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Pairing between *gypsy* Insulators Facilitates the Enhancer Action in *trans* throughout the *Drosophila* Genome

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The Suppressor of the Hairy wing [Su(Hw)] binding region within the *gypsy* retrotransposon is the best known chromatin insulator in *Drosophila melanogaster*. According to previous data, two copies of the *gypsy* insulator inserted between an enhancer and a promoter neutralize each other's actions, which is indicative of an interaction between the protein complexes bound to the insulators. We have investigated the role of pairing between the *gypsy* insulators located on homologous chromosomes in *trans* interaction between *yellow* enhancers and a promoter. It has been shown that *trans* activation of the *yellow* promoter strongly depends on the site of the transposon insertion, which is evidence for a role of surrounding chromatin in homologous pairing. The presence of the *gypsy* insulators in both homologous chromosomes even at a distance of 9 kb downstream from the promoter dramatically improves the *trans* activation of *yellow*. Moreover, the *gypsy* insulators have proved to stabilize *trans* activation between distantly located enhancers and a promoter. These data suggest that *gypsy* insulator pairing is involved in communication between loci in the *Drosophila* genome.

The enhancer-mediated activation is the basic mechanism of gene regulation in eukaryotes. Enhancers can act over large distances to activate transcription independently of their orientation and position relative to the promoter without affecting adjacent genes (6, 11). Insulators represent a class of DNA sequences that restrain regulatory interactions within eukaryotic genomes (21, 35, 36, 53, 63, 65). These elements restrict the enhancer and silencer functions, contributing to the establishment of independent gene regulation within heterochromatic and euchromatic domains.

The best known insulator was identified in *Drosophila melanogaster* within the 5′ untranslated region of the *gypsy* retrotransposon (42). It consists of 12 binding sites for the Su(Hw) protein (44, 62). Recently, another protein, CP190, was shown to bind to the *gypsy* insulator (54). These DNA-binding proteins are important for the *gypsy* insulator function, as mutations in the *su(Hw)* and *CP190* genes reverse the mutagenic effects of the *gypsy* retrotransposon (46, 54). Mutations in another gene, *mod(mdg4)*, alter the phenotypes of the *gypsy*-induced mutations (17, 18, 22). Previous studies indicate that the Mod(mdg4)-67.2 protein isoform interacts with the Su(Hw) and CP190 proteins (14, 25, 54). The prevalent model suggests that boundary elements, or insulators, subdivide eukaryotic chromosomes into functionally and structurally autonomous domains (65). The insulators determine the limits of higher-order "looped" chromatin domains by interacting either with each other or with some nuclear structures (8, 36, 65). This interaction might be responsible, at least partially, for the establishment of independent chromatin domains. In this context, it is noteworthy that the *gypsy* insulators have been found to coalesce into a few "insulator bodies" located at the periphery of the nucleus (19), which are partially or completely disrupted in the nuclei of *CP190* or *su(Hw)* or *mod(mdg4)* mutants (20, 54). The fact that duplication of the *gypsy* insulator neutralizes the enhancer-blocking activity is also indicative of the interaction between the protein complexes bound to the *gypsy* insulators (9, 51).

The properties of the *gypsy* insulator properties suggest its involvement in pairing between homologous chromosomes in germ and somatic cells. In dipterans, homologous chromosomes are intimately synapsed in somatic cells of different types (12, 13, 30, 31, 34, 57, 66) and massive pairing of sister chromatids and homologs is responsible for the precisely banded pattern of polytene chromosomes (40). In *Drosophila*, a number of loci have been found at which pairing significantly influences gene expression. Such an influence was first detected within the *bithorax* complex by E. B. Lewis, who coined the term "transvection" to describe it (39).

A useful system for studying the factors determining transvection is the *yellow* gene, which encodes the protein responsible for dark pigmentation of the cuticle in larvae and adult insects (52). The enhancers controlling *yellow* expression in the wings and body cuticle are located in the upstream gene region, whereas the enhancer controlling *yellow* expression in bristles resides in the intron (23, 43). The wing and body enhancers of one allele can *trans* activate the *yellow* promoter on the paired homologous chromosome (24, 47, 60). However, the *cis* preference of enhancers for their own promoter precludes their action in *trans* (49). According to recent studies, *yellow* transvection can occur at multiple genomic locations, and the *Drosophila* genome is generally permissive to the enhancer action in *trans* (10). It has also been shown that transvection between *yellow* alleles strongly depends on homologous pairing and does not take place between nonhomologous sites (10, 24).

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In this study, we used the transvection at *yellow* as a model system to analyze the role of the interaction of *gypsy* insulators in pairing between homologous chromosomes. Taking into account that *trans* activation of the *yellow* promoter is more effective in the presence of *gypsy* insulators on both homologs, we supposed that the proteins bound to the *gypsy* insulator may be involved in this process. Moreover, the *gypsy* insulators have proved to stabilize *trans* activation between distantly located enhancers and a promoter.

MATERIALS AND METHODS

Plasmid construction. The 8-kb fragment containing the *yellow* gene and the cDNA *yellow* clone were kindly provided by P. Geyer. The 3-kb SalI-BamHI fragment containing the *yellow* regulatory region (yr) was subcloned into BamHI- and XhoI-digested pGEM7 (yr plasmid). The 5-kb BamHI-BglII fragment containing the coding region (yc) was subcloned into CaSpeR2 (C2-yc) or CaSpeR3 (C3-yc). The 430-bp *gypsy* sequence containing the Su(Hw)-binding region was PCR amplified from the *gypsy* retrotransposon. To confirm its identity, the product after sequencing was subcloned into CaSpeR3 (C3-su) and pSK (pSK-su) and between the LOX and FRT sites (lox-su-lox and FRT-su-FRT).

