Differences in insulator properties revealed by enhancer blocking assays on episomes

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Insulators are genomic elements that define domains of transcriptional autonomy. Although a large number of insulators have been isolated, it is unclear whether these elements function by shared molecular mechanisms. Novel applications of FLP recombinase technology were used to dissect and compare the function of the *Drosophila* gypsy and scs insulators. Interactions between FLP monomers bound to chromosomally integrated FRT sites were unimpeded by either insulator, demonstrating that these insulators do not establish a chromosomal environment capable of disrupting all types of protein–protein interactions. The gypsy insulator blocked enhancer-activated transcription on FLP-generated extra-chromosomal episomes, whereas the scs insulator displayed silencing effects. These data indicate that these insulators differ in the mechanisms used to prevent enhancer function. That the gypsy insulator blocked enhancer–promoter communication within small episomes suggests that these effects may be accomplished without a global reorganization of chromatin structure. Instead, the gypsy insulator may disrupt enhancer-activated transcription by direct interference with transmission of the enhancer signal to the promoter.

Keywords: *Drosophila*/enhancers/gene expression/insulators/transcriptional regulation

Introduction

Eukaryotic genomes have large amounts of DNA, extending to lengths of nearly 2 m, housed within nuclei with diameters of ~5 µm. Extensive condensation is required for nuclear DNA placement, accomplished by assembling DNA into nucleosomes that form higher order chromatin structures, providing a compaction of 200- to 1000-fold (Lawrence *et al.*, 1990). The manner in which DNA is packaged and organized within the nucleus has an impact on cellular processes, such as transcription. Most transcriptional events are regulated by control elements that reside in large, complex regions that act over long distances to elicit changes in the activity of a target promoter. While packaging of DNA into chromatin may facilitate regulatory interactions by decreasing linear distances, a secondary consequence is an increased proximity of control elements that may lead to inappropriate cross-regulation. Recent studies suggest that eukaryotic genomes contain a class of sequences, known

as insulators, which restrict promiscuous interactions between long distance regulatory elements and may help to maintain transcriptional fidelity.

Insulators have been isolated from a number of organisms, including humans, mice, chickens and *Drosophila* (reviewed in Geyer, 1997; Kellum and Elgin, 1998; Bell and Felsenfeld, 1999; Dorsett, 1999; Udvardy, 1999). These sequences possess two defining properties. First, insulators protect gene expression from chromosomal position effects that result from ectopic placement of genes within the genome. Both positive and negative position effects are prevented by insulator action (Kellum and Schedl, 1991; Roseman *et al.*, 1993, 1995; Cuvier *et al.*, 1998). Secondly, insulators block enhancer function in a position-dependent manner. Enhancer-activated transcription is only impeded when an insulator is interposed between an enhancer and promoter, but not when the insulator is positioned upstream of an enhancer (Holdridge and Dorsett, 1991; Geyer and Corces, 1992; Kellum and Schedl, 1992; Cai and Levine, 1995). Disruption of enhancer–promoter interactions does not interfere with promoter function, leaving basal transcription of the target promoter intact (Roseman *et al.*, 1993; Scott and Geyer, 1995). Similarly, enhancer blocking is not accompanied by enhancer silencing (Cai and Levine, 1995; Scott and Geyer, 1995). Taken together, these data suggest that insulators interfere with the mechanism by which enhancers communicate with promoters. While most insulators display a general enhancer blocking capacity, at least one insulator shows enhancer specificity (Ohtsuki and Levine, 1998). The widespread distribution of insulators suggests that they might be fundamental components of eukaryotic genomes that organize functional domains within chromosomes.

Several insulators have been isolated from the *Drosophila* genome. The gypsy insulator was identified as the enhancer blocking component of the gypsy retrotransposon (Holdridge and Dorsett, 1991; Geyer and Corces, 1992). This insulator is unusual because it is composed of 12 binding sites for a single DNA-binding protein, Suppressor of Hairy-wing [Su(Hw)], which is essential for insulator function (Modolell *et al.*, 1983; Parkhurst *et al.*, 1988; Spana *et al.*, 1988). In some, but not all, cases the gypsy insulator requires a second protein, Modifier of mdg4 [Mod(mdg4)], for enhancer blocking (Georgiev and Gerasimova, 1989). The Mod(mdg4) protein is a non-DNA-binding protein that appears to be recruited to the insulator by the Su(Hw) protein (Dorn *et al.*, 1993; Cai and Levine, 1995; Gerasimova *et al.*, 1995; Georgiev and Kozycina, 1996; Gerasimova and Corces, 1998). Another set of *Drosophila* insulators are the specialized chromatin structures sequences, scs and scs' (Kellum and Schedl, 1991, 1992). These insulators were identified as regions of unusual chromatin structure that

demarcate the borders of decondensed chromatin that result from heat shock-induced transcription of the 87A7 *hsp70* genes (Udvardy *et al.*, 1985). *scs* and *scs'* contain multiple, partially redundant sequence elements that are responsible for insulator function (Vazquez and Schedl, 1994; Zhao *et al.*, 1995; Dunaway *et al.*, 1997). Several proteins associate with these insulators: *scs* binding protein/Zeste-white 5 (SBP/Zw5) with *scs* (Gaszner *et al.*, 1999) and boundary element-associated factors 32A and 32B (BEAF) and DREF with *scs'* (Hart *et al.*, 1997, 1999; Cuvier *et al.*, 1998). The gypsy and *scs/scs'* insulators are good models for understanding mechanisms of insulator function, as these insulators display robust enhancer blocking activity for a large number of enhancers in both *Drosophila* and other species.

To investigate the basis of insulator disruption of enhancer-activated transcription, we addressed two questions. First, we determined whether the insulators prevent interactions between any type of protein bound on different sides of an insulator-defined domain. We examined gypsy insulator effects on the function of the yeast FLP recombinase because this protein is involved in a non-transcriptional process that requires interactions between proteins bound at separate FLP recombination target sites (FRTs) for enzymatic function (Lee and Jayaram, 1997). Secondly, we examined whether chromosomal integration was needed for insulators to block enhancer function. While previous transient transfection studies suggested that the gypsy insulator prevented enhancer action on a plasmid (Holdridge and Dorsett, 1991), enhancer blocking was accompanied by a loss of promoter activity, leaving open the possibility that in this assay system unusual properties of the gypsy insulator were revealed. In our studies the off-chromosomal effects of the gypsy insulator were examined using a system to generate stable, single copy episomes during *Drosophila* development (Ahmad and Golic, 1996). For comparison, both assays were extended to the *scs* insulator.

