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Drosophila Su(Hw) Insulator Can Stimulate Transcription of a Weakened *yellow* Promoter Over a Distance

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ABSTRACT

The insulator element from the *gypsy* transposon is a DNA sequence that blocks activation of a promoter by a transcriptional enhancer when placed between them. The insulator contains reiterated binding sites for the Suppressor of Hairy-wing [Su(Hw)] zinc-finger protein. A protein encoded by another gene, *modifier of mdg4* [*mod(mdg4)*], is also required for the enhancer-blocking activity of the Su(Hw) insulator. Here we present evidence that the Su(Hw) insulator activates a weakened *yellow* promoter at a distance. Deletion of the upstream promoter region (UPR), located close by the TATA box, significantly reduces *yellow* expression. The Su(Hw) insulator placed at different positions relative to the *yellow* promoter partially compensates for loss of the UPR. Su(Hw) is able to stimulate *yellow* expression even if it is located at a 5-kb distance from the promoter. The stimulatory activity depends on the number of Su(Hw)-binding sites. Mutational analysis demonstrates that only the DNA-binding domain and adjacent regions of the Su(Hw) protein are required for stimulation of *yellow* transcription.

ENHANCER-mediated activation is a fundamental mechanism of gene activation in eukaryotes (Dorsett 1999; West *et al.* 2002). Enhancers can act over large distances to activate transcription, regardless of their orientation and position relative to the promoter, without affecting adjacent genes. Recently, sequences referred to as insulators have been found in different organisms to prevent activation or repression from extending across them to a promoter (Dorsett 1999; Sun and Elgin 1999; Udvardy 1999; Gerasimova and Corces 2001; Oki and Kamakaka 2002; West *et al.* 2002; Kuhn and Geyer 2003). The best-studied vertebrate insulator is the chicken β-globin insulator (Bell *et al.* 1999). Well-characterized insulators in Drosophila include the scs and scs′ sequences found at the boundary of the 87A heat-shock locus (Kellum and Schedl 1991; Zhao *et al.* 1995), Fab-7 and Fab-8 insulators from the *Abd-B* region (Hagstrom *et al.* 1996; Zhou *et al.* 1996, 1999; Barges *et al.* 2000) and the Suppressor of Hairy-wing [Su(Hw)] insulator identified in the *gypsy* retrotransposon (Spana *et al.* 1988; Mazo *et al.* 1989).

The properties of an insulator element may be exemplified by the Su(Hw) insulator, which can block diverse enhancers if inserted between an enhancer and a promoter (Holdridge and Dorsett 1991; Geyer and Corces 1992; Geyer and Clark 2002), but does not affect the intrinsic activity of the enhancer (Cai and Levine 1995; Scott and Geyer 1995). The Su(Hw) insulator can also function as a barrier blocking the silencing activity of the Polycomb group response element (Sigrist and Pirrotta 1997; Mallin *et al.* 1998) and partially protecting a transgene from silencing when inserted into heterochromatin (Roseman *et al.* 1993; 1995; van der Vlag *et al.* 2000).

Genetic and molecular approaches have led to identification and characterization of two proteins required for activity of the Su(Hw) insulator. One is Su(Hw), a 12-zinc-finger protein encoded by the *su(Hw)* gene, which binds to the repeated sequence motifs in the *gypsy* insulator (Dorsett 1990; Spana and Corces 1990). The enhancer-blocking activity of Su(Hw) requires 9 of its 12 zinc fingers and a domain of ~150 amino acids including the C-terminal leucine zipper (Harrison *et al.* 1993; Kim *et al.* 1996).

Mutations in another gene, *modifier of mdg4* [*mod(mdg4)*], alter the phenotypes of *gypsy*-induced mutations, indicating that the product of this gene is also involved in the function of the Su(Hw) insulator (Georgiev and Gerasimova 1989; Gerasimova *et al.* 1995; Georgiev and Kozycina 1996; Cai and Levine 1997; Gdula and Corces 1997). The *mod(mdg4)* gene, also known as *E(var)3-93D*, encodes a large set of individual protein isoforms with specific functions in regulating the chromatin structure of different genes (Gerasimova *et al.* 1995; Buchner *et al.* 2000). The available genetic data suggest that Mod(mdg4) is required for the enhancer-blocking activity (Gerasimova *et al.* 1995; Georgiev and Kozycina 1996; Gdula and Corces 1997). Biochemical studies using purified Su(Hw) and

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Mod(mdg4) proteins indicate that one protein isoform, Mod(mdg4)-67.2, interacts with the enhancer-blocking domain of the Su(Hw) protein (Gause *et al.* 2001; Ghosh *et al.* 2001).