(i) (E)(Y)W. The *yellow* regulatory region includes the body enhancer and the wing enhancer located between bp −700 and −1868 and between bp −1868 and −2873 relative to the transcription start site of the *yellow* gene, respectively (23). The *yellow* enhancers were PCR amplified with primers 5′ TAT GCA ACT GAC GAT GGC TTA AG (between bp −2808 and −2786 relative to the *yellow* start site) and 5′ AAT TGG AAC TCG TGC TCG 3′ (between −711 and −728) and cloned between the FRT sites (y-en). The promoter and first exon of the *yellow* gene were PCR amplified with primers 5′ TAC AAG GAA ACA CCT GC 3′ (bp −702 and −686) and 5′ TCG TCT GTA CTA GAT TAA AAT 3′ (between bp +599 and +579) and cloned between the LOX sites (y-pr). The latter primer carried a point mutation in the final PCR product, which disrupted the site for SpeI endonuclease normally located in the *yellow* gene intron. The (y-pr) fragment was ligated into C2-yc cleaved with SpeI and XbaI [C2-yc-(y-pr)]. The (y-en) fragment was ligated into C2-yc-(y-pr) cleaved with XhoI and XbaI.

(ii) (E)(Y)SW. The SphI-XbaI DNA fragment was cut from (E)(Y)W and ligated into C3-su cleaved with SphI and XbaI.

(iii) (E)(Y)WS. The SphI-XbaI DNA fragment was cloned into EyeSYWS cleaved with SphI and XbaI. The EyeSYWS was earlier described by Muravyova et al. (51).

The fragment of the *yellow* wing and body enhancers SalI-Eco47III was cloned into FRT-su-FRT to obtain the FRT-E-su-FRT plasmid. The fragment FRT-E-su-FRT was ligated into yr which was digested with the SalI and Eco47III enzymes. Its correct orientation in the resulting yr-FRT-su-FRT plasmid was confirmed by PCR analysis.

(iv) (ES)Y(SW). The single lox site was first ligated into C2-yc digested with NruI in the intron of the *white* gene. Then we cloned a lox-su fragment from the lox-su-lox plasmid between the *yellow* and *white* genes to produce C2-yc-lox-su-w-lox. Finally, we combined the C2-ys-lox-su-w-lox and yr-FRT-su-FRT fragments obtained with XbaI and BamHI endonucleases.

(v) (ES)(Y)W and (ES)(Y)SW. The lox site was introduced into the SpeI site of the *yellow* gene intron within the C2-yc plasmid. We added another lox site or the lox site together with the *gypsy* insulator (su-lox) between the *yellow* and *white* genes to the BglII site to obtain plasmids C2-yc-lox-y-lox and C2-yc-lox-y-lox-su, respectively. To obtain the final constructs (ES)(Y)W and (ES)(Y)SW, the plasmids were cleaved with XbaI and BamHI and combined with the yr-FRT-su-FRT fragment.

***Drosophila* strains, transformation, and genetic crosses.** Flies were cultured on the standard yeast medium at 25°C. Five females were mated with two males in vials and brooded every second day. The temperature and crowding were carefully controlled, as both factors affect pigmentation. The mutant alleles and chromosomes used in this work and balancer chromosomes are described elsewhere (41).

The transposon constructs together with P25.7wc, the *P* element containing defectively inverted repeats that was used as a transposase source (32), were injected into *y ac* w^{1118} preblastoderm embryos. The resulting flies were crossed with the *y ac* w^{1118} flies, and the transgenic progeny were identified by their eye color. In all transgenic lines, the flies had dark-yellow to dark-orange eyes, which indicated that chromatin surrounding the transgenes was permissive to transcription. To check transposon integrity and copy number, the transformed lines were examined by Southern blot hybridization. Chromosome localization of various transgene insertions was determined by crossing the transformants with the *y ac* w^{1118} balancer stock containing dominant markers, *In(2RL),CyO* for chromosome 2, and *In(3LR)TM3*,Sb^{-} for chromosome 3. The precise sites of transgene insertions were determined by inverse PCR (iPCR) (http://www.fruitfly.org/methods). Genomic DNA from two flies was digested by RsaI or FspBI. After heat inactivation of the endonucleases, DNA fragments were self-ligated at 4°C for 24 h in 0.4 ml reaction mixture. For iPCR, we used the primers from the *P* element, 5′ AAG ATT CGC AGT GGA AGG CTG CAC 3′ and 5′ TCC GCA CAC AAC CTT TCC TCT CAA C 3′. The successfully amplified products were cloned in a Bluescript plasmid (Stratagene, La Jolla, CA) and sequenced. We used fluorescence in situ hybridization (FISH) (37) to map the insertions that, according to iPCR data, were located in repetitive elements of the genome. The full-length *yellow* gene labeled by the Bionick labeling kit (Life Technologies) served as a probe.

The selected lines were crossed on a $su(Hw)^{v}/su(Hw)^{f}$ and $mod(mdg4)^{u1}$ mutant background (17) to determine the contribution of the *gypsy* insulator to the *yellow* phenotype.

The enhancerless or promoterless lines were obtained by crossing the flies bearing the transposons with the Flp (w^{1118}; S^{2} *CyO hsFLP ISA*/*Sco*; +) or Cre (y^{1} w^{i}; *CyO P*[*w*+,*cre*]/*Sco*; +) recombinase-expressing lines. A high level of F recombinase was produced by exposing late embryos and second or third instar larvae to heat shock at 37°C for 2 h. All excisions were confirmed by Southern blot hybridization and/or PCR analysis. The details of the crosses used for genetic analysis and for excision of functional elements are available upon request.

Analysis of *yellow* phenotypes. Pigmentation of the wing blades and body cuticle in the abdominal stripes (below, referred to as wing and body pigmentation) was scored in 3- to 4-day-old females by using a five-grade pigmentation scale (48), with pigmentation scores of 1 and 5 corresponding to the null phenotype and the wild-type or almost wild-type state, respectively. Pigmentation scores were assigned by comparing the progeny of the flies obtained from parallel controls. Intermediate scores were determined relative to the pigmentation levels of $y^{1\#8}/y^{1\#8}$, y^{2}/y^{2}, $y^{82f29}/y^{1\#8}$, and $y^{2}/y^{1\#8}$ females, which corresponded to scores of 1, 1; 1, 1–2; 3, 3; and 4, 4 in the wing and body, respectively (10, 48). The pigmentation scores were independently determined by two investigators, who examined 30 to 50 female flies from each of the two independent crosses. Small variations in pigmentation depending on culture conditions did not exceed 0.5 points. The average phenotype was determined by averaging the pigmentation scores (http://www.igb.ac.ru/Kravchenko-Suppl.pdf).

