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Drosophila *mini-white* model system: new insights into positive position effects and the role of transcriptional terminators and *gypsy* insulator in transgene shielding

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ABSTRACT

The *white* gene, which is responsible for eye pigmentation, is widely used to study position effects in *Drosophila*. As a result of insertion of P-element vectors containing *mini-white* without enhancers into random chromosomal sites, flies with different eye color phenotypes appear, which is usually explained by the influence of positive/negative regulatory elements located around the insertion site. We found that, in more than 70% of cases when *mini-white* expression was subject to positive position effects, deletion of the *white* promoter had no effect on eye pigmentation; in these cases, the transposon was inserted into the transcribed regions of genes. Therefore, transcription through the *mini-white* gene could be responsible for high levels of its expression in most of chromosomal sites. Consistently with this conclusion, transcriptional terminators proved to be efficient in protecting *mini-white* expression from positive position effects. On the other hand, the best characterized *Drosophila gypsy* insulator was poorly effective in terminating transcription and, as a consequence, only partially protected *mini-white* expression from these effects. Thus, to ensure maximum protection of a transgene from position effects, a perfect boundary/insulator element should combine three activities: to block enhancers, to provide a barrier between active and repressed chromatin, and to terminate transcription.

INTRODUCTION

Enhancer-mediated activation is a fundamental mechanism of gene activation in eukaryotes (1,2). Enhancers can act over large distances to activate transcription of a certain gene, regardless of their orientation and position relative to the promoter and without affecting adjacent genes. According to recent data, enhancers interact directly with tagged genes by looping out the intervening sequences (3–6). The assumed ability of enhancers to stimulate unrelated promoters provided a basis for the model suggesting the existence of a specific class of regulatory elements that form independent transcriptional domains and preclude undesirable interactions between enhancers and promoters (7).

The sequences referred to as insulators due to their ability to prevent activation or repression signals from passing across them to a promoter have been found in different organisms (8–13). Insulators are defined by two properties: these nucleoprotein complexes can block enhancer action on a promoter when interposed between them and can protect transgenes they flank from chromosomal position effects.

Over many years, the *white* gene has been widely used as a model system for analyzing the enhancer-blocking and boundary activities of insulators in *Drosophila* (14–20). The reasons for this are several. The *white* gene, being well characterized molecularly, is not essential for fly viability (21–24). A tissue-specific enhancer is responsible for *white* activation in the eyes (25), and the level of eye pigmentation is a sensitive indicator of the amount of *white* transcription. In test systems, the *mini-white* gene of the CaSpeR vector has usually been used (26). This gene contains ~300 bp of 5′ and 630 bp of 3′ flanking DNA

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and has the greater part of the first intron deleted. Transformants carrying the *mini-white* gene show a range of eye coloration from pale yellow to red, depending on the position of *mini-white* insertion into the genome (21,23,24). To explain the high sensitivity of the *mini-white* gene to chromosomal position effects, it has been speculated that the *white* promoter can function as an enhancer trap, meaning that enhancers located either 5′ or 3′ of the transposon are able to stimulate transcription of the *mini-white* gene.

Using the *mini-white* model, it was shown that insulators could protect *white* expression from the influence of nearby enhancers (14,16,18). With regard to the proposed property of insulators to protect a transgene promoter from unspecific functional interactions with neighboring enhancers, we re-examined the ability of the *mini-white* gene to be activated by non-specific enhancers in random genomic positions and the functioning of the *gypsy* insulator as a boundary element.

MATERIALS AND METHODS

Plasmids and constructs

Promoterless gene *mini-white* was PCR-amplified with primers 5′-ctccaagcggtttacgcc-3′ and 5′-cagccgaatgaa ttctagttcc-3′ from the pCaSpeRΔ700 plasmid (27), digested with *Eco*RI, and subcloned into the P-elements-containing pCT plasmid digested with *Sma*I–*Eco*RI (pCTdW). The promoter of the *white* gene (Prw) was obtained from pCaSpeR2 digested with *Eco*47III and *Afl*II; T^{ASC} was PCR-amplified with primers 5′-gttaccga caaacgacagtccac-3′ and 5′-cggcgtgtgctacttgtcttagg-3′ from genomic DNA; T^{SV40} was obtained from pUASt plasmid digested with *Bam*HI and *Xba*I; a 340-bp fragment containing the Su(Hw)-binding region (Gy) was PCR-amplified from the *gypsy* retrotransposon. An 8-kb fragment containing the *yellow* gene and the cDNA *yellow* clone were kindly provided by P. Geyer. A 3-kb *Sal*I–*Bam*HI fragment containing the *yellow* regulatory region (yr) was subcloned into pGEM7 cleaved with *Bam*HI–*Xho*I (yr plasmid). A 5-kb *Bam*HI–*Bgl*II fragment containing the *yellow* coding region (yc) was subcloned into pCaSpeR2 (C2-yc). The *Eco47*III–*Sca*I fragment of the *yellow* coding region (fragment from −893 to +4774 bp relative to the *yellow* transcription start site) was cloned into pCTdW digested with *Eco*RI (pCTdWY).

