked. (C) Pattern of *lacZ* expression obtained with the NEE–SU–H1 fusion gene in a wild-type embryo. The proximal H1 directs intense expression in the head, while the distal NEE is attenuated (compare with D). (D) Same as (C), except that the fusion gene is expressed in a *mod(mdg4)^{u1}* mutant. The distal NEE is derepressed, as indicated by the strong lateral stripes. The proximal H1 continues to direct strong expression in the head. These results suggest that the gypsy insulator is disrupted in the *mod(mdg4)* mutant. (E) The *white* expression pattern obtained with the NEE–λ1.4–H1 fusion gene in an embryo derived from a *mod(mdg4)^{u1}* homozygous female. The proximal NEE and distal H1 direct an additive pattern of expression, although the distal H1 might be slightly attenuated by the large λ spacer DNA. (F) Same as (E), except that the fusion gene is expressed in a wild-type embryo. The pattern is essentially identical to that observed in the mutant. (G) The *lacZ* staining pattern obtained with the fusion gene in a wild-type embryo. Both enhancers work to give a robust composite staining pattern. (H) Same as (G), except that the fusion gene is expressed in a *mod(mdg4)^{u1}* mutant. There is no discernible change in the staining pattern as compared with wild-type.

enhancer ('2PE') and E3 are presented in Figure 3. These enhancers normally direct a composite staining pattern consisting of a central segmentation stripe and a band of staining along the ventral surface (Figure 3F and G). As seen for E2–E3 fusion genes, the gypsy insulator selectively blocks distal, but not proximal, enhancers (Figure 3B and C). The insulator blocks interactions between the distal E3 and *white* promoter, so that expression is mediated only by the proximal 2PE enhancer in the ventral mesoderm (Figure 3B). Conversely, the *eve/lacZ* reporter gene is expressed within the limits of stripe 3, while the distal 2PE is blocked (Figure 3C).

The activities of this fusion promoter are altered in *mod(mdg4)^{u1}* mutants (Figure 3A and D). As before, there is a general repression of *white*, so that both the distal E3 and proximal 2PE fail to direct normal levels of expression (Figure 3A). The weak stripe 3 pattern suggests that the distal E3 is derepressed, although it is difficult to assess E3–*white* interactions since E3 is a weak enhancer that is quite sensitive to general spacing effects (see below). Nonetheless, reduced expression of the proximal 2PE enhancer indicates a general repression of the *white* promoter. In contrast, the rightward *lacZ* reporter gene is fully active, so that both the proximal E3 and the distal 2PE direct a composite staining pattern consisting of a central stripe and a ventral band (Figure 3D). The

mod(mdg4)^{u1} mutation causes a nearly complete derepression of the distal 2PE enhancer, in that staining in ventral regions is nearly as intense as that observed for fusion promoters lacking the insulator (Figure 3D, compare with H; see below).

Control experiments involved the use of a large, 1.4 kb, spacer DNA from λ (see diagram below Figure 3E–H). Unfortunately, this spacer causes a reduction in E3–*white* interactions, leading to a relatively weak stripe 3 pattern (Figure 3F). This spacer does not reduce 2PE–*eve/lacZ* interactions on the *lacZ* reporter gene (Figure 3G). Moreover, the same spacer does not impede interactions between *white* and a different enhancer, *hairy* H1 (see below). These results, as well as previous studies (e.g. Cai and Levine, 1995), suggest that E3 is inherently 'weak' and easily blocked. There is no significant change in the composite staining pattern when this fusion gene is expressed in embryos derived from *mod(mdg4)^{u1}* females (Figure 3E and H; compare with F and G, respectively). These observations suggest that the selective repression of *white* observed in *mod(mdg4)^{u1}* mutants depends on the presence of the gypsy insulator.