RESULTS

The *gypsy* insulator facilitates *trans* activation of the *yellow* promoter. Previous studies showed that *yellow* transvection could occur at multiple genomic locations (10). The *yellow* region used in this work contained approximately 7.5 kb of 5′ flanking DNA and 2.1 kb of 3′ flanking DNA, which might include the sequences involved in homologous pairing. For this reason, we reexamined the *trans* action of the *yellow* enhancers without the flanking DNA. The *yellow* coding region was flanked by a 3-kb region that included only wing and body enhancers on the 5′ side and by a 200-bp region on the 3′ side. In the (E)(Y)W construct (Fig. 1), the wing and body enhancers were flanked by FRT sites, while the *yellow* promoter was flanked by LOX sites. The mini-*white* gene was used as a marker.

Twelve transgenic lines with the (E)(Y)W transposon were established. Heterozygous (E)(Y)W/+ females (Table 1) displayed wing and body pigmentation ranging from almost wild type (score 4–5) to weak (score 2). The difference in *yellow* expression might be explained by the effect of surrounding chromatin.

Enhancerless derivatives, ($^{\Delta}$E)(Y)W, were obtained by crossing (E)(Y)W flies with those carrying a Flp recombinase transgene and selecting the progeny with reduced cuticular pigmentation. All heterozygous ($^{\Delta}$E)(Y)W/+ females had yellow body cuticle and wing blades (a score of 1, 1) and wild-type

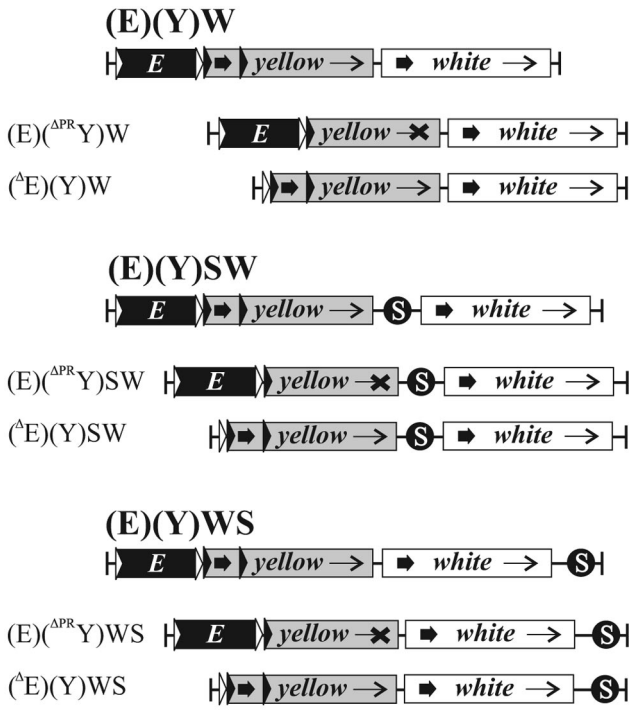

FIG. 1. Structures of (E)(Y)WS, (E)(Y)SW, and (E)(Y)W transgenic constructs and their derivatives. The derivative pairs used in complementation tests for each construct are shown. Black boxes marked with an E are enhancers of the *yellow* gene. Coding regions of the *yellow* gene and the mini-*white* reporter gene are shown as gray and white boxes, respectively. Thick, short arrows in the boxes indicate promoters of *yellow* or mini-*white*. Thin arrows in the boxes indicate the direction of transcription. Black crossing indicates disruption of transcription resulting from excision of the promoter or part of the coding region. White and black triangles are FRT and LOX sites, respectively. Black circles marked with an S are the *gypsy* insulators.

bristle pigmentation, indicating that the *yellow* promoter was not influenced by the surrounding enhancers or repressive chromatin. Promoterless derivatives, (E)($^{\Delta PR}$Y)W, were produced by crossing (E)(Y)W flies with those carrying a Cre recombinase transgene and selecting progeny with a y^1-like phenotype and pigmented eyes.

The flies carrying a ($^{\Delta}$E)(Y)W allele at an ectopic site were crossed with those carrying the corresponding (E)($^{\Delta PR}$Y)W allele at the same site; the level of the wing and body pigmentation in the female progeny was evaluated to determine whether interallelic complementation was possible (Table 1). At all 12 genomic sites, ($^{\Delta}$E)(Y)W/(E)($^{\Delta PR}$Y)W females had darker wing and body pigmentation than the heterozygous ($^{\Delta}$E)(Y)W/+ females. The pigmentation score of *yellow* phenotypes averaged 2.4/2.5 (wing/body). Interestingly, the strength of *trans* activation did not correlate with the level of the *yellow* activation in *cis* [Table 1, compare lines (E)(Y)W no. 3 and 8 with no. 11 and 12]. It seems possible that the *trans* activation level is influenced by homolog pairing efficiency depending on insertion site.

To check pairing between the *gypsy* insulators for the role in the improvement of activation in *trans*, the *gypsy* insulator was inserted between the *yellow* and mini-*white* genes in the (E)(Y)W construct (Fig. 1). Table 1 shows characteristics of 10 established (E)(Y)SW transgenic lines. The enhancerless and promoterless derivatives were obtained in the same ways as the (E)(Y)W transgenic lines. Pigmentation in the body cuticle and wings of all heterozygous ($^{\Delta}$E)(Y)W/+ females scored 1, 1 (with one exception: line no. 22 had the score 2–3, 2). The average score of *y* phenotypes for ($^{\Delta}$E)(Y)SW/(E)($^{\Delta PR}$Y)SW transheterozygous females was 3.7, 3.4 (wing, body). A comparison of the results obtained with (E)(Y)W and (E)(Y)SW transgenic lines showed that the pairing between the *gypsy* improved *trans* activation of the *yellow* promoter (Table 1).