Recently it has been found that the Su(Hw) insulator can stimulate transcription from the alcohol dehydrogenase gene (*Adh*) promoter in a distance-dependent manner (Wei and Brennan 2001). Since the Su(Hw) insulator failed to stimulate the *Adh* promoter with the GATA-binding site deleted or the *white* promoter lacking this site in the larval fat body, it was suggested that the Su(Hw) insulator facilitates the access of the GATA transcription factor to the *Adh* promoter. Here we examined the role of the Su(Hw) insulator in stimulating transcription of the *yellow* gene. The *yellow* gene is required for larval and adult cuticle pigmentation (Nash and Yarkin 1974). The temporal and spatial pattern of its expression is controlled by at least five independent, tissue-specific transcriptional enhancers (Geyer and Corces 1987; Martin *et al.* 1989). The enhancers that control *yellow* expression in the wings and body cuticle are located in the 5′ upstream region of the *yellow* gene, whereas the enhancers controlling its expression in the tarsal claw and bristles reside in the intron of the gene.

Previously we found that a particular *yellow* sequence upstream of the TATA promoter is critical for the *yellow* transcription during pupal development (Belenkaya *et al.* 1998). Deletion of the upstream promoter region (UPR) leads to pronounced reduction of *yellow* expression. Here we show that the Su(Hw) insulator in many transgenic lines partially or completely restores *yellow* expression in the absence of the UPR. Like a distance-independent enhancer, the Su(Hw) insulator can stimulate the *yellow* promoter over at least 5 kb. At the same time, the Su(Hw) insulator fails to compensate for deletion of the bristle enhancer; that is, it does not work as a transcriptional enhancer.

MATERIALS AND METHODS

Drosophila strains: All flies were maintained at 25° on a standard yeast medium. The lines bearing mutations in the *su(Hw)* gene were obtained from V. Corces. The structure and origin of the *su(Hw)* mutations were described by Harrison *et al.* (1993). Drosophila lines carrying combinations of *mod(mdg4)*^{u1} with *su(Hw)*^{j} and *su(Hw)*^{v} were previously obtained (Georgiev and Kozycina 1996). All other mutant alleles and chromosomes used in this work and all balancer chromosomes are described in Lindsley and Zimm (1992).

DNA constructs: The 8-kb fragment containing the *yellow* gene and the cDNA *yellow* clone were kindly provided by P. Geyer. The 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).

The 430-bp *gypsy* sequence containing the Su(Hw)-binding region was PCR amplified from the *gypsy* retrotransposon. After sequencing to confirm its identity, the product was inserted in the CaSpeR2 vector (C2-su). The 5-kb *Bam*HI-*Bgl*II fragment containing the *yellow* coding region (yc) was subcloned into CaSpeR3 (C3-yc) or CaSpeR2-su (C3-su-yc).

The deletion of the regulatory region (yr) between positions −438 and −70 relative to the transcription start site (Geyer *et al.* 1986) was generated by PCR amplification of the yr plasmid between primers y6, 5′-CATTGGCCTGTCTTCGTC TTCGG-3′, and y7, 5′-CAGGAGGCTCGTGCATAGAATGC-3′. The PCR products were blunted, self-ligated, and used for transformation. One of the successfully mutagenized clones was sequenced to confirm that no unwanted changes had been introduced into the *yellow* sequence (Δyr).

The four (S^{x4}) and eight (S^{x8}) reiterated Su(Hw)-binding sites and five binding sites from the Su(Hw) insulator (S^{x5g}) were obtained from E. Savitskaya.

(S)dY(S)W: The Su(Hw) insulator flanked by the frt [frt(su)] sites was inserted in the Δyr plasmid treated with *Eco*47III. The Δyr-frt(su) fragment was ligated into C2-lox(su)-yc treated with *Xba*I and *Bam*HI.

($S^{\times 8}$)dY(S)W: The eight Su(Hw)-binding sites flanked by Flippase recombinase target (FRT) sites [frt(S^{x8})] were inserted in the Δyr plasmid treated with *Eco*47III. The Δyr-frt(S^{x8}) fragment was ligated into C2-lox(su)-yc treated with *Xba*I and *Bam*HI.

(S^{x4})dYW: The four reiterated Su(Hw)-binding sites flanked by locus of X-over P (LOX) sites [lox(S^{x4})] were inserted in the Δyr plasmid treated with *Eco*47III. The Δyr-lox(S^{x4}) fragment was ligated into C3-yc treated with *Xba*I and *Bam*HI.

(S^{g5})(S^{x4})dYW: The five Su(Hw)-binding sites from the *gypsy* insulator flanked by FRTs [frt(S^{g5})] and four reiterated Su(Hw)-binding sites flanked by LOXs [lox(S^{x4})] were ligated together. The [frt(S^{g5}) + lox(S^{x4})] fragment was inserted in the Δyr plasmid treated with *Eco*47III. The Δyr-[frt(S^{g5}) + lox(S^{x4})] fragment was ligated into C3-yc treated with *Xba*I and *Bam*HI.

To obtain the constructs bearing the intronless *yellow* gene, the *Bam*HI-*Bgl*II fragment containing the cDNA *yellow* region (Yil) was subcloned into CaSpeR3 (C3-Yil) or CaSpeR2-su (C2-su-Yil). The Su(Hw) insulator was inserted in the yr plasmid treated with *Eco*47III (yr-su). The yr and yr-su fragments were correspondingly subcloned into C2-su-Yil (YilSW) and C3-Yil (SYilW).