We also analyzed a fusion promoter that contains the 330 bp *rhomboid* lateral stripe enhancer ('NEE'; Ip *et al.*, 1992) and a 200 bp *hairy* stripe 1 enhancer ('H1'; see Zhou *et al.*, 1996). The two enhancers direct a composite

Fig. 5. The gypsy insulator can mediate long-range repression. Transgenic embryos contain the fusion gene diagrammed below. The *eve* stripe 3 enhancer (E3) is located 5' of both the *white* and *lacZ* test promoters. A 700 bp DNA fragment was placed downstream of the *lacZ* transcription unit. This fragment contains both the *rhomboid* NEE and a copy of the 340 bp gypsy insulator. The NEE is inactive and not shown in the diagram of the fusion gene. Embryos were hybridized and oriented as described in the preceding figures. (A) *white* staining pattern obtained in a wild-type embryo. A strong stripe 3 pattern is observed; there is also a weak stripe 7 pattern. (B) Same as (A), except that the fusion gene is expressed in an embryo derived from a *mod(mdg4)^{u1}* homozygous female. There is a slight, but consistent, reduction in the stripe 3 pattern. (C) The *lacZ* staining pattern in a wild-type embryo. Strong expression is observed within the limits of stripe 3. There is also a weaker stripe 7 pattern and staining in the head due to vector sequences. (D) Same as (C), except that the fusion gene is expressed in the *mod(mdg4)^{u1}* mutant. There is no change in the pattern relative to the wild-type control.

pattern of expression, consisting of a head stripe (H1) and lateral stripes (NEE; e.g. Figure 4G). The gypsy insulator selectively blocks or attenuates the distal enhancer so that the NEE directs lateral stripes of *white* expression (Figure 4B), while H1 directs an intense head stripe of *lacZ*; the distal NEE is attenuated, but not completely blocked (Figure 4C). In this regard, we note that the NEE is most resistant to inhibition by the insulator among the various enhancers that have been tested in this assay.

As before, the *mod(mdg4)^{u1}* mutation exerts differential effects on the *white* and *lacZ* staining patterns. Both proximal and distal enhancers interact with the *eve/lacZ* gene, thereby resulting in a composite *lacZ* staining pattern which includes intense NEE lateral stripes (Figure 4D). In contrast, there is a general repression of *white*, so that the proximal NEE directs weaker lateral stripes in the mutant background as compared with normal embryos (Figure 4A, compare with B). The use of the 1.4 kb spacer sequence indicates that the repression of *white* depends on the presence of the gypsy insulator (Figure 4A and E).

***gypsy* can function as a long-range repressor**

Additional experiments were conducted to determine whether the gypsy insulator can work over long distances to repress *white*. For this purpose, we examined a fusion gene containing E3 positioned between the leftward *white* gene and rightward *lacZ* gene. The insulator was placed downstream of the *lacZ* transcription unit, >4 kb away from the *eve/lacZ* transcription start site, and even farther from the *white* promoter (see diagram below Figure 5).

The insulator does not interfere with the activities of E3 in wild-type embryos (Figure 5A and B), so that both reporter genes are expressed within the limits of stripe 3. The weak stripe 7 pattern is mediated by regulatory sequences contained within E3 (Small *et al.*, 1996; see Figure 2).

The *lacZ* staining pattern is not altered when this fusion gene is expressed in embryos derived from *mod(mdg4)^{u1}* homozygous females (Figure 5D; compare with B). In contrast, there is a subtle, but reproducible reduction in *white* expression (Figure 5C; compare with A). Control stainings were done with a comparable fusion gene lacking the insulator DNA; no reduction in stripe 3 expression was observed (data not shown). While it appears that the insulator can silence *white* over long distances, we note that this repression is far less efficient than that obtained when the insulator is located near *white* (e.g. Figure 2A).