TABLE 1. Pigmentation scores in (E)(Y)W, (E)(Y)SW, and (E)(Y)WS transgenic lines and their transheterozygous enhancerless and promoterless derivatives

Transgenic line (localization)	Cuticle pigmentation score (wing/body)[a]			
	(E)(Y)/+	($^{\Delta}$E)(Y)/(E)($^{\Delta PR}$Y)		
		+/+	*su(Hw)*$^{-}$	*mod(mdg4)*^{ul}
(E)(Y)W				
1 (3)	5/4	**3–4/3–4**	ND	ND
2 (3)	4–5/3–4	**1/2–3**	ND	ND
3[c] (3)	4/4	**3–4/3**	ND	ND
4 (3)	3–4/2–3	**3–4/1–2**	ND	ND
5 (3)	3–4/2	**2/2**	ND	ND
6 (3)	4/3–4	**2–3/2**	ND	ND
7 (3)	4/3	**3–4/4**	ND	ND
8 (2)	4/2–3	**3/3**	**3/2–3**	**3/2**
9 (3)	3/2–3	**1/2**	ND	ND
10 (2)	4–5/3–4	**2/2**	**2/1–2**	**2/2**
11 (3)	4–5/4–5	**1/2**	ND	ND
12 (2)	5/3–4	**2–3/2**	**2–3/2**	**2/2**
APS[b]	4.1/3.3	2.4/2.5	ND	ND
(E)(Y)SW				
13 28E9	5/5	**3–4/3**	**2/2**	ND
14 64B12	4–5/4	**4/3–4**	ND	ND
15 13A5	4–5/4	**4–5/4**	**3–4/2–3**	ND
16 (2)	4/3–4	**3–4/3–4**	**2/2**	ND
17 52B2	4–5/4	**3–4/3**	**2/2**	**2/2**
18 64C9	3–4/3–2	**3–4/3**	ND	ND
19 (2)	4–5/5	**4–5/3–4**	**3/3**	**3–4/3**
20 58F[d]	4/3–4	**2–3/2–3**	**1–2/1–2**	ND
21[c] (2)	2–3/3	**2–3/3**	ND	ND
22 (3)	5/4–5	**5/4–5**	ND	ND
APS	4.2/3.9	3.7/3.4	2.3/2.2	ND
(E)(Y)WS				
23 20E	3–4/3–4	**3/2**	ND	ND
24 (3)	4/3–4	**4/3**	ND	ND
25 23A3	4/3	**3–4/3**	**1–2/1–2**	**3–4/2**
26 39E3	5/5	**5/4–5**	**4/3**	**5/4**
27 29D4	5/5	**5/3–4**	**3/2**	**3–4/2**
28 75B5	4–5/4	**4/3–4**	ND	ND
29 100B3	5/4	**4/3**	ND	ND
30 68C13	4/3	**3/2–3**	ND	ND
31[c] 74A1	4/3	**4/3**	ND	ND
32 35DE[d]	4/4	**3–4/3–4**	**1/1**	**3–4/3–4**
33 (3)	4–5/4	**3–4/3**	ND	ND
34 28D2	4–5/4	**4–5/3**	**3/2**	**4–5/3**
APS	4.3/3.8	3.9/3.1	2.5/1.9	3.8/2.9

[a] Pigmentation levels are shown for heterozygous (E)(Y)WS, (E)(Y)SW and (E)(Y)W females [(E)(Y)/+] and for transheterozygous females carrying the promoterless and enhancerless derivatives of the constructs on the +/+, *su(Hw)*$^{-}$, or *mod(mdg4)*^{ul} background [($^{\Delta}$E)(Y)/(E)($^{\Delta PR}$Y)]. Bold data indicate complementation between *y* alleles. ND, not determined.

[b] APS, average pigmentation scores assigned to each transgenic construct. Statistical data are available at http://www.igb.ac.ru/Kravchenko-Suppl.pdf.

[c] Flies in these transgenic lines displayed variegated pigmentation of bristles.

[d] Localization of these insertions was determined by FISH.

The improvement of *trans* activation in the presence of the *gypsy* insulator could be explained by the blocking of *cis* interaction between the *yellow* enhancers and the *white* promoter. To check this assumption, we inserted the *gypsy* insulator into the (E)(Y)WS construct downstream from the *white* gene (Fig. 1). The enhancerless and promoterless derivatives were obtained for 12 transgenic lines. In a complementation test, transheterozygous ($^{\Delta}$E)(Y)WS/(E)($^{\Delta PR}$Y)WS females had the average pigmentation scores similar to that in the ($^{\Delta}$E)(Y)SW/(E)($^{\Delta PR}$Y)SW females carrying the insulator between the *yellow* and *white* genes: 3.9, 3.1 and 3.7, 3.4, respectively (Table 1). As the *gypsy* insulator did not separate the *yellow* and *white* genes in the (E)(Y)WS transgenic lines, *trans* activation improvement in its presence could not be explained by the block of interaction between the *yellow* enhancers and the *white* promoter in *cis*. It is noteworthy that the *gypsy* insulator located even at a distance of 9 kb from the *yellow* promoter is capable of improving *trans* activation.

To demonstrate the role of the *gypsy* insulator in *trans* activation, six pairs of the (E)(Y)SW and five pairs of the (E)(Y)WS derivatives located on the X and second chromosomes were tested on the *su(Hw)*$^{-}$ background. Inactivation of the *su(Hw)* gene had no influence on the pigmentation of the ($^{\Delta}$E)(Y)SW/+ and ($^{\Delta}$E)(Y)WS/+ females (data not shown). At the same time, the tested ($^{\Delta}$E)(Y)SW/(E)($^{\Delta PR}$Y)SW and ($^{\Delta}$E)(Y) WS/(E)($^{\Delta PR}$Y)WS females crossed on the *su(Hw)*$^{-}$ background displayed similar transvection as the ($^{\Delta}$E)(Y)W/(E)($^{\Delta PR}$Y)W females.

To test whether the *su(Hw)* mutation has a general effect, we crossed the flies of the three (E)(Y)W lines without the *gypsy* insulator on the *su(Hw)*$^{-}$ background. In a complementation test, no significant changes in *trans* activation were revealed in ($^{\Delta}$E)(Y)W/(E)($^{\Delta PR}$Y)W transheterozygous females (Table 1); therefore, the Su(Hw) protein is unlikely to have a general effect on *trans* activation.

The second component of the *gypsy* insulator, Mod(mdg4) protein, had a weaker effect on the transvection level. We observed only slight changes of pigmentation in transheterozygous ($^{\Delta}$E)(Y)SW/(E)($^{\Delta PR}$Y)SW and ($^{\Delta}$E)(Y)WS/(E)($^{\Delta PR}$Y)WS females on the *mod(mdg4)*^{u1} background (Table 1). Interestingly, all effects manifested in this case consisted in *trans* activation decrease.