We found that neither the gypsy nor the *scs* insulator impeded FLP-mediated recombination. These data show that insulator activity is not absolute, because conditions exist whereby interactions between proteins cannot be prevented. Interestingly, the episomal properties of the gypsy and *scs* insulators were distinct. The gypsy insulator blocked enhancer activity outside a chromosomal context, whereas the *scs* insulator showed silencing effects. These observations indicate that these insulators may impart enhancer blocking by different mechanisms. The implications of our findings are discussed in the context of prevailing models of insulator function.

Results

The gypsy insulator does not disrupt interactions between pairs of FLP monomers

An FLP recombinase assay system was used to determine whether the gypsy insulator prevented long range interactions between proteins that are not transcriptional regulators (Figure 1; Golic and Lindquist, 1989). FLP catalyzes efficient recombination in *Drosophila* without utilization of any accessory proteins (Golic and Lindquist, 1989). This recombinase is well suited for a protein interaction assay because recombination requires inter-

Fig. 1. The FLP recombinase assay. (A) The details of the FLP recombination catalytic reaction are illustrated. FLP recombination (FLP assembly) involves association of a pair of FLP monomers (yellow circles) to the FRT sites (half arrows) on the *white* reporter gene (red rectangle). Pairs of FLP monomers interact to form an active quaternary complex (FLP interaction), allowing excision of the intervening *white* gene onto an episome (Excision). A solo FRT remains in the chromosome (half arrow in wavy line). Excision of the reporter gene in mitotically active cells leads to its loss (noted as the X). (B) Representative eye phenotypes are shown for flies carrying the *white* reporter gene and that either did not (-FLP) or did express (+FLP) FLP recombinase early in development. (C) Levels of FLP recombinase activity in transformed lines carrying FRT-flanked *white* reporter genes with different insulator insertions. (Left) The structures of the reporter genes are shown, with the positions of the gypsy (black triangle) and *scs* (spotted triangle) insulators indicated. (Right) Summary of the level of FLP recombination in independent transformed lines examined in *su(Hw)*⁺ (gray bar) and *su(Hw)*⁻ (black bar) backgrounds. FLP recombinase levels were measured as the percentage of flies with mosaic eyes relative to the total number of flies scored per cross. Each line was assayed three times and the mean plotted; thin lines represent standard deviations of the mean (<15%). Values for P[Gypsy-In] and P[Scs-In] were not significantly different from those for P[Gypsy-Out] ($P = 0.116$ and 0.081 , respectively).

action between pairs of FLP monomers bound at distant FRT sites to form a quaternary protein complex that comprises the active enzyme (Figure 1A; Lee and Jayaram, 1997). We reasoned that if the gypsy insulator prevented general protein-protein interactions, then FLP recombination levels would decrease when a gypsy insulator was located between FRTs.

Levels of FLP recombination were monitored using a *hsp70-white* gene flanked by FRTs. The *white* gene encodes a transport protein required for the import of pigment precursors (Dreesen *et al.*, 1988) and is an

advantageous reporter gene for two reasons. First, its expression is cell autonomous, allowing assessment of *white* activity in individual cells. Secondly, the level of eye pigmentation is a sensitive indicator of the level of *white* transcription. High levels of transcription produce red eyed flies, whereas low levels of transcription produce yellow eyed flies.

Two *white* reporter transgenes were generated that carried insertions of the gypsy insulator either outside the FRT sites flanking the *white* gene (*P[Gypsy-Out]*) or between FRTs within the first *white* intron (*P[Gypsy-In]*, Figure 1C). These transposons were injected into γ^-w^- embryos and transformants were identified by the restoration of eye coloration. Only transformed lines carrying single, independent insertions were analyzed further.

To assay FLP recombinase activity, males carrying the FRT-flanked *white* reporter gene were crossed to females carrying a heat shock-inducible source of FLP, *P[hsFLP]1* (Golic and Lindquist, 1989). FLP recombinase was induced early in development, causing excision of the *white* gene as an episome in a portion of the cells. This episome was subsequently lost, due to insufficient replication and segregation. Recombination levels were quantified by calculating the percentage of adult flies that showed patches of white eye tissue on a pigmented background, which represented an indirect measure of FLP protein–protein interactions (Figure 1B).

Levels of *white* excision in flies carrying the *P[Gypsy-Out]* transposon were examined to determine FLP recombinase activity when the insulator was located in a position outside the FRT sites. Five independent transformed lines were studied. The percentage of adult flies with mosaic eyes ranged from 42 to 64% (average 54%, Figure 1C). This variable level of recombination may reflect differences in FLP accessibility to FRTs caused by distinct chromatin structures assembled at different genomic insertion sites (Ahmad and Golic, 1996). To confirm that the gypsy insulator in *P[Gypsy-Out]* had no effect on FLP-induced excision, the level of recombination in a *P[Gypsy-Out]* line was tested in a *su(Hw)* mutant background. If the gypsy insulator did not interfere with recombination, then excision levels should be unchanged in the absence of the insulator protein. We found that the recombination frequency in *P[hsFLP]1; P[Gypsy-Out]; su(Hw)* flies was the same as that obtained in wild-type flies (Figure 1C). These results demonstrate that the outside location of the gypsy insulator did not limit recombinase activity, even though this insulator was in close proximity to an FRT.

The level of FLP recombination in *P[Gypsy-In]* flies was determined to assess the effects of placing the pairs of FLP monomers in independent gypsy insulator-defined domains. In the four independent lines analyzed, the percentage of adult flies that had mosaic eyes ranged from 36 to 56% (average 43%, Figure 1C), which was similar to the range obtained for *P[Gypsy-Out]*. These data indicate that the gypsy insulator does not interfere with FLP recombination. This conclusion was verified by testing the level of FLP recombination for a *P[Gypsy-In]* line in a *su(Hw)* mutant background. The level of FLP recombination was unchanged in *P[hsFLP]1; P[Gypsy-In]; su(Hw)* flies (Figure 1C), establishing that the gypsy insulator

protein does not block interactions between distantly bound pairs of FLP monomers.