Analysis of a mod(mdg4) null mutant

mod(mdg4) encodes a nuclear protein with a BTB domain that mediates protein-protein interactions, including interactions with su(Hw) (Dorn *et al.*, 1993; Gerasimova *et al.*, 1995). The *mod(mdg4)^{u1}* allele is caused by the insertion of a Stalker transposon into C-terminal sequences of the coding region (Gerasimova *et al.*, 1995). This insertion could result in the synthesis of truncated proteins, which might function in a dominant-negative or neomorphic fashion. To investigate this possibility, we examined a null mutation that lacks the promoter and first exon of the coding region.

Fig. 6. Reduced levels of *mod(mdg4)*⁺ gene activity disrupt insulator function. Embryos express the transgene shown in the diagram. It is the same as that used in Figure 2A–D. The fusion gene contains the *eve* stripe 3 (E3) and stripe 2 (E2) enhancers separated by the 340 bp gypsy insulator DNA. Embryos are presented as described in the preceding figures. (A) The *white* expression pattern in an embryo derived from a heterozygous female carrying one copy of the wild-type gene and one copy of the *mod(mdg4)*^{u1} mutation. The proximal E3 directs a stripe 3 pattern, while E2 is blocked. (B) Same as (A), except that the embryo was stained to reveal the *lacZ* pattern. The proximal E2 directs a strong stripe 2 pattern, whereas the distal E3 is severely attenuated. (C) The *white* expression pattern in an embryo derived from a *mod(mdg4)*^{u1/mod(mdg4)}^{u1} homozygous female (same embryo as in Figure 2A). The staining pattern is weak, presumably due to attenuated interactions of both enhancers. The weak stripe 2 pattern suggests a derepression of the distal E2. (D) Same as (C), except that the *lacZ* pattern is being monitored. Stripes 2 and 3 are observed, indicating a derepression of the distal E3 due to a breakdown in insulator activity. Because stripe 3 is somewhat weaker than stripe 2, it would appear that the insulator retains some activity. (E) The *white* staining pattern observed in an embryo derived from a transheterozygous female containing a *mod(mdg4)*^{u1} allele and the null allele *E(var)93D-P142.4D15*. There is a general repression of *white*, including attenuation of the proximal E3. (F) Same as (E), except that the *lacZ* pattern is visualized. There is a reduction in insulator activity so that both E2 and the distal E3 are active.

The insulator selectively blocks the interaction of the distal E2 with the leftward *white* reporter gene (Figure 6A), and attenuates the interaction of the distal E3 with the rightward *lacZ* reporter (Figure 6B). The insulator works equally well in wild-type embryos and embryos derived from *mod(mdg4)*^{u1/+} heterozygous females (Figure 6A; compare with Figure 2B). As shown previously, the *lacZ* staining pattern is derepressed and *white* expression attenuated in embryos derived from *mod(mdg4)*^{u1} homozygous females (Figure 6C and D). Very similar staining patterns are observed in embryos derived from transheterozygous females, which contain one copy of the *mod(mdg4)*^{u1} allele and one copy of the null allele (Figure 6E and F). Once again, there is a derepression of the distal E3 on the *lacZ* gene (Figure 6F) and a general repression of *white* (Figure 6E). Similar staining patterns were observed in embryos derived from null homozygous oocytes (data not shown), which were produced as germ line clones using the flip–frt method (Chou and Perrimon, 1992). These results suggest that it is the reduction in the levels of the *mod(mdg4)* protein, and not a change in the nature of the protein, which alters the behavior of the gypsy insulator in *mod(mdg4)*^{u1} mutants.

It would appear that *mod(mdg4)* is required for the normal function of the gypsy insulator DNA since

mod(mdg4) mutants result in a derepression of distal enhancers on the *eve/lacZ* promoter (e.g. Figure 6D and F). A similar loss of insulator function is observed when the same fusion gene is expressed in embryos carrying a transheterozygous combination of different *su(Hw)* hypomorphic alleles. In these mutants, both the E3 and E2 enhancers are active on both the *white* and *lacZ* promoters (Cai and Levine, 1995; data not shown). Thus, both *su(Hw)* and *mod(mdg4)* are required for normal insulator activity; however, *mod(mdg4)* mutants result in the repression of the *white* promoter, while *su(Hw)* mutants do not.