(Prw)dW, (T^{ASC})dW and (Gy)dW. Fragments Prw, T^{ASC} and Gy were flanked by loxP sites and inserted into the pCTdWY plasmid digested with *Xba*I.

$Y^{\Delta-893}$ and (UAS)$Y^{\Delta-893}$. The *yellow* enhancers were deleted from the yr plasmid by digestion with *Eco47*III and *Nco*I. The resulting DNA fragment yrΔ was cloned into the yc plasmid ($Y^{\Delta-893}$). The UAS promoter digested with *Bam*HI and *Hpa*I from the pUASt plasmid was cloned between the loxP sites (UAS). The resulting DNA fragment was inserted into yr digested with *Eco47*III and *Nco*I [yrΔ(UAS)]. The yrΔ(UAS) fragment was cloned into the yc plasmid.

(Ee)(T^{ASC})W and (Ee)(T^{SV40})W. The *white* eye enhancer (Ee; fragment from −1465 to −1084 bp relative to the *white* transcription start site (24) flanked by frt sites was inserted into pCaSPeR2 digested with *Xba*I [(Ee)W]. Thereafter, T^{ASC} and T^{SV40} fragments flanked by loxP sites were inserted between the eye enhancer and *white* promoter at the *Hpa*I restriction site.

UAS(T^{ASC})dW, UAS(T^{SV40})dW and UAS(Gy)dW. The UAS promoter digested with *Bam*HI and *Hpa*I from the pUASt plasmid was inserted into pSK–*lacZ* digested with *Bam*HI and *Eco*RV. A 2-kb UAS–lz fragment digested with *Bam*HI and *Hinc*II was then subcloned into pCTdWY digested with *Xba*I. Fragments T^{ASC}, T^{SV40} and Gy flanked by loxP sites were cloned into pCTUAS–lz–dWY between the UAS promoter and the *white* coding region at the *Xho*I restriction site.

(T^{ASC})W and (T^{SV40})W. T^{ASC} and T^{SV40} fragments flanked by loxP sites were inserted into the pCaSPeR2 plasmid digested with *Hpa*I.

(Gy)W(Gy). One *gypsy* insulator (Gy) flanked by frt sites was inserted into the pCaSPeRΔ700 plasmid digested with *Eco*RI [W(Gy)]. The second *gypsy* insulator (Gy) flanked by loxP sites was inserted into the W(Gy) plasmid digested with *Xba*I.

Y(T^{ASC})W. The *Eco47*III–*Sca*I fragment of the *yellow* coding region was cloned into (T^{ASC})W digested with *Xba*I.

Generation and analysis of transgenic lines

The construct and P25.7wc plasmid were injected into *$yacw^{1118}$* preblastoderm embryos (28). The resultant flies were crossed with *$yacw^{1118}$* flies, and the transgenic progeny were identified by their eye color or bristle pigmentation. Chromosome localization of various transgene insertions was determined by crossing the transformants with the *$yacw^{1118}$* balancer stock containing dominant markers, *In(2RL), CyO* for chromosome 2 and *In(3LR)TM3, Sb* for chromosome 3.

To determine the levels of *yellow* and *white* expressions, we visually estimated the degree of pigmentation in the abdominal cuticle and wing blades (*yellow*) and in the eyes (*white*) of 3–5-day-old males developing at 25°C. For *yellow*, a five-grade scale was used, with grade 1 corresponding to the total loss of *yellow* expression and grade 5 corresponding to wild-type pigmentation. Identical data were obtained for the wing and body pigmentation in all experiments. On the nine-grade scale for *white*, bright red (R) and white (W) eyes corresponded to the wild type and the total loss of *white* expression, respectively. Intermediate levels of eye pigmentation, in the order of decreasing gene expression, were brownish red (BrR), brown (Br), dark orange (dOr), orange (Or), dark yellow (dY), yellow (Y) and pale yellow (pY). The pigmentation scores were independently determined by two investigators. These scores (every unit representing one

line) were entered into the corresponding table and used to assess changes in gene expression.

The lines with DNA fragment excisions were obtained by crossing the transposon-bearing flies with the Flp (w^{1118}; *S2CyO, hsFLP, ISA/Sco;+*) or Cre (*yw; CyO, P[w+,cre]/Sco;+*) recombinase-expressing lines (29,30). The Cre recombinase induces 100% excisions in the next generation. The high level of FLP recombinase (almost 90% efficiency) was produced by daily heat shock treatment for 2 h during the first three days after hatching. All excisions were confirmed by PCR analysis with the pairs of primers flanking the −893 insertion site (5′-atccagttgattttcagggacca-3′ and 5′-ttggcaggtgattttgagca tac-3′) relative to the *yellow* transcription start site and the insertion site upstream from the *white* gene promoter (5′-gattaacccttagcatgtccg-3′ and 5′-tttcacactttcccctgc-3′).

To induce GAL4 expression, we used the modified yw^{1118}; *P[w⁻, tubGAL4]117/TM3,Sb* line (Bloomington Center #5138), in which the marker *mini-white* gene was deleted as described (31).