For comparison, the effects of the *scs* insulator were examined. We constructed *P[Scs-In]*, which carried a *scs* insulator inserted at the same site in the *white* intron that was used for the gypsy insulator. In the four independent lines analyzed, recombination levels were similar to those obtained for *P[Gypsy-In]*, ranging from 32 to 54% (average 42%, Figure 1C). These experiments demonstrate that chromosomally integrated *scs* insulators do not impede FLP recombination.

Enhancer action is supported on an episome

An episomal enhancer blocking assay was developed to test whether the gypsy insulator required chromosomal integration to disrupt enhancer–promoter communication. Episomes containing the *white* gene were generated using FLP recombinase, as described above, except that episomes were produced in post-mitotic cells, where they remain stable and can be transcribed (Ahmad and Golic, 1996). Expression of only excised *white* genes was examined because the chromosomally integrated *P* transposons contained a rearranged *white* gene in which 3' *white* sequences were cloned upstream of 5' sequences, making it non-functional (Figure 2A; Ahmad and Golic, 1996). Recombination between two strategically placed FRTs excised the *white* gene onto an episome, regenerating its structural organization and restoring *white* gene expression (Figure 2A).

A second reporter gene, *vermillion* (Fridell and Searles, 1991), was carried on the *P* transposons that carried the rearranged *white* gene (hereafter referred to as episomal transposons). This gene was located outside the FRTs and was not included in the FLP-excised DNA. The episomal transposons were injected into $v^-;ry^-$ embryos and transformants were identified by restoration of *vermillion* function. Only independent transformed lines carrying a single transposon were characterized. In all cases, evaluation of *white* gene expression was accomplished by crossing males carrying the chromosomally integrated episomal transposon to w^- , *P[hsFLP]1* females. Heat shocks were administered during late larval/early pupal development and the eye phenotype of the resulting adult male progeny was assessed.

Previous studies of *white* gene expression on an episome included *hsp70*–*white* fusion genes that lacked the eye enhancer (Ahmad and Golic, 1996), making it uncertain whether small episomes supported enhancer-activated transcription. To address this issue we generated three transposons: *P[mini-Off]*, *P[EnOff]* and *P[EnYellowOff]* (Figure 2B). The name of each transposon reflects the order of its components, such as the eye enhancer (En) and *yellow* DNA (Yellow), relative to the *white* promoter. Off indicates that *white* gene expression was studied on an episome. The mini-Off episome contained a *mini-white* gene with 300 bp of 5' flanking DNA and was expected to direct a basal level of *white* expression (Roseman *et al.*, 1993). The EnOff and EnYellowOff episomes carried a *white* gene with the eye enhancer inserted 0.3 or 5.5 kb 5' of the promoter, respectively. In the EnYellowOff episome, the 5.5 kb separation of the enhancer and promoter was achieved by insertion of the intronless *yellow* gene. This gene served as

Fig. 2. Effects of the eye enhancer on expression of an episomal *white* gene. (A) Shown is a diagram of the strategy employed to produce episomal *white* reporter genes. The episomal transposons carried two genes: a rearranged *white* gene and the *vermilion* gene (brown rectangle). Chromosomally integrated transposons produce no eye pigmentation (white eyed fly). FLP recombinase excises the *white* gene, restoring its structure and function. Expression of FLP recombinase in post-mitotic cells generates stable episomes that are able to direct productive *white* transcription (red eyed fly). (B) Representative examples of eye phenotypes of flies carrying *white* episomes are shown above the corresponding structure of the FLP-generated episome. Each episome carries a *white* gene with an intronic insertion of an FRT site (arrowhead). Additional DNA sequences included on some episomes are the *white* eye enhancer (red oval) and the intronless *yellow* gene with its corresponding wing and body enhancers (yellow ovals and rectangle). Other symbols are as described in Figure 1.

neutral spacer DNA in this episome, as *yellow* regulatory sequences do not affect *white* expression (data not shown). It was predicted that both the EnOff and EnYellowOff episomes should direct enhancer-activated transcription. Furthermore, if episomes supported enhancer function from a distance, then flies carrying the EnYellowOff and EnOff episomes should have the same eye phenotype.

Four independent *P[mini-Off]* transformed lines were analyzed. In all cases, flies carrying the FLP-induced mini-Off episome had an orange eye color (Figure 2B; Table I). This eye color was identical to that seen in flies carrying a chromosomal insertion of the enhancerless *mini-white* (Roseman *et al.*, 1993), confirming that the mini-Off episome directs a basal level of *white* transcription. The expression of *white* in flies carrying the EnOff episomes was examined in seven independent transformed lines. In six lines EnOff flies had red eyes, while flies from the seventh line had brown eyes (Figure 2B; Table I). These eye colors were the same as those observed in flies that carried chromosomally integrated enhancer-activated

Table I. Summary of eye color phenotypes obtained from independent transformed lines carrying an FLP-generated episome

Construct	Red ^a	Brown ^b	Orange ^c	Yellow ^d	Total
mini-Off	0	0	4	0	4
EnOff	6	1	0	0	7
EnYellowOff	5	0	0	0	5
GypEnOff	2	0	0	0	2
EnGypOff	9	3	0	0	12
GypEnGypOff	0	0	3	0	3
EnGypGypOff	4	0	0	0	4
GypGypEnOff	2	0	0	0	2
GypYellowEnGypOff	0	0	4	0	4
ScsEnOff	0	3	0	0	3
EnScsOff	0	0	3	0	3
ScsEnScsOff	0	0	0	3	3
Scs-mini-Off	0	0	1	3	4
ScsEnGypOff	0	3	0	0	3

^aWild-type *white* gene expression.

^bNear wild-type expression.

^cBasal expression.

^dBelow basal expression.

white transgenes flanked by gypsy insulators (Roseman *et al.*, 1993), suggesting that the red and brown eye colors represent the wild-type and near wild-type levels of gene transcription, respectively. These data demonstrate that the episomal eye enhancer is capable of enhancer-activated transcription. That enhancer action occurred at a distance was confirmed by examination of the eye phenotypes produced from five independent transformed lines of *P[EnYellowOff]*. In all cases, flies carrying the EnYellowOff episome had a red eye color (Figure 2B; Table I). These findings validate the approach of using FLP-induced episomes to study the effects of insulators on enhancer-promoter interactions.