Discussion

The gypsy retrotransposon disrupts gene function by blocking the interactions of distal enhancers with target promoters. Extragenic modifiers of gypsy-induced mutations have been identified, and among these the *mod(mdg4)* gene is the most paradoxical since it suppresses the effects of some gypsy insertions, but enhances others. We have presented evidence that *mod(mdg4)* is essential for normal insulator activity. Moreover, the gypsy insulator functions as a promoter-specific silencer in *mod(mdg4)*^{u1} mutant embryos. Selective repression of *white*, but not *lacZ*, is observed even when the insulator is positioned far from both promoters. Repression of *white* does not

Fig. 7. Summary of su(Hw)–mod(mdg4) interactions. wt refers to the gypsy insulator complex formed in a wild-type embryo. Functional interactions between su(Hw) and mod(mdg4) cause this bidirectional transcription complex to be divided into separate domains, whereby leftward enhancers (E1) interact with the leftward promoter and rightward enhancers (E2) interact with the rightward promoter. mod[−] refers to the situation observed in embryos containing reduced levels of mod(mdg4) protein. The su(Hw)–mod(mdg4) insulator complex is disrupted so that both enhancers E1 and E2 can interact with both the leftward and rightward promoters. In addition, it is possible that the su(Hw) protein can, in the absence of competing mod(mdg4), interact with an unknown repressor protein ('?'; see Discussion). This repressor selectively silences some promoters (in this case the leftward promoter), but not others (e.g. the rightward promoter).

influence the expression of a closely linked *eve/lacZ* gene, suggesting that the insulator does not occlude binding of upstream activators through general changes in chromatin structure such as chromosome condensation. Instead, promoter-specific repression can explain why *mod(mdg4)^{ul}* suppresses the *ct⁶* mutation but enhances *y²*.

Mechanism of insulator function

A model summarizing the results of this study is presented in Figure 7. As shown previously, the zinc finger su(Hw) protein binds to multiple sites within the 340 bp gypsy insulator DNA (Spana *et al.*, 1988; Harrison *et al.*, 1993; Kim *et al.*, 1996). It would appear that mod(mdg4) lacks a DNA-binding domain and requires specific protein–protein interactions with su(Hw) in order to interact with the insulator (Gerasimova *et al.*, 1995). The su(Hw)–mod(mdg4) protein complex divides the bidirectional *white*–*lacZ* gene complex into two domains. Enhancers to the left of the insulator (e.g. 'E1'; see Figure 7) interact with the leftward target promoter, while enhancers to the right of the insulator complex ('E2') interact with the rightward promoter. As discussed earlier, it is unclear whether this separation of the leftward and rightward transcription units into separate domains involves the formation of chromatin loops. It has been proposed that endogenous insulators located near the site of integration would interact with the su(Hw)–mod(mdg4) complex to separate the two genes into distinct nuclear compartments (Corces, 1995). An alternative view is that the su(Hw)–mod(mdg4) complex mimics the basal promoter region, for example by bending the DNA, similar to the structural changes induced by the binding of the TFIID complex to TATA (Oelgeschläger *et al.*, 1996).

Reduction or loss of *mod(mdg4)* activity causes the su(Hw) insulator to mediate promoter-specific repression

of the *white* gene. In the absence of *mod(mdg4)* function, it is possible that su(Hw) interacts with a different, unknown protein ('?'), which selectively represses *white*. Moreover, because *mod(mdg4)* is absent, the insulator no longer blocks distal enhancers, so both the E1 and E2 enhancers can interact with both promoters, particularly the rightward *eve/lacZ* reporter gene.