Construct insertion sites in transgenic lines were determined with inverse PCR technique. Genomic DNA extracted from transgenic flies was treated with *Rsa*I or *Mbo*I endonuclease. The cleaved DNA was ligated and PCR-amplified with primers 5′-aagattcgcagtggaaggctg cac-3′ and 5′-tccgcacacaacctttcctctcaac-3′ (after *Rsa*I cleavage) or 5′-cccttagcatgtccgtggggtttg-3′ and 5′-cgctgtct cactcagactcaatacgacac-3′ (after *Mbo*I cleavage). The PCR products were sequenced, and the coordinates and directions of insertions were determined with the Flybase R5.13 database.

RESULTS

Stimulation of the *mini-white* gene in transgenic lines is determined in most cases by transcription through the transposon

As shown previously, transformants carrying the *mini-white* gene frequently showed eye pigmentation in the range of orange to red, which indicated elevation of *mini-white* expression above the basal level (pale yellow to dark yellow eyes) (21,23,24). There are two explanations of the *mini-white* stimulation in some genomic positions. On the one hand, *mini-white* expression can be stimulated (or repressed) by surrounding regulatory elements such as enhancers or silencers or due to local chromatin organization. On the other hand, transposon insertion in the transcribed region can lead to an increase in the amount of mRNA products transcribed through the *white* coding region.

To decide between these models, we flanked the *white* promoter from −110 to + 276 by sites for Cre recombinase (loxP) and reinserted it into the promoterless *mini-white* gene [(Prw)dW, Figure 1]. For selecting transgenic lines, the marker *yellow* gene (responsible for cuticle and bristle pigmentation) was inserted on the 3′ side of the *mini-white* gene. The Wari insulator (27) located on the 3′ side of the *mini-white* gene was deleted from the construct. We obtained 154 independent transgenic lines carrying a single copy of the (Prw)dW construct (Figure 1). Flies in these lines had eye phenotypes ranging from pale yellow to red, which confirmed previous observations that *mini-white* expression is sensitive to position effects.

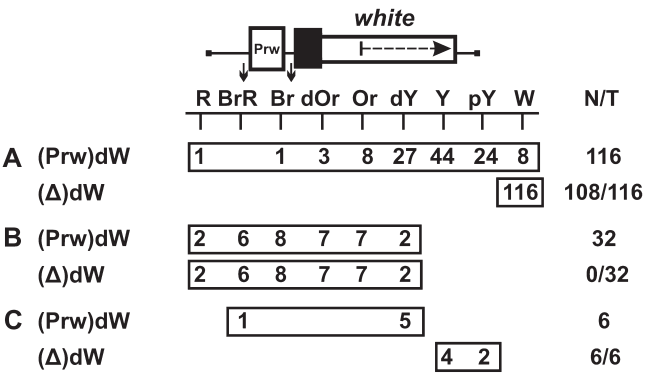

Figure 1. Stimulation of the *mini-white* gene in transgenic lines. Levels of eye pigmentation in transgenic lines in which the deletion of the *white* promoter (**A**) resulted in the white color of the eyes, (**B**) had no effect on eye pigmentation, or (**C**) partially reduced it. The *white* gene is shown as a white rectangle with the arrow indicating the direction of transcription; the white square marked 'Prw' represents the *white* promoter. The black box shows the promoter deletion in *mini-white*. Downward arrows indicate cleavage sites for Cre recombinase; in construct names, the corresponding excisable element is parenthesized. The 'white' columns show the numbers of transgenic lines with the *white* eye pigmentation levels. *N* is the number of lines in which flies acquired a new *w* phenotype relative to the initial lines. *T* is the total number of lines examined for each particular construct.

Next, we deleted the *white* promoter by inducing recombination between the loxP sites. In 116 transgenic lines (Figure 1A), the deletion of the promoter resulted in the white color of the eyes, indicating the loss of *mini-white* expression. In 95 out of these 116 transgenic lines, the eye color in flies ranged from pale yellow to dark yellow, corresponding to the basal level of the *mini-white* expression driven by the promoter alone. In 11% (13 out of 116) of the transgenic lines, flies had darker eye pigmentation, which could be indicative of *mini-white* activation by a regulatory element located outside the transposon.

In 38 out of 154 transgenic lines, the deletion of the *white* promoter either had no effect on *mini-white* expression (32 lines, Figure 1B) or partially reduced it (six lines, Figure 1C). In 30 out of 32 transgenic lines in which the pigmentation level remained unchanged after the deletion of the promoter, the eye color in flies was in the range from orange to red, indicating that *mini-white* expression was above the basal level.

To gain an insight into the nature of *mini-white* activation at different genomic positions, we determined its chromosomal insertion sites in 23 transgenic lines. Table 1 shows positions of the *mini-white* gene relative to neighboring genes in these lines.

In eight transgenic lines in which flies acquired white eyes after the deletion of the promoter, the transposon was inserted either into intergenic regions (five lines) or into genes oriented opposite to the direction of transcription (three lines).