Block of an episomal enhancer requires two gypsy insulators flanking the eye enhancer

The effects of insertion of a single gypsy insulator on the episome were tested. A gypsy insulator (Gyp) was inserted either upstream of the enhancer, *P[GypEnOff]*, or between the eye enhancer and *white* promoter, *P[EnGypOff]*. Two independent *P[GypEnOff]* lines were analyzed. In both cases flies carrying the GypEnOff episome had red eyes (Figure 3; Table I). This phenotype indicates that the upstream gypsy insulator does not prevent enhancer-activated transcription. Insertion of the gypsy insulator between the eye enhancer and promoter also failed to block the enhancer. Of the 12 *P[EnGypOff]* transformed lines studied, flies from nine lines had red eyes and flies from the three remaining lines had brown eyes (Figure 3; Table I), reflecting wild-type or near wild-type levels of gene expression. To confirm that the gypsy insulator positioned between the enhancer and promoter had no effect on enhancer function, *white* expression on the episome was examined in three *P[EnGypOff]* lines in a *su(Hw)*⁻ background. In all cases, the eye phenotypes of *P[EnGypOff]*, *su(Hw)*⁻ flies were the same as those observed for parental *su(Hw)*⁺ flies, demonstrating that a single insulator did not prevent enhancer-activated transcription (data not shown).

We reasoned that the lack of effect of a single insulator inserted between the eye enhancer and promoter was

Fig. 3. Enhancer blocking effects of the gypsy insulator on the episome. Representative examples of eye phenotypes of flies carrying *white* episomes with insertion of the gypsy insulator are shown above the corresponding structure of the FLP-generated episome. The three columns on the left indicate eye phenotypes in a *su(Hw)*⁺ background, whereas the last column indicates eye phenotypes in a *su(Hw)*[−] background.

caused by enhancer action in the 3′ direction (counter-clockwise) relative to the *white* promoter. This possibility was addressed by construction of a *P[GypEnGypOff]* transposon that contained the eye enhancer flanked by gypsy insulators. Three independent transformed lines were obtained and tested. In all cases, the eye color of flies carrying the GypEnGypOff episome was orange (Figure 3A; Table I), a phenotype similar to that found in flies carrying the enhancerless mini-Off episome (Figure 2B). From these data we infer that flanking the enhancer with gypsy insulators completely blocked enhancer-activated transcription. To verify that the low level of *white* expression on the GypEnGypOff episome required the Su(Hw) protein, the level of *white* expression in a *su(Hw)* mutant background was examined for two *P[GypEnGypOff]* lines. The eye color of *su(Hw)*[−] flies carrying the GypEnGypOff episome increased to the level observed in flies carrying the EnOff episome, confirming the Su(Hw) protein requirement (Figures 2 and 3).

We tested the possibility that the placement of two gypsy insulators on the episome may indirectly reduce episomal transcription because of the large amount of Su(Hw) protein bound, an effect that would be lost in a *su(Hw)* mutant background. We reasoned that if the amount of bound Su(Hw) protein was responsible for the low level of *white* expression in GypEnGypOff flies, then transcription should be similarly decreased in any episome carrying two gypsy insulators, irrespective of their location. To this end we constructed *P[EnGypGypOff]* and *P[GypGypEnOff]* transposons. Four *P[EnGypGypOff]* and

two *P[GypGypEnOff]* transformed lines were obtained and analyzed (Figure 3; Table I). Flies carrying either episome had a red eye color that was indistinguishable from flies carrying the EnOff episome, demonstrating that high levels of transcription on the episome remain possible when two insulators are present. These data verify that the episomal block of enhancer-activated transcription requires that gypsy insulators flank the enhancer. Furthermore, they show that the gypsy insulator does not require chromosomal integration to prevent enhancer–promoter communication.

An increased episome size does not influence enhancer blocking

The size of the GypEnGypOff episome was 5.5 kb. We wondered whether the structure or size of the excised DNA would influence enhancer blocking. To address this issue, a *P[GypYellowEnGypOff]* transposon was constructed. This episome carries an insertion of the intronless *yellow* gene between the two gypsy insulators, increasing the episome size to 10 kb (Figure 3A). Four *P[GypYellowEnGypOff]* transformed lines were obtained and tested. In all cases, flies carrying a *P[GypYellowEnGypOff]* episome had an orange eye color, indicating that robust enhancer blocking was maintained (Figure 3; Table I). The enhancer block was lost in *su(Hw)*[−] flies carrying the GypYellowEnGypOff episome, confirming that decreased expression required the Su(Hw) protein and was not caused by inclusion of the *yellow* gene between insulators (Figure 3). These data show that off-chromosomal enhancer blocking by the gypsy insulator is not affected by the size or structure of the episome.

The episomal behavior of the *scs* insulator differs from that of the gypsy insulator

The effect of the *scs* insulator in our episomal enhancer blocking assay was determined. For these studies two transposons were constructed that contained a single *scs* insulator inserted either upstream of the enhancer (*P[ScsEnOff]*) or between the eye enhancer and promoter (*P[EnScsOff]*).

Three independent *P[ScsEnOff]* lines were obtained and studied. In all cases, flies carrying the ScsEnOff episome had a brown eye color (Figure 4; Table I), indicating a near wild-type level of *white* expression. Surprisingly, no line showed a wild-type, red eye color. These results contrast with those obtained for the EnOff, GypEnOff and GypGypEnOff episomes, wherein 10 of 11 lines showed a red eye color (Table I). We postulated that this difference might reflect a slight repressive effect of the 5′ *scs* insulator. This supposition was supported by our results on the effects of a single *scs* insulator inserted between the enhancer and promoter. Three independent transformed lines of *P[EnScsOff]* were studied (Figure 4; Table I). In all cases, flies carrying an EnScsOff episome had an orange eye phenotype, suggesting that a single *scs* insulator inserted between the eye enhancer and promoter dramatically reduced *white* expression, even though the eye enhancer was unimpeded in the 3′ direction.