The promoter-specific repression of *white* might explain why the *mod(mdg4)^{ul}* mutation enhances *y²*, but suppresses *ct⁶*. Perhaps the *y* and *white* promoters are repressed, while *cut* and *eve/lacZ* are refractory to gypsy-mediated silencing. It is possible that proximal sequences in the *white* and *y* promoters are responsible for mediating this repression.

Modes of repression

Previous studies suggest that there may be two basic forms of repression in the early *Drosophila* embryo, short-range and long-range. Short-range repressors function over distances of just 50–100 bp to inhibit, or quench, neighboring activators bound within an enhancer (Gray *et al.*, 1994; Arnosti *et al.*, 1996; Gray and Levine, 1996). This form of repression permits enhancer autonomy in modular promoters, so that a short-range repressor bound to a given enhancer does not interfere with the activities of other enhancers contained within the same promoter region. Several spatially localized repressors have been shown to function in this manner, including the zinc finger repressor snail, which is a mesoderm determinant, and the segmentation repressor Kruppel, which defines the posterior border of *eve* stripe 2. Short-range repressors do not exhibit promoter specificity. For example, Kruppel represses the stripe 2 enhancer and establishes the posterior stripe 2 border on both the *white* and *eve/lacZ* reporter genes (e.g. Gray and Levine, 1996).

Long-range repressors can function over distances of several kilobases to block the assembly or function of the basal transcription complex (for review, see Cai *et al.*, 1996). Such repressors function in a dominant fashion, and block the activities of multiple enhancers in modular promoters (Jiang *et al.*, 1992, 1993). The dorsal protein can mediate long-range repression through a 600 bp silencer element, the VRE, located within the promoter region of *zen*. The VRE contains several dorsal binding sites and closely linked negative response elements (NREs). Dorsal is inherently an activator, but mediates repression by recruiting 'co-repressors' to the NREs (Lehming *et al.*, 1994). The VRE can function over distances of several kilobases to repress the ventral expression of the *eve* stripe 2 and stripe 3 enhancers (Cai *et al.*, 1996). The long-range, dominant repression activity of the VRE does not appear to exhibit promoter specificity. Thus, the VRE represses the ventral expression of both *eve* stripes when monitoring either the *white* reporter gene or *lacZ*.

The repression mediated by the gypsy insulator seems to be distinct from previously characterized short-range and long-range repressors. It can function over long distances, >4 kb, to attenuate E3–*white* interactions (see Figure 6). However, unlike the *zen* VRE, the gypsy insulator appears to function in a promoter-specific fashion, and does not interfere with *lacZ* expression.

Mechanisms of repression

The gypsy-mediated silencing observed in *mod(mdg4)^{ul}* does not appear to involve general changes in chromatin structure such as chromosome condensation. For example, the *lacZ* reporter gene is fully active in situations where the nearby *white* promoter is repressed. This result suggests that the insulator does not interfere with the binding of upstream activators to neighboring enhancers. Previous genetic studies have identified a number of suppressors of *mod(mdg4)* mutants, including *su(var)205* (Dorn *et al.*, 1993), which encodes HP1, a constituent of heterochromatin (James *et al.*, 1989). Gerasimova *et al.* (1995) have proposed that HP1 mediates general silencing of the *y* promoter region in the absence of *mod(mdg4)* gene activity. Genetic tests are consistent with the notion that this repression of *y* involves a heterochromatin-mediated process. Our studies do not address the issue of heterochromatin, but strongly suggest that the conversion of the gypsy insulator DNA into a silencer does not involve long-range changes in chromatin structure.