In contrast, all transgenic lines displaying promoter-independent *mini-white* expression were generated by

Table 1. Sites of (Prw)dW construct insertion in transgenic lines

Transformant			
(Prw)dW	(Δ)dW	Location	Gene location
DY	w	X:250400	Between genes *CG3777* and *y*
Or	w	X:4100155	50 bp upstream of *Fas2* coding region
Or	w	2L:129261	Coding region in *CG3164*, opposite direction
Or	w	3R:229119	Coding region in *growl*, opposite direction
DY	w	3R:25081082	Coding region in *stg*, opposite direction
DOr	w	3L: 8681912	Between genes *h* and *SrpRbeta*
Or	w	Transposon *invader4*	480 bp from the 5′-end
Y	w	Transposon *diver*	370 bp from the 5′-end
DOr	dOr	2R:3074855	Coding region in *pk*, codirectional
Br	Br	2R:6421873	Coding region in *lola*, codirectional
BrR	BrR	2R:6422634	Coding region in *lola*, codirectional
R	pY	2R:13152978	Promoter of *mb1*, codirectional
Or	Or	2R:14059049	Coding region in *Dgp-1*, codirectional
DY	dY	2R:17090276	Coding region in *Glycogenin*, codirectional
Or	Or	2R:18100720	Coding region in *ari-2*, codirectional
Or	Or	2L:267546	Coding region in *CG3645*, codirectional
Br	Br	3R:12106070	Coding region in *gish*, codirectional
Or	Or	3L:131776	Coding region in *Pk61C*, codirectional
Or	Or	3L:543606	Coding region in *CG17090*, codirectional
DOr	dOr	3L:7361721	Coding region in *Sh3beta*, codirectional
DOr	dOr	3L:9394976	Coding region in *eIF-4E*, codirectional
Br	Br	3L:10679976	Coding region in *simj*, codirectional
DOr	dOr	3L:21016912	Coding region in *skd*, codirectional

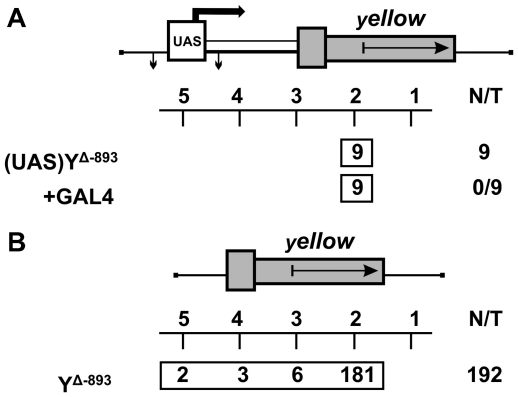

Figure 2. Stimulation of the enhancerless *yellow* gene in transgenic lines. (**A**) Testing the ability of the UAS promoter located at –893 to produce the functional Yellow protein. The UAS promoter is represented by the white rectangle marked 'UAS'. The 'yellow' columns show the numbers of transgenic lines with different levels of body and wing pigmentation. '+GAL4' indicates that yellow phenotypes in transgenic lines were examined after induction of GAL4 expression. (**B**) Testing the frequency of *yellow* stimulation in random genomic positions. For other designations, see Figure 1.

insertions into genes whose transcription direction coincided with that of the *mini-white* gene. According to available data (NCBI GEO), some of these genes are specifically active in the eye imaginal discs or are expressed throughout development in all tissues. Thus, *mini-white* expression could be due to transcription driven by the promoter of a tagged gene. Since the deletion of the *white* promoter did not alter *mini-white* expression (except for the line marked grey in Table 1), it seems likely that transcription through the *mini-white* gene resulted in inactivation of the *white* promoter, as was previously shown for *Drosophila* genes *Ubx*, *Abd-A* (32,33) and *dihydrofolate reductase* (34).

Taken together, these results suggest that only in 13 out of 154 (8%) transgenic lines could the high level of *mini-white* expression be due to stimulation of the *white* promoter by neighboring enhancers or transcriptionally active chromatin.

The enhancerless *yellow* gene is rarely activated by surrounding regulatory elements

We extended our study to one more tissue specific gene, *yellow,* that is required for larval and adult cuticle pigmentation. The spatiotemporal pattern of its expression is controlled by at least five independent, tissue-specific transcriptional enhancers (35,36). As shown previously, the intensity of cuticle pigmentation correlates with the level of *yellow* gene expression (37). At first, we tested whether transcription from upstream promoters can stimulate *yellow* expression, as in the case of the *mini-white* gene. The UAS promoter flanked by lox sites was cloned at –893 relative to the *yellow* transcription start sites (Figure 2A). The *yellow* wing and body enhancers were deleted from the construct. We obtained nine independent transgenic lines, each carrying a single copy of the construct. To express the GAL4 protein, we used the transgenic line carrying the GAL4 gene under control of the ubiquitous *tubulin* promoter (*tubGAL4*). In transgenic lines carrying the enhancerless *yellow* gene fused with GAL4-binding sites, *tubGAL4* stimulates *yellow* transcription in all cuticle structures (data not shown). At the same time, induction of the UAS promoter by GAL4 expression did not change wing and body pigmentation in transgenic flies, indicating that transcripts generated from the upstream promoter failed to produce the functional Yellow protein. Thus, in contrast to the situation with the *mini-white* gene, an increase in *yellow* expression could be attributed only to stimulation of the promoter by regulatory elements located near the site of the *yellow* transgene insertion.