The *P[ScsEnScsOff]* transposon was constructed to resolve whether the *scs* insulator in the EnScsOff episome actually silenced *white* expression or whether it simply conferred a complete enhancer block. We reasoned that if

Fig. 4. Effects of the scs insulator on enhancer blocking on the episome. Representative examples of eye phenotypes of flies carrying *white* episomes with insertion of the scs insulator alone or in combination with the gypsy insulator are shown above the structure of the corresponding FLP-generated episome.

a total enhancer block was enforced, then placement of a second insulator upstream of the enhancer should not further reduce *white* expression. If this prediction was met, then flies carrying a ScsEnScsOff episome should have an eye color similar to flies carrying EnScsOff episomes. Three independent *P[ScsEnScsOff]* lines were obtained and analyzed. In all cases, flies with the ScsEnScsOff episome had a yellow eye color, implying that *white* expression was repressed to a level lower than basal transcription (Figure 4; Table I).

To explore further the silencing effects of the scs insulator on an episome, a *P[Scs-mini-Off]* transposon was constructed, wherein a single scs insulator was inserted upstream of the minimal *white* promoter. In three of the four lines analyzed, Scs-mini-Off episomal flies had a yellow eye color, while the fourth line had an orange eye color (Figure 4; Table I). These data suggest that a single scs insulator shifts the level of enhancerless *white* transcription to below basal levels, again implying silencing.

A final experiment was undertaken to test the effects of a single scs insulator on the episome. These experiments were based on previous observations that two different insulators can work in concert to define independent domains of gene function (Kellum and Schedl, 1991, 1992; Hagstrom *et al.*, 1996; Namciu *et al.*, 1998). We postulated that the gypsy and scs insulators might similarly collaborate, such that if the scs insulator could confer enhancer blocking on an episome, then it should block the 3' enhancer activity present in the EnGypOff episomes. As the eye phenotype associated with enhancer blocking (in the GypEnGypOff episome) was substantially lighter than that observed in flies carrying an insertion of the scs

insulator upstream of the eye enhancer (in the ScsEnOff episome), we reasoned that cooperative enhancer blocking by both insulators should be discernible. For this reason, *P[ScsEnGypOff]* was constructed. Three independent lines were isolated and examined. In contrast to our prediction, flies from all three lines had a brown eye phenotype, indicating a slightly reduced level of *white* expression, as seen in flies with the ScsEnOff episome (Figure 4; Table I). From these data we infer that the gypsy and scs insulators did not cooperate to confer enhancer blocking. Considering all of the scs episomal results, we suggest that the scs insulator displays novel properties on an episome, causing silencing of *white* gene expression. In addition, we found no evidence for enhancer blocking by the episomal scs insulator.

Discussion

A large number of insulators have been isolated from several eukaryotic genomes. It is unclear whether all of these insulators use identical molecular mechanisms to confer regulatory independence. To address this question, two novel applications of FLP technology were used to elucidate additional properties of the *Drosophila* gypsy and scs insulators.

The gypsy and scs insulators fail to block general protein–protein interactions

The FLP recombination assay examined whether insulators generally prevent interactions between proteins bound in different insulator-defined chromosomal domains. We found that FLP protein–protein interactions were unimpeded by insertion of either the gypsy or scs insulator between FRT sites (Figure 1). Our observations extend those of a previous plasmid-based *Xenopus* oocyte assay of scs function (Dunaway *et al.*, 1997), demonstrating that even within a chromosomal context, the scs insulator does not affect levels of FLP recombination. Recombination between FRT sites on homologous chromosomes occurs predominantly in the G₁ and G₂ phases of the cell cycle (Beumer *et al.*, 1998), the periods when active transcription occurs. These data imply that the failure of the gypsy and scs insulators to block recombination does not reflect differences in the cell cycle timing between recombination and transcription.

While the effects of the gypsy and scs insulators on enhancer–promoter interactions appear general, several examples of enhancer bypass of an insulator have recently been reported (Barolo and Levine, 1997; Morris *et al.*, 1998; Scott *et al.*, 1999; Zhou and Levine, 1999). One determinant of bypass is enhancer strength, with stronger enhancers able to direct transcription in the presence of an intervening insulator (Vazquez and Schedl, 1994; Scott *et al.*, 1999). For this reason it is possible that FLP protein interactions are so strong that they overcome the gypsy and scs insulators. However, we note that FLP recombinase represents the only non-transcriptional protein to be directly assayed for effects of insulators on protein–protein interactions. Thus, an alternative explanation for the failure of the gypsy and scs insulators to block FLP recombination may be that these insulators are specialized for disrupting interactions between transcription factors. This premise is compatible with observations that the

gypsy insulator protects a chromosomal DNA replication origin from chromosomal position effects, because the chromosomal modifiers of this origin were not characterized (Lu and Tower, 1997). Additionally, transcriptional activators have been found to play a role in regulating the function of a number of replication origins (van der Vliet, 1996). Regardless of which proposal is correct, our data show that insulator activity is not absolute, because conditions exist whereby interactions between proteins cannot be prevented.

The chromatin structure of an episome is sufficient to establish a gypsy insulator block of enhancer-activated transcription

An episomal blocking assay was used to evaluate the requirement for chromosomal integration for function of the gypsy and scs insulators (Figure 2). Episomes carrying the *white* gene were generated by FLP recombinase in post-mitotic cells. One strength of this system is that effects of the gypsy and scs insulators were studied in nuclei containing a single episome. In this way we avoided difficulties in interpretation associated with studies involving transient transfection of multiple templates, wherein only a fraction are transcriptionally active. Additionally, gene expression was assessed in tissues that normally express the *white* gene.

We found that the gypsy insulator prevented enhancer-activated transcription on the episome. Gypsy insulator function was not influenced by either the size or structure of the episome (Figure 3). Interestingly, the off-chromosomal requirements for enhancer blocking differed from those established for chromosomally integrated genes. On the episome, two insulators needed to flank the eye enhancer to prevent enhancer-activated transcription (Figure 3). We hypothesize that this difference is explained by the known bi-directional nature of enhancers, a supposition supported by studies of the chicken β -globin insulator (Recillas-Targa *et al.*, 1999). In circular plasmids, the β -globin insulator provided robust enhancer blocking only when two insulators flanked the enhancer, whereas in a linearized plasmid only a single β -globin insulator, placed between the enhancer and promoter, was required. These data demonstrate that within the context of an episome, an enhancer can direct transcription from either the 5' or 3' direction relative to the target promoter, causing an apparent change in the episomal insulator requirement.