The *gypsy* insulator promotes transvection between the *yellow* enhancer and promoter located in nonhomologous loci. In complementation tests, the *gypsy* insulator proved to facilitate *trans* activation of the *yellow* promoter between homologous derivatives of the transgene, and the next question was whether it could stabilize transvection between nonhomologous derivatives. In the control experiment, we determined whether the transvection was supported by the enhancerless ($^{\Delta}$E)(Y)W allele and the promoterless (E)($^{\Delta PR}$Y)W allele from different ectopic sites. To this end, ($^{\Delta}$E)(Y)W males from twelve transgenic lines were mated to (E)($^{\Delta PR}$Y)W females from these lines. Phenotypic analysis of transheterozygous females obtained in 132 crosses revealed yellow pigmentation of the body cuticle and wing blades (data not shown). This result is in good agreement with those obtained by Chen et al. (10) and suggests that transvection between nonhomologous sites does not occur in the absence of the *gypsy* insulators.

TABLE 2. Complementation scores for the nonhomologous transheterozygotes of the (E)(Y)SW and (E)(Y)WS derivatives[a]

Pairs of transgenic lines [(E)($^{\Delta PR}$Y)/($^{\Delta}$E)(Y)] and distance between them, (kb) or locations	Cuticle pigmentation score (wing/body) [($^{\Delta}$E)(Y)/(E)($^{\Delta PR}$Y)]		
	+/+	*su(Hw)*$^{-}$	*mod(mdg4)*^{ul}
(E)(Y)SW			
14/18, 617	**2–3/1**	ND	ND
18/14	**3/2**	ND	ND
13/15, 2L and X	**3/1**	ND	ND
15/13	**3–4/1–2**	ND	ND
(E)(Y) WS			
27/34, 667	**3/2**	**1–2/1**	ND
34/27	**3/2**	ND	ND
26/32, ~5,334	**4/2**	**1/1**	**2–3/2**
32/26	**3–4/2**	ND	**3/2**
27/32, ~7,755	**3–4/1–2**	**1/1**	**2/1**
32/27	**3–4/1–2**	ND	**2/1**
27/26, 13,010	**2–3/1**	**1–2/1**	**1–2/1**
26/27	**2–3/1**	**2/1**	**1–2/1**
26/34, 13,677	**3/1**	**1/1**	**1/1**

[a] Bold data indicate complementation between *y* alleles. ND, not determined.

To analyze the effect of the *gypsy* insulator, we combined the nonhomologous enhancerless and promoterless derivatives of the 9 (E)(Y)SW and 12 (E)(Y)WS transgenic lines in an interallelic complementation test. Altogether, the phenotypes of 338 allele combinations were tested. Transheterozygous females obtained in 13 out of 338 crosses had visible activation of *yellow* expression, predominantly in the wings. In general, the transvection level was lower than that in complementation between the derivatives of the same insertion site. We observed one transvection event between nonhomologous insertions 14/18, both located on the third chromosome; one event between insertions 15/13 located on the X and second chromosomes, respectively; and five events between insertions located on the second chromosome (no. 27/34, no. 27/32, no. 27/26, no. 32/26, and no. 34/26) (Table 2). In most transvection events, *trans* activation occurred in both cases: the first allele provided the *yellow* promoter and the second provided the *yellow* enhancer, or vice versa. In the no. 34/26 event, however, *trans* activation occurred only between the no. 34 enhancerless and no. 26 promoterless derivatives. To clarify the circumstances of transvection, we determined the precise sites of insertions for most of the (E)(Y)SW and (E)(Y)WS transgenic lines (Table 1; Fig. 2). This analysis showed that insertions 34 and 26 were more than 20 Mb apart, which could account for limitation of the transvection to only one direction. Nevertheless, the relative distances between nonhomologous insertions could not be regarded as the determinants of tranvection efficiency. It was found that the complementation test between derivatives of no. 31 and no. 28 insertions located 814 kb away from each other did not give rise to *yellow trans* activation; conversely, comparable distances of 617 kb between insertions 14/18 and 667 kb between no. 27/34 did not prevent the tranvection of their enhancerless and promoterless derivatives. Likewise, a relatively short distance between insertions was not necessarily prerequisite for transvection. Thus, the no. 13 transgene insertion was between insertions 27 and 34 at distances of 308 and 359 kb from them, respectively. However, the

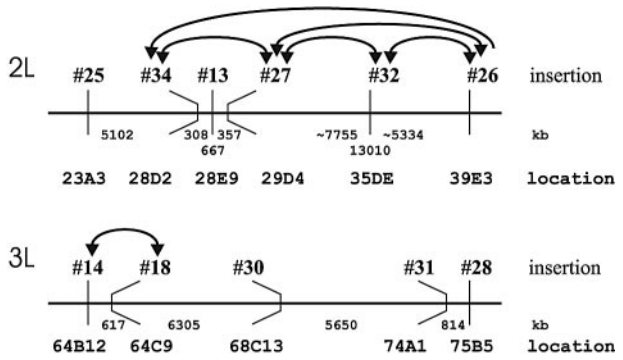

FIG. 2. *trans* activation between transposon derivatives of nonhomologous loci. Positions of the (E)(Y)WS and (E)(Y)SW insertions on the 2L and 3R chromosomes and distances between neighbors are shown. Arcs join the insertions whose derivatives can complement each other. Arrowheads at the ends of arcs indicate the direction of *trans* activation from enhancer to promoter.

no. 13 transgene derivatives could complement neither no. 27 nor no. 34 derivatives, whereas the latter two could support each other's transvections. It should also be noted that the no. 13 insertion did not represent a closed, inaccessible locus, as its derivatives show transvection with the no. 15 derivatives located on the X chromosome. In our opinion, the transvection efficiency depends mainly on the spatial arrangement of insertions within the nuclear architecture rather than on the linear chromosome distances between insertions sites. In a number of cases, transvection occurred when the distances between insertions were great: 7.16 Mb between no. 27/32, 5.33 Mb between no. 26/32, and 13.01 Mb between no. 27/26 (Fig. 2).