Next, we used the construct containing the *yellow* sequences from −893 to +5204, including those with the bristle enhancer located in the intron. On the whole, we obtained 192 transgenic lines, each carrying a single insertion. Among them, only 11 lines showed a weak (six lines) or strong (five lines) increase in *yellow* expression (Figure 2B). Thus, in only a minor part of the transgenic lines was the *yellow* promoter activated by an enhancer or chromatin located outside the construct. These results confirm our main conclusion that enhancer–promoter interactions are specific and that incorrect stimulation of a promoter by a wrong enhancer is a relatively rare event.

Transcriptional terminators can protect *mini-white* expression from activating chromosomal position effects

If the high level of the *mini-white* expression in most genomic positions is accounted for by transcription through the gene, transcriptional terminators should function like boundaries. To test this assumption, we

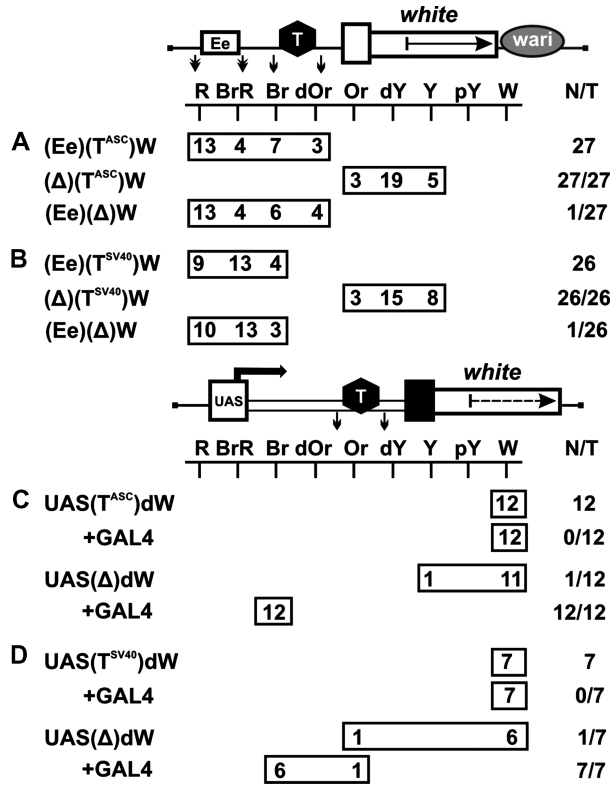

Figure 3. Functional activity of selected terminators in the eyes. Testing the enhancer-blocking activity (**A**) of the terminator found in the regulatory region of the *Achaete–Scute* gene complex (T^{ASC}) and (**B**) of the SV40 terminator (T^{SV40}). A terminator (T) is shown as a black hexagon; in construct names, terminators are indicated in superscript. The eye enhancer is represented by the rectangle marked 'Ee'. The Wari insulator is shown as a gray oval marked 'wari'. Downward arrows indicate cleavage sites for Cre or FLP recombinase. Testing the activity of (**C**) T^{ASC} and (**D**) T^{SV40} terminators in the eyes. '+GAL4' indicates that eye phenotypes in transgenic lines were examined after induction of GAL4 expression. In this case, *N* is the number of lines in which flies acquired a new *w* phenotype upon induction of GAL4. For other designations, see Figures 1 and 2.

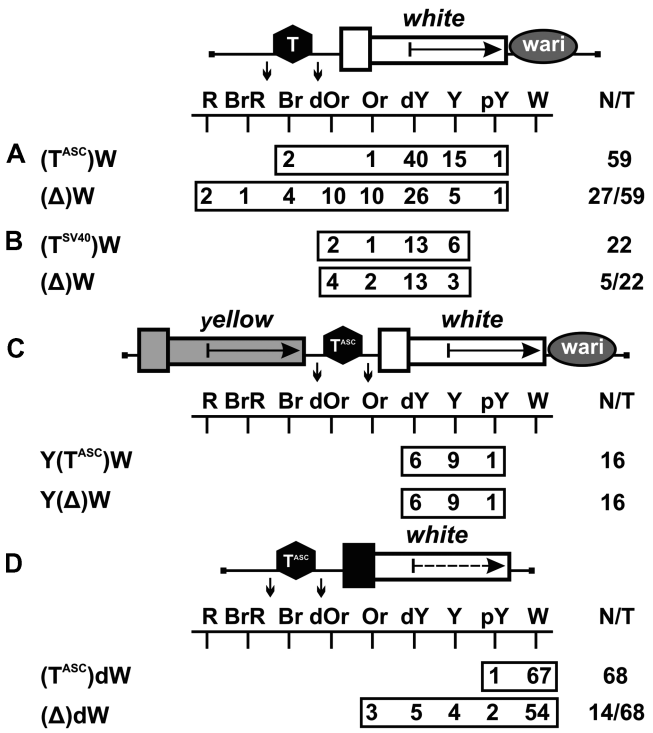

Figure 4. Testing (**A**) T^{ASC} and (**B**) T^{SV40} terminators for the ability to protect *mini-white* expression from positive position effects. (**C**) Testing T^{ASC} for the negative influence on *mini-white* expression. The *yellow* gene is shown as a gray rectangle with the arrow indicating the direction of transcription. (**D**) Testing T^{ASC} for the ability to terminate endogenous transcription at different genomic sites. For other designations, see Figures 1–3.

chose the well-studied SV40 terminator (designated T^{SV40}) and the terminator named T^{ASC} that was previously identified in the regulatory region of the *Achaete–Scute* gene complex (O.Maksimenko and P.Georgiev, unpublished data).