Our episomal data for the gypsy insulator contrast with those obtained in a previous study (Holdridge and Dorsett, 1991). In these prior transient transfection experiments, only a single gypsy insulator, placed between the *hsp70* heat shock elements (HSEs) and promoter, was required to block enhancer-activated transcription of the *hsp70* promoter. That a second gypsy insulator was not required may be explained if the HSEs were unable to direct long range activation of transcription, precluding any modulation of transcription from the 3' direction. Alternatively, the properties of the gypsy insulator may be distinct in this assay system. The gypsy insulator-induced block of *hsp70* transcription was coupled with repression of basal *hsp70* transcription. While this effect is not normally associated with the wild-type gypsy insulator (Geyer and Corces, 1992; Cai and Levine, 1995; Scott and Geyer, 1995),

certain genetic backgrounds can change the character of the gypsy insulator to that of a promoter-specific silencer (Gerasimova *et al.*, 1995; Georgiev and Kozycina, 1996; Cai and Levine, 1997). For example, mutations in *mod(mdg4)*, the gene encoding a second gypsy insulator component, cause targeted promoter repression. For this reason we postulate that the different gypsy insulator requirements observed in the transient assay may reflect that the insulator protein complex was incompletely assembled or altered in some way, possibly due to a limiting amount of Mod(mdg4) protein, causing it to convert to a silencer.

Our findings demonstrate that the chromatin structure assembled on circular DNAs as small as 5.5 kb is sufficient for enhancer blocking by the gypsy insulator. From these observations we infer that higher order chromatin structures may not be essential for this process. This conclusion is based on the following rationale. First, higher order chromatin structures associated with heterochromatic regions do not appear to be maintained on FLP-excised episomes. This supposition is based on findings that heterochromatic repression can be relieved when the affected gene is excised onto a similarly sized episome by FLP recombinase (Ahmad and Golic, 1996) and that repressive chromatin structures associated with chromosomally integrated genes are not passively maintained on the episome (Cheng *et al.*, 1998). Secondly, the putative chromatin domains defined by the gypsy insulator are predicted to be very small. For example, in the GypEnGypOff episome the gypsy insulators could delimit putative domains of 1.1 and 4.4 kb that associate with ~6–22 nucleosomes, respectively. Thus, even the largest insulator-defined region on the GypEnGypOff episome could form only a minimal domain of higher order structure, encompassing no more than 3.5 turns of a solenoid, the proposed basis of the 30 nm fiber. Thirdly, enhancer blocking on the episome is position dependent, requiring that the two insulators flank the enhancer. These observations are inconsistent with proposals that suggest that higher order chromatin structures are imparted by localization of the episome into an insulator-specific nuclear compartment, as it is unclear how this mechanism would impart position dependence to the insulator block. For these reasons we suggest that the gypsy insulator does not prevent enhancer–promoter communication by promoting global reorganization of chromatin structures and propose that these data support models suggesting that the gypsy insulator may disrupt enhancer-activated transcription by direct interference in transcriptional processes (see below).

Properties of the episomal scs insulator differ from those defined for the gypsy insulator

The episomal properties of the gypsy and scs insulators were distinct. We found that a single scs insulator significantly decreased *white* gene expression on the episome. While placement of one scs insulator upstream of the *white* promoter may have caused only a slight reduction in *white* gene expression (ScsEnOff, Figure 4), dramatic *white* repression was observed when a single scs insulator was inserted between the eye enhancer and promoter (Figure 4, EnScsOff). Several experiments were conducted to determine the basis for these repressive

effects (Figure 4). In all cases, insertion of the scs insulator on the episome reduced *white* gene expression below that seen in similar gypsy insulator episomes.

The behavior of the scs insulator on the episome may be explained by loss of the scs-containing episomes or, alternatively, by scs possessing silencer, not enhancer blocker, activity on the episome. We favor the latter explanation for the following reasons. First, the eye phenotypes of flies carrying episomes with a single scs insulator are significantly different (compare ScsEnOff and EnScsOff, Figure 4). A difference in the degree of silencing between these episomes is not explained by episome loss, but is consistent with the suggestion that the scs insulator confers distance-sensitive promoter silencing. Secondly, flies displaying reduced eye pigmentation had a uniform level of pigmentation across the eye. Episome loss would be predicted to produce flies with a mottled eye phenotype (Ahmad and Golic, 1996). For these reasons we conclude that the scs insulator represses gene expression on the episome.

It is possible that the distinct episomal behavior of the scs insulator results from incomplete assembly of the protein components onto the episomal scs insulator, as discussed above for the gypsy insulator. Alternatively, these data might reflect differences in the mechanism employed by the scs insulator to block enhancer-activated transcription relative to that of the gypsy insulator. Interestingly, the scs insulator was identified based on its link to a structural chromosomal domain (Udvardy *et al.*, 1985), unlike the gypsy insulator, which was discovered based on its effects on transcription (Modolell *et al.*, 1983; Harrison *et al.*, 1989). This raises the possibility that the episomal effects of the scs insulator may reflect an inability to assemble higher order chromatin domains on the episomes, thereby causing it to exert unusual effects on gene expression.

Our data contrast with those obtained from experiments testing the effects of the scs insulator in plasmid-based assays in heterologous systems, such as *Xenopus* oocytes and human U-2 osteosarcoma cells (Dunaway *et al.*, 1997; van der Vlag *et al.*, 2000). In both studies, scs behaved as an insulator outside a chromosomal context, blocking enhancer function and chromatin-associated repressors, respectively. Several explanations may account for this distinction. First, it is possible that the promoters used in these assay systems were not sensitive to scs repression. Secondly, the components of the *Drosophila* scs insulator that are responsible for silencing may be missing in these heterologous systems. For example, the only scs insulator protein identified to date (SBP/Zw5) does not appear to have homologs in other organisms (Gaszner *et al.*, 1999). A more precise understanding of scs insulator function should be forthcoming as additional protein components are identified.