To confirm the role of the *gypsy* insulator in *trans* activation between nonhomologous enhancerless and promoterless derivatives, we tested *trans*-activated allele combinations on the *su(Hw)*$^{-}$ or *mod(mdg4)*^{u1} background (Table 2). Both mutants had a strongly reduced level of *trans* activation. Surprisingly, the *mod(mdg4)*^{u1} mutation affected transvection between nonhomologous insertions much more strongly than transvection between homologous insertions (Tables 1 and 2); apparently, the long-range *trans* activation is more sensitive to the *gypsy* insulator's components. On the other hand, its effect on both homologous and nonhomologous *trans* activation was much less severe than that of the *su(Hw)*$^{-}$ mutation (Tables 1 and 2). This agrees with the extent of instability of insulator bodies on the *su(Hw)*$^{-}$ and *mod(mdg4)*^{u1} backgrounds: they are completely destroyed in the former case but only partially affected in the latter case (20).

Thus, the *gypsy* insulator supports transvection between nonhomologous loci, and the efficiency of *trans* activation mainly depends on the relative arrangement of the loci in the nuclear architecture rather than on the linear distances between them on the chromosomes.

Efficient pairing-dependent *yellow* activation requires the presence of the *gypsy* insulator in both homologous chromosomes. The *gypsy* insulator contributes to various functions in the *Drosophila* nuclei, as follows from its interactions with GAGA factor or Mcp elements (45) and stimulation of the transcription of some genes (28, 64); moreover, there are multiple sites of the Su(Hw) protein localization on polytene chromosomes (20). Hence, the effect on *trans* activation caused by the *su(Hw)* mutation (see above) might be indirect, reflecting general changes in chromatin. To confirm that the Su(Hw) protein plays a role in transvection stabilization by participating in the direct interaction between the insulators located on different chromosomes, we created a construct yielding derivatives with or without the *gypsy* insulators.

In the (ES)Y(SW) transposon (Fig. 3), the *yellow* gene was flanked by the *gypsy* insulators inserted at −893 bp relative to the *yellow* transcription start and on the 3′ side of the *yellow*. The DNA fragment including the *gypsy* insulator at −893 bp and the *yellow* enhancers was flanked by FRT sites. The LOX

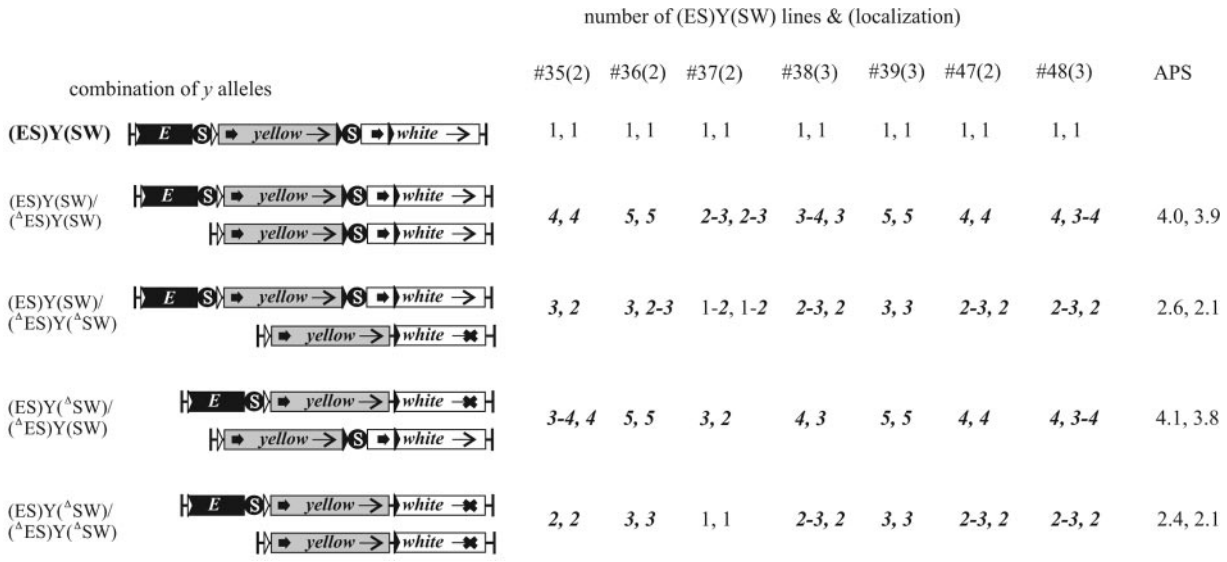

FIG. 3. Complementation tests for (ES)Y(SW) construct and derivatives. Pairs of derivatives and pigmentation scores for seven different transgenic insertions are shown. Bold, italic numbers indicate the level of complementation between *y* alleles. For other designations, see Fig. 1.