To verify their potential effect on the interaction between the eye enhancer and the *white* promoter, we made constructs in which these terminators flanked by lox sites were inserted between the eye enhancer flanked by frt sites and the *white* promoter (Figure 3A and B). As in common enhancer-blocking assay with the *mini-white* gene, the Wari insulator (27) was left intact in these constructs. The flies of all resultant transgenic lines displayed strong eye pigmentation that remained unchanged upon deletion of the terminators but decreased considerably upon deletion of the eye enhancer. Therefore, the terminators failed to influence enhancer–promoter communication.

To test the above terminators for the ability to arrest transcription elongation in the eyes, we used a model system that contained the UAS promoter, 2-kb spacer from the *lacZ* gene, and the promoterless *mini-white* gene with deleted Wari insulator (Figure 3C and D). The terminators flanked by lox sites were inserted into the spacer. The flies of all resultant transgenic lines had white eyes, with eye pigmentation remaining unchanged upon induction of the UAS promoter by crossing with the *tubGAL4* line. In derivative transgenic lines obtained by deleting the terminators, induction of the UAS promoter by GAL4 expression resulted in the brown eye color of transgenic flies, which was indicative of strong *mini-white* activation. These results confirm that the *mini-white* gene is effectively translated when containing an additional sequence of at least 2 kb at the 5′-end and that the terminators are able to effectively terminate transcription in the eyes in all transgenic lines tested.

Next, we examined whether the terminators can function as boundaries and effectively protect *mini-white* expression from the positive effects of surrounding sequences. The terminators flanked by loxP sites were inserted in front of the *mini-white* gene (Figure 4A and B). These constructs contained the Wari insulator (27) on the 3′ side of the *mini-white* gene.

As a result, we obtained 59 lines carrying T^{ASC} (Figure 4A) and 22 lines carrying T^{SV40} (Figure 4B). In 75 transgenic lines, flies had eye color ranging from pale yellow to dark yellow, which corresponded to the basal

level of *mini-white* expression. A stronger eye pigmentation was observed in only six transgenic lines flies, confirming our previous observation that activation of the *white* promoter by a neighboring enhancer or/and chromatin is a rare event. Deletion of the terminators resulted in a higher level of eye pigmentation in 32 out of 81 transgenic lines, providing evidence that the *mini-white* gene is often stimulated by transcription initiated upstream of this gene.

To test T^{ASC} for the negative influence on *mini-white* expression, we made the construct Y(T^{ASC})W in which the *yellow* gene with the regulatory region was inserted upstream of the *mini-white* gene in (T^{ASC})W (Figure 4C). In such a transgene, the *yellow* gene functioned as a buffer protecting the *mini-white* gene from the position effects of sequences located upstream of the transposon insertion site. In 16 transgenic lines, the eye color of flies ranged from yellow to dark yellow, with pigmentation level remaining unchanged upon the deletion of T^{ASC}. Thus, it appears unlikely that T^{ASC} directly affects the *mini-white* expression.

To obtain additional evidence that T^{ASC} can terminate endogenous transcription, thereby protecting *mini-white* expression, we used the (T^{ASC})dW construct containing the promoterless *white* gene (Figure 4D). The flies of 68 resultant transgenic lines had white eyes; after deletion of T^{ASC}, the flies of 14 lines acquired the eye color ranging from pale yellow to orange. Thus, T^{ASC} could effectively terminate endogenous transcription at different genomic sites.

Taken together, these results show that most of position effects on *mini-white* expression are caused by transcription through the transgene. Therefore, any regulatory element capable of terminating transcription can protect *mini-white* expression, acting like a boundary.

Flanking the *mini-white* gene by *gypsy* insulators only partially protects it from position effects

The best studied *Drosophila* insulator was found in the regulatory region of the *gypsy* retrotransposon (38,39). The authors of previous studies (14,15) observed that the *gypsy* insulator completely insulated *mini-white* expression. However, they performed experiments with the *yellow* gene inserted in the opposite orientation upstream of the *mini-white* gene, and its presence could strongly reduce the ability of transcripts to pass through the transgene to produce the functional White protein. Accordingly, we did not observe a high level of *mini-white* expression in any of Y(Δ)W transgenic lines carrying the construct in which the *yellow* gene was inserted upstream of the *mini-white* gene (Figure 4C). For this reason, we tested whether the *gypsy* insulator could protect *mini-white* expression from position effects.