Models of gypsy insulator function

Several models have been proposed to account for insulator function (Geyer, 1997; Bell and Felsenfeld, 1999; Dorsett, 1999; Udvardy, 1999). These models fall into two general classes, categorized as structural and transcriptional models. Given the large number of insulators identified to date, it is likely that insulators establish

Fig. 5. Transcriptional models of gypsy insulator function. Possible mechanisms employed by the gypsy insulator to disrupt enhancer (large gray ovals) communication with complexes of transcription factors assembled at the promoter (small group of ovals). Three alternatives are indicated. (A) Looping. Enhancers may transduce signals to the promoter complex by looping out the intervening DNA. If this occurs, insulators may engage in a dynamic interaction with enhancer-binding proteins by assembling a complex of proteins (small group of rectangles) that mimic the complexes of proteins bound near promoters, thereby decoying the enhancer into a non-productive interaction that diffuses the enhancer signal. (B) Tracking. Insulators may block the propagation of an enhancer signal (series of arrows) along the DNA, thereby acting as a physical barrier to prevent the signal from reaching the promoter complex. (C) Linking. Enhancers may recruit facilitator proteins that may interact or link up with each other and/or protein complexes nucleated along the chromatin fiber to decrease the apparent distance required for transmission of the enhancer signal (shown as loops). If this occurs, insulators may directly target facilitator proteins, disrupting their ability to shorten the enhancer–promoter distance, thereby interfering with receipt of the signal.

regulatory autonomy using mechanisms associated with both classes.

Structural models propose that insulators organize and define distinct chromatin domains that are physically inaccessible to each other, linking global chromosome organization with the functional properties of insulators. In this way, the transcriptional effects are an indirect consequence of chromosome organization. One class of structural models proposes that DNA within an insulator-defined domain is folded into higher order chromatin structures that preclude associations between proteins in different domains (Kellum and Schedl, 1991; Vazquez and Schedl, 1994; Udvardy, 1999). Our data that the gypsy insulator blocks enhancer-activated transcription outside a chromosomal context argue that the formation of higher order chromatin structures may not be required. A second class of structural models suggests that insulators interact with specific sub-nuclear structures promoting compartmentalization of insulated genes, which is responsible for establishment of functional domains (Gerasimova and Corces, 1999). Our observation that enhancer blocking on the episome remains position dependent fails to support models that suggest that the episomal gypsy insulator block is due to specialized partitioning of the episome into an insulator compartment.

Transcriptional models suggest that insulators interfere directly with the transmission or reception of the enhancer signal by a promoter (Figure 5; Geyer, 1997; Bell and Felsenfeld, 1999; Dorsett, 1999). Data described herein are more consistent with this class of models. First, the failure of the gypsy insulator to prevent FLP protein interactions is consistent with transcriptional models, because they predict that insulator effects should be limited to transcriptional factors. Secondly, the demonstration that the gypsy insulator functions outside a

chromosomal context weakens the connection between gypsy insulator function and global reorganization of chromatin structures.

Further support that the gypsy insulator directly interferes with the transcriptional process comes from several recent observations. First, two proteins necessary for enhancer function, Chip and Nipped-B, have been implicated as direct targets of the gypsy insulator (Morcillo *et al.*, 1997; Rollins *et al.*, 1999). Chip interacts with LIM domain nuclear proteins, acting to facilitate the DNA-binding capacity of some transcription factors (Breen *et al.*, 1998). The Nipped-B protein shows similarity to yeast and fungal proteins that function in chromosome condensation and DNA repair (Rollins *et al.*, 1999). As Chip and Nipped both modulate enhancer activity, perhaps as putative enhancer facilitators, these observations strengthen the assertion that the gypsy insulator directly disrupts transmission of the enhancer signal to the promoter. Secondly, enhancer strength is an important determinant of gypsy insulator function, demonstrating a link between transcription and insulation (Scott *et al.*, 1999).

The mechanisms by which the gypsy insulator might impede transduction of the enhancer signal depend upon the mechanics of enhancer–promoter interactions, a poorly understood process (Figure 5). If enhancers interact with the promoter complex through a looping mechanism (Figure 5A; Ptashne, 1988), then the gypsy insulator could block transmission of the enhancer signal by docking or decoying the enhancer into a non-productive interaction. If the enhancer assembles a protein complex that tracks down the DNA to the promoter (Figure 5B; Courey *et al.*, 1986), then the gypsy insulator could act as a physical impediment to prevent transmission of the signal. Finally, if the enhancer interacts with the promoter through a linking mechanism (Figure 5C; Bulger and Groudine, 1999; Dorsett, 1999), then the gypsy insulator could block receipt of the enhancer signal by interfering with facilitator proteins that mediate transmission of the signal. Further studies are required to distinguish between these possibilities. The proposal that insulators may directly disrupt enhancer–promoter communication suggests that understanding mechanisms of insulator function will provide insights into the fundamental question of how enhancers activate transcription.

Materials and methods

Drosophila stocks

Flies were raised on standard corn meal/agar medium at 25°C, 70% humidity. *P* element transposons were introduced into flies by germline transformation (Rubin and Spradling, 1982). The FLP recombinase reporter transgenes were introduced into the γ -*ac*-*w*¹¹¹⁸ stock, while the episomal reporter genes were introduced into the ν ^{36f}; *ry*⁵⁰⁶ stock (Lindsley and Zimm, 1992). Each independent line was analyzed by Southern blotting to determine the number of insertions and confirm the integrity of the transposon. Only transgenic lines carrying single *P* transposons were analyzed.

The effects of a *su(Hw)* mutant background on gypsy insulator function were tested as described previously (Roseman *et al.*, 1995). The heteroallelic combination of *su(Hw)*^Y/*su(Hw)*^f was used in these studies, as this combination is female fertile and completely suppresses the somatic effects of gypsy insertions.