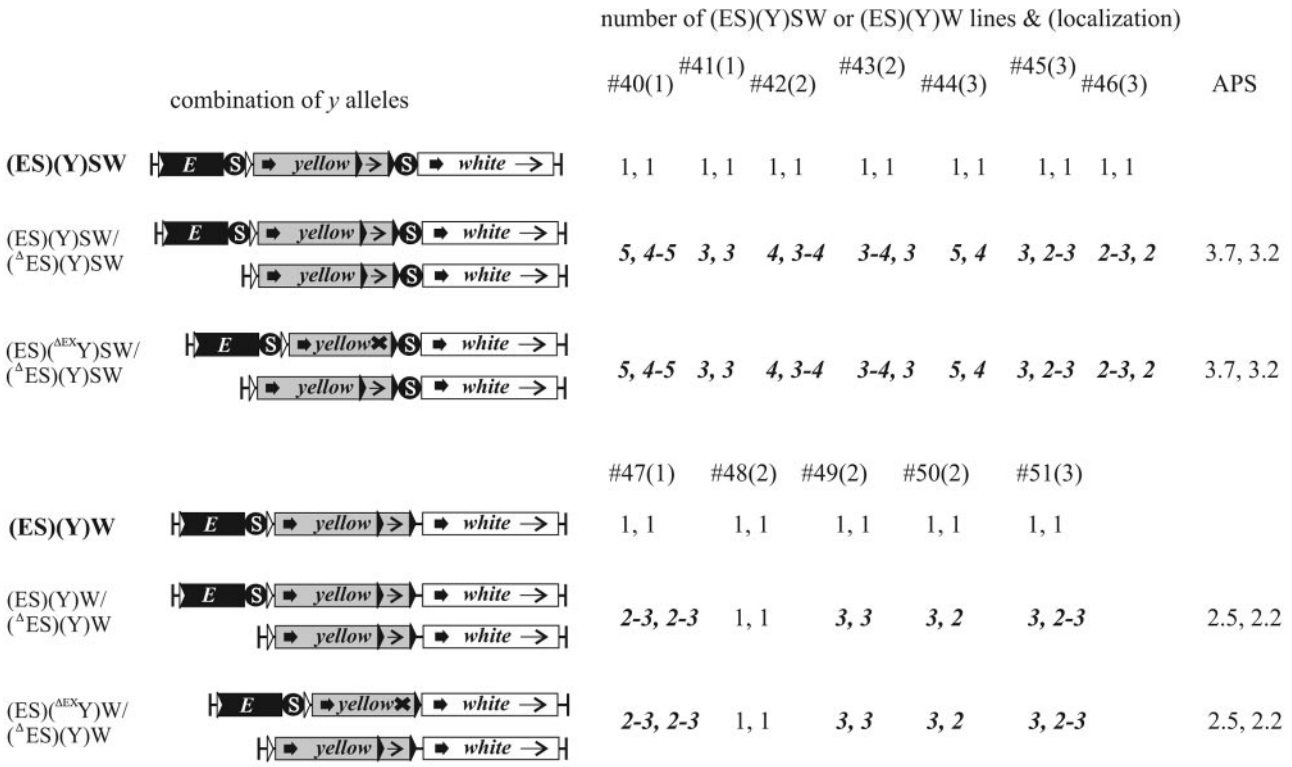

FIG. 4. Complementation tests for (ES)(Y)SW and (ES)(Y)W constructs and derivatives. For designations, see the legends to Fig. 1 and 3.

sites flanked the DNA fragment containing the *gypsy* insulator located on the 3′ side of the *yellow* and the *white* promoter.

We obtained seven (ES)Y(SW) transgenic lines with the *gypsy* insulator blocking the *cis* interaction between the *yellow* enhancers and the promoter, which accounted for the yellow pigmentation of the body cuticle and wing blades (Fig. 3). As the deletion of the *yellow* enhancers and the upstream *gypsy* insulator did not change the phenotype of flies, ($^{\Delta}$ES)Y(SW) derivatives were selected by PCR analysis of individual flies obtained in the progeny of the cross with the flies carrying a Flp recombinase transgene (data not shown). The flies with the deleted 3′ *gypsy* insulator and the *white* promoter, (ES)Y($^{\Delta}$SW) or with deletions of the two DNA fragments, ($^{\Delta}$ES)Y($^{\Delta}$SW) were selected by their white eyes and pigmented bristles. Pigmentation of the wings and body cuticle in heterozygous (ES)Y(SW)/+ flies and their derivatives was weak (approximately score 1) due to the blocking of *yellow* enhancers in the (ES)Y(SW) and (ES)Y($^{\Delta}$SW) transgenes and their deletion in the ($^{\Delta}$ES)Y(SW) and ($^{\Delta}$ES)Y($^{\Delta}$SW) transgenes.

In all seven transgenic lines, transheterozygous ($^{\Delta}$ES)Y(SW)/(ES)Y(SW) and ($^{\Delta}$ES)Y(SW)/(ES)Y($^{\Delta}$SW) females displayed activation of the *yellow* gene (Fig. 3). As in (ES)Y(SW) and (ES)Y($^{\Delta}$SW) alleles, the *gypsy* insulator blocked the *yellow* enhancer action on the *yellow* promoter in *cis*; we suggested that the *yellow* enhancers *trans*-activated the *yellow* promoter of the ($^{\Delta}$ES)Y(SW) construct. As the *gypsy* insulators may interact in *cis* (51), it may appear that the level of transvection may be affected by *cis* interactions if one homolog carries two *gypsy* copies. In fact, this is not the case: transheterozygous ($^{\Delta}$ES)Y(SW)/(ES)Y(SW) and ($^{\Delta}$ES)Y(SW)/(ES)Y($^{\Delta}$SW) females displayed the same level of *yellow* activation.

If only one of the paired *y* alleles contained one or two copies of the *gypsy* insulator, as in ($^{\Delta}$ES)Y($^{\Delta}$SW)/(ES)Y(SW) or ($^{\Delta}$ES)Y($^{\Delta}$SW)/(ES)Y($^{\Delta}$SW) transheterozygotes, the wing and body pigmentations were lower than in the transheterozygotes with *gypsy* insulators in both *y* alleles (Fig. 3). This is evidence that *gypsy* insulators in both paired homologs are necessary for efficient transvection.

The *gypsy* insulator facilitates *trans* activation of the *yellow* promoter. According to the results of studies on transvection between (ES)Y(SW) derivatives, the *gypsy* insulator inserted between the *yellow* enhancers and the promoter allowed the enhancers to activate the *yellow* promoter located on the homologous chromosome. This observation contradicts the previous data that the *yellow* enhancers isolated by a *gypsy* insertion in the y^2 or y^{69} alleles failed in *trans* activation of the *yellow* promoter in the enhancerless y^{82f29} allele (47). The conflicting results might be explained by earlier finding that the pairing between homologous sequences sometimes promoted the *gypsy* insulator bypass in *cis* (48). Therefore, such kind of pairing between the (ES)Y(SW) derivatives may allow the *yellow* enhancers to bypass the *gypsy* insulator and act on the promoter in *cis*.

To verify this assumption, the (ES)(Y)SW and (ES)(Y)W transposons were constructed (Fig. 4). As in the previous (ES)Y(SW) transposon, the *gypsy* insulator at −893 bp and the *yellow* enhancers were flanked by the FRT sites. To inactivate the *yellow* function, the second exon of the *yellow* gene was

flanked by LOX sites to promote its deletion from transgenic flies. To compare transvection in the presence or absence of the *gypsy* insulators in both homologs, the second *gypsy* insulator was inserted between the *yellow* and *white* genes in the (ES)(Y)SW construct [but not in the (ES)(Y)W construct].