Materials and methods

Drosophila strains and genetic crosses

yw⁶⁷ flies were used for all P-transformation injections. This strain was also used as the wild-type stock for control stainings. Females of genotype *y² sc¹ w^{AG} c^P*; *mod(mdg4)^{ul}* (kindly provided by Tatiana Gerasimova and Victor Corces, see also Gerasimova *et al.*, 1995) were mated with *yw⁶⁷* males to generate *mod(mdg4)^{ul}* heterozygous and *mod(mdg4)⁺* flies carrying gypsy insertional mutations on the X chromosome. To generate a transheterozygous strain for *mod(mdg4)^{ul}* and the *E(var)93D* deficiency, the above stock was crossed with *E(var)93D-P142.4D15 TM3 Sb/TM6* (kindly provided by Manfred Frasch, see Azpiazu and Frasch, 1993) which contains a 1 kb deletion removing the promoter and first exon of the gene. Virgin females of the indicated genotype were mated with wild-type males carrying the P-transposon. Embryos were collected and fixed as described previously (Jiang *et al.*, 1992).

Cuticle preparations

Wings were dissected from wild-type and mutant flies, and mounted in a drop of glycerol. Notum was boiled in 10% KOH for 10 min and washed in H₂O (Ashburner, 1989). Following serial dehydration in 50, 70 and 100% ethanol, they were rinsed in xylene and mounted in Permount (Fisher, SP15-100). The mounted cuticles were photographed using Nomarski optics.

P-element transformation and whole mount in situ hybridization

Fusion genes were made by inserting test enhancers into the unique *EcoRI* site of a P-element transformation vector that contains the *eve* basal promoter (from -42 bp), a 100 bp untranslated leader sequence and the first 22 codons of the coding region fused in-frame to the *lacZ* gene (pEb vector; Small *et al.*, 1992; S.Small, unpublished data). This CaSpeR derivative (Thummel *et al.*, 1988) contains the *mini-white* gene transcribed in the opposite orientation relative to the linked *eve/lacZ* fusion gene. P-element fusion genes were introduced into the *Drosophila* germ line as described in Small *et al.* (1992). Multiple transformant lines were generated for each construct, and at least three independent lines were tested. Hybridizations using digoxigenin-UTP-labeled antisense RNA probes to *lacZ* or *white* were performed exactly as described by Jiang *et al.* (1991).

Construction of *eve/lacZ* P-element transposons

The E3-SU-E2 fusion gene shown in Figures 2 and 6 is described in Cai and Levine (1995). The E3-T0.4-E2 gene containing a 400 bp DNA fragment from the *twist* promoter region (located between the DE and PE *cis* regulatory elements; see Jiang *et al.*, 1991) was kindly provided by Steve Small. The 2PE-SU-E3 gene (Figure 3) was prepared with a 500 bp *eve* stripe 3 enhancer (Small *et al.*, 1993) and a 560 bp DNA fragment containing two tandem copies of the *twist* PE ('proximal

element'; see Jiang *et al.*, 1991; Jiang and Levine, 1993). These DNAs were co-ligated into the unique *EcoRI* site in the pEb vector, creating a new *NotI* site between the two enhancers. The correct orientation of the enhancers was confirmed by DNA sequencing. Either the 340 bp gypsy insulator DNA or a 1.4 kb *EcoRI* fragment from λ was inserted into the *NotI* site using a *NotI* linker. The NEE-SU-H1 and NEE- λ 1.4-H1 fusion genes (Figure 4) were prepared with the 330 bp *rhomboid* NEE (Ip *et al.*, 1992; Gray *et al.*, 1994; Gray and Levine, 1996) and a 200 bp *hairly* stripe 1 enhancer (kindly provided by Gary Struhl; see Zhou *et al.*, 1996). These DNAs were co-ligated into the unique *EcoRI* site of the pEb vector, creating a new *NotI* site between the two enhancers. The position and orientation of the enhancers were confirmed by DNA sequencing. The 340 bp gypsy insulator or 1.4 kb λ spacer was inserted into the *NotI* site. The E3-SU fusion gene shown in Figure 5 was prepared with a 700 bp DNA fragment containing both the 340 bp gypsy insulator and the 330 bp NEE. This DNA was ligated into the unique *XbaI* site located just downstream of the *lacZ* transcription unit. The NEE is not active on either the *white* or *lacZ* genes from this distant location.

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