At first, we examined whether the *gypsy* insulator could terminate transcription in the eyes. The *gypsy* insulator flanked by lox sites was inserted in the direct orientation, as in the *gypsy* retrotransposon, in the spacer between the UAS promoter and the promoterless *mini-white* gene (Figure 5A). In all transgenic lines tested, flies had brown eyes after induction of the UAS promoter by GAL4

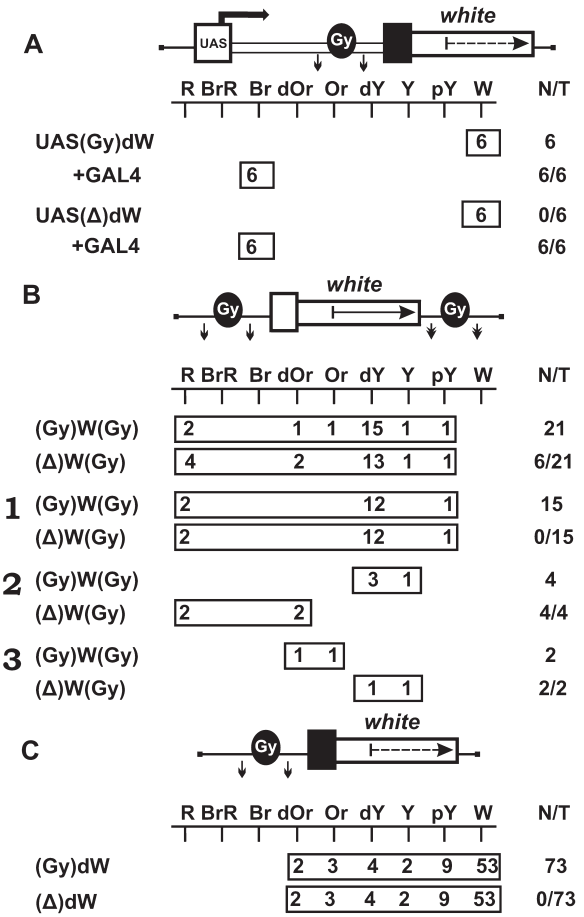

Figure 5. (**A**) Testing the *gypsy* insulator (Gy, black oval) for the ability to terminate transcription in the eyes. (**B**) Testing the *gypsy* insulator for the ability to protect *mini-white* expression from positive position effects. Numbers on the left (1–3) indicate groups of transgenic lines in which eye pigmentation (1) remained unchanged, (2) increased, or (3) decreased after the deletion of the insulator. (**C**) Testing the *gypsy* insulator for the ability to function as a transcriptional terminator in different genomic positions. For other designations, see Figures 1 and 2.

expression. Deletion of the insulator had no influence on eye pigmentation, suggesting that this insulator failed to terminate transcription in the eyes, despite the presence of AATAAA repeats in its sequence. The *gypsy* insulator in all subsequent constructs was inserted in the direct orientation.

Next, we made the construct in which the *mini-white* gene was inserted between the loxP- and frt-flanked *gypsy* insulators (Figure 5B). In this construct, the Wari insulator (27) was deleted from the *mini-white* gene. We obtained a total of 21 independent lines, each containing a single copy of the construct in which the *mini-white* gene was flanked by the *gypsy* insulators. The eye color phenotypes of these transgenic lines varied from pale yellow to red, which contradicted the results obtained by Roseman *et al*. (14). Thus, the presence of the *yellow* gene proved to shield the *mini-white* gene from positive position effects. After the deletion of the *gypsy* insulator located upstream of the *mini-white* gene, the eye color phenotypes

Table 2. Sites of (Gy)W(Gy) construct insertion in transgenic lines

Transformant			
(Gy)W(Gy)	(Δ)W(Gy)	Location	Gene location
R	R	X:18265579	Coding region in *CrebB-17A*, codirectional
R	R	3R:3718330	Coding region in *mRpS9*, codirectional
Y	R	3R:18759108	Coding region in *ftz-f1*, codirectional
dY	R	3R:24714644	Coding region in *Doa*, codirectional
dY	dOr	2L:12507930	Coding region in *bun*, opposite direction
dY	dOr	3R:466875	Coding region in *CG9775*, opposite direction
Or	Y	2R:5936940	Between *CG18445* and *CG2249*
dOr	dY	3L:637935	Between *Reg-2* and *ban*

remained unchanged in 15 transgenic lines [Figure 5B (1)], with eye pigmentation increasing [Figure 5B (2)] or decreasing [Figure 5B (3)] in four and two transgenic lines, respectively.

We determined chromosomal insertion sites for the four transgenic lines in which flies had a red eye color after deletion of the *gypsy* insulator. The results showed that the *mini-white* gene was colinearly inserted in the coding regions of the *CrebB-17A*, *mRpS9*, *Doa* and *ftz-f1* genes, which are strongly expressed in the eye imaginal discs (NCBI GEO) (Table 2). In two lines carrying insertions in the *Doa* and *ftz-f1* genes, the *white* expression was considerably reduced in the presence of the *gypsy* insulator. In both lines, we found potential polyA sites immediately upstream of the insertion sites. In the other two lines in which *gypsy* had no influence on the *white* expression, we did not find any signal for transcription termination in the vicinity of the transposon insertion sites. As shown previously, the *gypsy* insulator can potentiate weak polyadenylation signals but fails to terminate transcription by itself (39–42). Thus, it appears that the *gypsy* insulator potentiated termination at weak polyA sites in the *Doa* and *ftz-f1* genes but did not affect transcription in the *CrebB-17A* and *mRpS9* genes.