We obtained seven (ES)(Y)SW and five (ES)(Y)W transgenic lines. The body and wings were yellow due to the block of *yellow* enhancers by the *gypsy* insulator. Derivatives with deletions in the regulatory region were selected by PCR analysis as before. Derivatives with deletions in the coding region were selected by the *yellow* null phenotype. All genomic sites with the two series of transgenic lines had identical transvection levels in transheterozygotes carrying one *y* allele with the enhancer region deleted [($^{\Delta}$ES)(Y)SW or ($^{\Delta}$ES)(Y)W] and the other allele with the functional *yellow* gene [(ES)(Y)SW or (ES)(Y)W], as well as in transheterozygotes carrying the same *y* allele with the enhancer region deleted together with the *y* allele containing a nonfunctional *yellow* gene [(ES)($Y^{\Delta EX}$)SW or (ES)($Y^{\Delta EX}$)W] (Fig. 4). This result provides evidence against the insulator bypass and suggests that the Su(Hw) insulator does not prevent the *yellow* enhancers from *trans*-activating the *yellow* promoter on the homologous chromosome. As a whole, the *trans* activation levels were higher in the allelic combinations derived from the (ES)(Y)SW transgenic lines than in those derived from the (ES)(Y)W lines (Fig. 4), thus confirming the role of pairing between the *gypsy* insulators located in homologous chromosomes in providing for efficient *trans* interaction between the *yellow* enhancers and the promoter.

Thus, we obtained objective evidence for *trans* activation in the cases of transposon insertions at many ectopic sites. In this context, previous data that the *yellow* enhancers isolated by a *gypsy* insertion into the y^2 or y^{69} alleles failed in *trans* activation of the *yellow* promoter in the enhancerless y^{82f29} allele may be explained by some specific traits of the endogenous *yellow* locus. In particular, this locus contains the recently identified 1A-2 insulator, which is similar but not identical to the *gypsy* insulator (54). In addition, the y^2 allele contains the whole *gypsy* retrotransposon, which makes the relative arrangement of regulatory elements in the y^2/y^{82f29} allele combination different from that in our transgene derivatives.

DISCUSSION

We have demonstrated that the level of *trans* activation by the wing and body enhancers strongly depends on the site of insertion and does not correlate with the level of the *yellow* promoter activation in *cis*. Thus, the genomic regions do not provide identical *yellow* activation in *trans* that might be explained by their pairing strength between the homologs. Hence, there might be some specific elements facilitating the pairing between homologous chromosomes. The *gypsy* insulator inserted either 5 kb or 9 kb downstream from the *yellow* promoter improves its *trans* activation by the enhancers located on the homologous chromosome. The interaction between the *gypsy* insulators can improve the local pairing between homologous chromosomes. Recent cytological data (8) indicate that the *gypsy* insulators create chromatin loop domains by associating with the nuclear matrix. Two homologous chromosomes form only one loop (8), suggesting that the proteins present in the *gypsy* insulator and the nuclear matrix could maintain homologous chromosome pairing during the interphase.

It is noteworthy that the reported *yellow* sequences significant for efficient transvection between enhancerless and promoterless *y* alleles (10) include the 1A-2 insulator located on the 3′ side of the *yellow* gene (27, 55). The insulator containing two binding sites for the Su(Hw) protein was not present in our constructs. Thus, reliable and efficient *trans* activation observed previously (10) upon the transgene insertion at all seven genomic sites can be explained by the presence of the endogenous Su(Hw) insulator improving local homologous pairing. As the Su(Hw) protein binds to approximately 200 sites in the *Drosophila* genome (20), the Su(Hw) binding sites appear to play a role in the pairing between homologous chromosomes. Recent studies of the *scs* and *scs′* insulators confirm that they interact with each other (7) and, therefore, may also be involved in this process.

Although the examples of transvection are many, the nature of chromosome pairing during the interphase is still obscure. Today, only the Zeste protein is known to be involved in some transvection effects. The *zeste* gene encodes the sequence-specific DNA-binding protein with the binding sites distributed throughout genome (3, 4, 58). Inactivation of Zeste disrupts allelic pairing, thus enhancing the heteroallelic mutant phenotype (33, 39). Zeste also supports transvection-like effects in the *decapentaplegic* (15, 16), *white* (1, 16), *eyes absent* (38), and *Ubx* genes (26).

Polycomb response elements (PREs) are another class of regulatory elements that may facilitate pairing between homologous chromosomes. PREs are short DNA segments initiating the assembly of silencing complexes composed of the Polycomb group (PcG) proteins (56). The silencing of a PRE-containing transposon construct is often dramatically enhanced in flies homozygous for the transposon insertion (61). The interaction between two copies of PREs on the homologous chromosomes is supposed to improve the stability and silencing power of the PcG complex. At the same time, the interaction between the PcG complexes may support homologous chromosome pairing. The combination of the binding sites for the proteins like Su(Hw), Zeste, and PcG may generate a unique code for making this process more efficient.

The Zeste, Su(Hw), and PcG proteins, along with having the ability to strengthen homologous effects, are involved in the formation of higher-order nuclear structures. Thus, Zeste can form high-order aggregates (5), which suggests that it can hold together certain DNA regions. Likewise, PcG proteins are organized into discrete nuclear bodies that may be the sites of the PRE-mediated silencing (59). The well-defined Fab-7 cellular memory module also leads to association of transgenes even when inserted into different chromosomes (2). The same was shown for the Mcp element, which contains an insulator and PRE (29, 50). Such long-distance interactions depend on the PcG proteins, at least partially (2, 50). Here, we have shown that the *gypsy* insulator provides for *trans* activation between selected genomic loci at distances exceeding 13 Mb. Together with previous data on the punctuated distribution of *gypsy* insulator proteins in the nucleus (20), the results of this study suggest their involvement in the arrangement of the chromatin fiber within the nucleus and in organization of communication between distant loci in the *Drosophila* genome.

Thus, the interaction between the *gypsy* insulators facilitates *trans* activation of the *yellow* promoter, which is evidence for the involvement of *gypsy* insulators in the regulation of homologous chromosome pairing and communication between distant loci. Further investigations are required to find out whether the transvection stabilization by *gypsy* insulator in homologous and distant locations relies on the same mechanism. The model system utilizing the effects of transvection between *yellow* transgenes provides a powerful tool for the analysis or identification of the proteins supporting interactions between loci.

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