Constructs used in the FLP recombinase assay

The effects of insulators on FLP-induced recombination were assessed using a modified *P*[>*w*^{hs}>] transposon (Golic and Lindquist, 1989). The

430 bp gypsy insulator, containing 12 Su(Hw) binding sites, was inserted into this vector by blunt end ligation at either an *A*fIII site (located in the first intron of the *white* gene ~1 kb downstream of the 5' FRT site) for *P*[*Gyp-In*] or an *Eco*RI site (~1.7 kb downstream of the 3' FRT site) for *P*[*Gyp-Out*]. The *P*[*Scs-In*] transposon was generated by inserting a 1.8 kb *Bam*HI–*Bg*II fragment, corresponding to the *scs* insulator, into the intronic *A*fIII site.

Analysis of FLP recombinase levels

FLP recombinase was produced by heat shock induction of a transgene, called *hsFLP*, which carried FLP coding sequences fused to the *hsp70* promoter (Golic and Lindquist, 1989). Prior to our insulator studies we tested several independent transgenic *hsFLP* lines to ascertain the level of FLP recombinase that was produced following heat shock induction. Relative levels of FLP activity were determined by measuring the efficiency of deletion of the *white* gene within the *P*[>*w*^{hs}>] transposon (Golic and Lindquist, 1989). This efficiency was judged both by the percentage of total progeny showing excision events and by the average loss of the *white* gene displayed in each eye. These efficiencies ranged between the lines tested, with one line showing 100% of the progeny with excision events that included an average of 50% loss of the *white* gene per eye. *P*[*ry*⁺, *hsFLP*]/1 was chosen for our insulator studies because it produced 30–60% of flies with excision events that included an average of 20% loss of the *white* gene per eye. These data demonstrated that *P*[*ry*⁺, *hsFLP*]/1 was the weakest available FLP source and suggested that this line did not produce an excessive amount of FLP recombinase.

To determine insulator effects on FLP recombinase activity, several transgenic lines were assayed simultaneously. For each line tested, two or three males carrying the FLP reporter transgene were mated to six or seven *w*¹¹¹⁸, *P*[*ry*⁺, *hsFLP*]/1 virgin females (Golic and Lindquist, 1989). After 3 days of mating, parents were transferred into new vials and subsequently transferred daily, three additional times. FLP recombinase was induced in progeny 16–40 h old by immersing them in a 37°C circulating water bath for 1 h. The eye phenotype of adult progeny was scored for the presence of white patches on a pigmented background. Percentages of mosaic flies were calculated by dividing the number of flies with mosaic patches by the total number of scored progeny. Experiments were performed a minimum of three times.

Constructs used in the episomal enhancer blocking assay

The off-chromosomal effects of insulators were examined using a modified *PX96* transposon (Ahmad and Golic, 1996). The base transposon, *P*[*mini-Off*], was generated from *PX96* by replacing the *hsp70* promoter as a *Not*I–*A*fIII fragment with the natural *white* promoter from the vector pCaSpeR3 (Pirrotta, 1988). The 670 bp *white* eye enhancer was isolated from the vector CaSpeR3ET and inserted upstream of the *mini-white* promoter to generate *P*[*EnOff*]. The gypsy and *scs* insulator fragments were subsequently cloned into the *P*[*EnOff*] transposon immediately up- or downstream of the eye enhancer to generate *P*[*GypEnOff*], *P*[*EnGypOff*], *P*[*GypGypEnOff*], *P*[*EnGypGypOff*], *P*[*ScsEnOff*] and *P*[*EnScsOff*]. For transposons *P*[*GypEnGypOff*], *P*[*ScsEnScsOff*] and *P*[*ScsEnGypOff*], the upstream insulator was inserted an additional 500 bp upstream of the eye enhancer into a unique *Spe*I site at the 3'-end of the *white* gene. The *P*[*Scs-mini-Off*] construct was made by inserting the *scs* insulator upstream of the promoter of *P*[*mini-Off*]. In *P*[*EnGypOff*], *P*[*ScsEnGypOff*] and *P*[*GypEnGypOff*], the gypsy insulators inserted between the eye enhancer and promoter were cloned in the same orientation, while the second gypsy insulator in *P*[*GypEnGypOff*] was cloned in an inverted orientation. The orientation of the *scs* insulator was the same for *P*[*ScsEnOff*], *P*[*ScsEnGypOff*], *P*[*EnScsOff*] and *P*[*Scs-mini-Off*]. In *P*[*ScsEnScsOff*], the *scs* insulator inserted between the enhancer and promoter was in the same orientation as *P*[*EnScsOff*], while the upstream *scs* insulator was cloned in an inverted orientation.

In the larger episomal transposons, *P*[*EnYellowOff*] and *P*[*GypYellow-EnGypOff*], the eye enhancer and gypsy insulator fragments were first inserted downstream of the 5 kb intronless *yellow* gene in a modified pBluescript vector. Subsequently, the entire *yellow* gene with either the eye enhancer or insulator was cloned into the unique *Spe*I site of *P*[*mini-Off*] and *P*[*EnGypOff*], respectively.

Production and phenotypic analysis of flies carrying episomes

Flies carrying FLP-induced episomes were produced by mating males carrying the episomal transposon to between 7 and 10 *w*¹¹¹⁸, *P*[*ry*⁺, *hsFLP*]/1 virgin females. After 3 days of mating, parents were transferred into new vials and subsequently transferred daily three additional times.

FLP recombinase activity was induced by immersing vials containing 6- to 7-day-old progeny in a 37°C circulating water bath for 1 h. Adult male progeny were collected immediately after eclosion and allowed to age for 3 days prior to phenotypic analysis of the eye color.

Phenotypic analysis of flies heat shocked at the appropriate stage was important, as the time of excision of the *white* gene influenced the resultant eye color (Ahmad and Golic, 1996). Early heat shocks lead to a loss of the episome because of continuing mitoses, while late heat shocks produce a lighter eye coloration because the cells accumulate *white* mRNA for a shorter period of time. To ensure that appropriate progeny were screened, the following criteria were established. Only phenotypes of flies carrying eyes with white anterior rims were scored, as this suggests that the majority of excision events occurred just as the morphogenetic furrow was finishing its movement through the eye disc. Additionally, only flies that showed a uniform coloration across the pigmented portion of the eye and not a salt and pepper coloration were scored, suggesting that excision was uniform. At least 50 male progeny were scored for each cross. Independent heat inductions were conducted at least three times, to demonstrate the reproducibility of the observed phenotype.

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