The chromosomal insertion sites were also identified for transgenic lines in which eye pigmentation increased from dark yellow to dark orange after the deletion of the *gypsy* insulator (Figure 5B). In these cases, the transposon was mapped in the coding regions of genes oriented opposite to the transcription direction, suggesting that the *gypsy* insulator in these transgenic lines could block communication between a nearby enhancer and the *white* promoter.

In two transgenic lines, the deletion of the *gypsy* insulator resulted in decreasing *mini-white* expression. Transposon insertions in these lines were mapped to intergenic regions, suggesting that, in all likelihood, the above phenomenon is explained by the boundary activity of the *gypsy* insulator, which protected *mini-white* expression from the repressive chromatin structures.

To further test the ability of the *gypsy* insulator to function as a transcriptional terminator in different genomic positions, we inserted the lox-flanked *gypsy* insulator in front of the promoterless *mini-white* gene lacking the endogenous Wari insulator (Figure 5C). In 20 out of 73 transgenic lines carrying single copies of the construct, flies had eye pigmentation in the range from pale yellow to orange. The deletion of the *gypsy* insulator had no effect on eye pigmentation in any of the transgenic lines examined. These results confirm that, in most cases, the *gypsy* insulator fails to terminate transcription.

DISCUSSION

We have shown here that, in most genomic positions of the *mini-white* transgene, positive position effects are caused by transcription through the gene. It seems likely that the *mini-white* gene has an internal site for translation initiation, which allows the functional White protein to be produced from transcripts initiated from promoters located upstream of the *white* coding region.

The possibility that a heterologous enhancer is able to stimulate *mini-white* expression has been observed (but not proved) in only 8% of transgenic lines. Infrequent *white* stimulation by surrounding chromatin may be explained by inactivation of the *white* promoter in most of genomic sites due to transcription through the gene. In such a case, the ability of enhancers to stimulate the *white* promoter does not manifest itself in many transcriptionally active regions. However, stimulation of the *white* gene shielded by a transcription terminator still proved to be a relatively rare event: eye pigmentation was within an orange–brown range in only six out of 81 transgenic lines (7%). On the other hand, deletion of the terminators resulted in stimulation of *white* expression in 40% of transgenic lines. Thus, *white* expression in many transcriptionally active regions was not stimulated by surrounding chromatin or regulatory elements.

The results of our experiments with another tissue-specific gene, *yellow,* are similar: it was activated by surrounding regulatory elements in only ~6% of transgenic lines. The *yellow* promoter is insensitive to transcription going through the gene (D. Chetverina, unpublished data). However, in contrast to the situation with *white*, the transcripts started upstream from the *yellow* promoter fail to produce the functional Yellow protein. Thus, we could not estimate the percentage of transcriptionally active regions in which the *yellow* promoter is insensitive to surrounding chromatin.

Taken together, these results indicate that tissue-specific promoters are infrequently activated by surrounding enhancers in genomic context at pupal–adult stages of *Drosophila* development. In embryos, conversely, extremely diverse, position-dependent expression patterns observed in various 'enhancer trap' experiments suggest that different endogenous enhancers can unspecifically activate a weak promoter located in the transposon (43–46). A probable explanation to these conflicting data is that specificity of enhancer–promoter

interactions increases in the course of *Drosophila* development.

A major putative function of insulators is to protect integrated reporter genes from positive or negative effects of the surrounding chromatin (9–11). The best-characterized boundary elements such as *Drosophila* scs insulator (16) and vertebrate HS4 insulator (18) were initially identified by their ability to protect the *white* gene from positive effects of the surrounding chromatin, with the resultant eye pigmentation being consistently lighter. Different regions of the SF1 insulator were shown to be required for enhancer blocking in embryos and for shielding the *mini-white* gene from chromosomal position effects (47). MAR elements were also shown to protect *white* expression from the positive position effects (19,20).

Here, we have shown that transcriptional terminators can effectively shield *mini-white* expression from position effects. Thus, A/T-rich MAR elements, 1.8-kb scs, 1.2-kb HS4 and SF1 insulators appear to protect *mini-white* expression by terminating transcription through the transgene. In contrast, the *gypsy* insulator only partially protects *mini-white* expression from position effects. We cannot exclude, however, that the *mini-white* transgene flanked by the combination of *gypsy* and Wari insulators is better protected from position effects, as was shown in previous studies (14,15). The *gypsy* insulator is effective in blocking enhancer stimulation (39,48–51), PRE-mediated silencing (51,52) and heterochromatin repression (14,15). At the same time, the *gypsy* insulator fails to shield the transgene expression from the effects of transcription initiated in the vicinity of the transgene insertion sites.

In conclusion, our results show that the position effects generated by transcription through a transgene are most frequent and that transcriptional terminators, compared to the classical *gypsy* insulator, provide better protection from these effects.

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