The CaSpeR2 plasmid with the 1.3-kb *Pst*I-to-*Xho*I fragment containing the *su(Hw)* gene promoter [pCsu(Hw)Pr] and the plasmids containing cDNAs of the *su(Hw)* and *mod(mdg4)* genes (C4Su(Hw)DC and C2-mod(mdg4)-2.2) were obtained from D. Dorsett. The chimeric gene expressing Su(Hw)$^{Mod(mdg4)}$ was constructed by ligation of the *Ehe*I-*Bam*HI fragment from C4Su(Hw)DC and the *Eco*RI-*Eco*72I DNA fragment from C2-mod(mdg4)-2.2. To obtain the final *P* transposon with a gene expressing Su(Hw)$^{Mod(mdg4)}$, the *Bam*HI-*Eco*RI fragment containing parts of the *su(Hw)* and *mod(mdg4)* genes was ligated into pCsu(Hw)Pr treated with *Xho*I and *Bam*HI.

Germline transformation and genetic crosses: The construct, together with a *P* element with defective inverted repeats used as a transposase source, P25.7wc (Kares and Rubin 1984), was injected into *y ac* w^{1118} preblastoderm embryos (Rubin and Spradling 1982; Spradling and Rubin 1982). The resulting flies were crossed with *y ac* w^{1118} flies, and transgenic progeny were identified by their eye color. 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 two and *In(3LR)TM3,Sb* for chromosome three. The transformed lines were examined by Southern blot hybridization (Sambrook *et al.* 1989) to check for transposon integrity and copy number.

The lines with excisions of the Su(Hw)-binding sites were obtained by crossing flies bearing the transposons with Flp or Cre recombinase-expressing lines w^{1118}; *CyO, FLP, ISA/Sco*;+ and y^{1}, w^{i}; *CyO, P[*w^{+}*,cre]/Sco*; +. All excisions were confirmed by PCR analysis.

To test the effects of Su(Hw) protein on *yellow* gene expression, lines containing the *yellow* transposons were crossed into a $su(Hw)^{v}/su(Hw)^{f}$ mutant background. This combination of the *su(Hw)* alleles reverses the phenotypes associated with *gypsy* insertions and is female fertile. $Su(Hw)^{v}$ is a deletion of the *su(Hw)* gene (Harrison *et al.* 1993), whereas $su(Hw)^{f}$ is a point mutation in the tenth zinc finger finger that retains some ability to bind DNA (Harrison *et al.* 1993).

The mutations in the *su(Hw)* and *mod(mdg4)* genes were combined with *P(y)* constructs as previously described (Georgiev and Kozycina 1996). Details of the crosses used for genetic analysis and for excision of functional elements are available upon request.

Pigmentation scale: To determine the *yellow* phenotype, the extent of pigmentation in bristles of adult flies was estimated visually in 3- to 5-day-old males developing at 25°. The degree of variegation in bristles of the thorax and head was scored using a five-point scale, where 1 denotes loss of pigmentation in all bristles at thorax and head; e-v, extreme variegation (only one to three bristles on the thorax and head are pigmented); m-v, moderate variegation (about half of the bristles are yellow); w-v, weak variegation (only one to three bristles on thorax and head are yellow); and 5, pigmentation of all bristles as in wild-type flies. At least 50 flies were scored independently by two people for each *y* line.

RESULTS

The Su(Hw) insulator can stimulate *yellow* transcription when the upstream promoter region is deleted: As all previous studies (Geyer *et al.* 1986; Parkhurst and Corces 1986; Geyer and Corces 1992; Georgiev and Kozycina 1996) showed that the Su(Hw) insulator does not activate the wild-type *yellow* promoter, we used the deletion derivatives of the latter. Belenkaya *et al.* (1998) showed that the *yellow* sequence located between positions −146 and −70 relative to the transcription start site is required for the function of the *yellow* promoter. Deletion of this 77-bp sequence, named the upstream promoter sequence (UPR), strongly reduces *yellow* expression in the body cuticle, wing blades, and bristles. To further weaken the *yellow* promoter, we deleted the region from position −438 to −70 (dY). This 368-bp deletion included the UPR and one of the larval enhancers previously mapped to the region between −294 and −92 (Martin *et al.* 1989). The control construct, dYW (Figure 1), contained the *white* gene as a marker for selecting successful insertions in the genome of the *y ac* w^{1118} strain. All 14 independently obtained transformants had strongly decreased pigmentation of the body cuticle, wing blades, and bristles (Table 1). Flies of 10 independent lines homozygous or heterozygous for the transgene displayed a y^{1}-like phenotype, which suggests almost complete inactivation of *yellow*. In two homozygous dYW lines, flies had yellow body cuticle and wing blades, and extremely variegated pigmentation of the head and thoracic bristles: only one to three bristles were pigmented. Flies displayed a weak pigmentation of the body cuticle and wing blades, and moderate variegation of bristle pigmentation in only two homozygous dYW lines; about half of the bristles were pigmented.

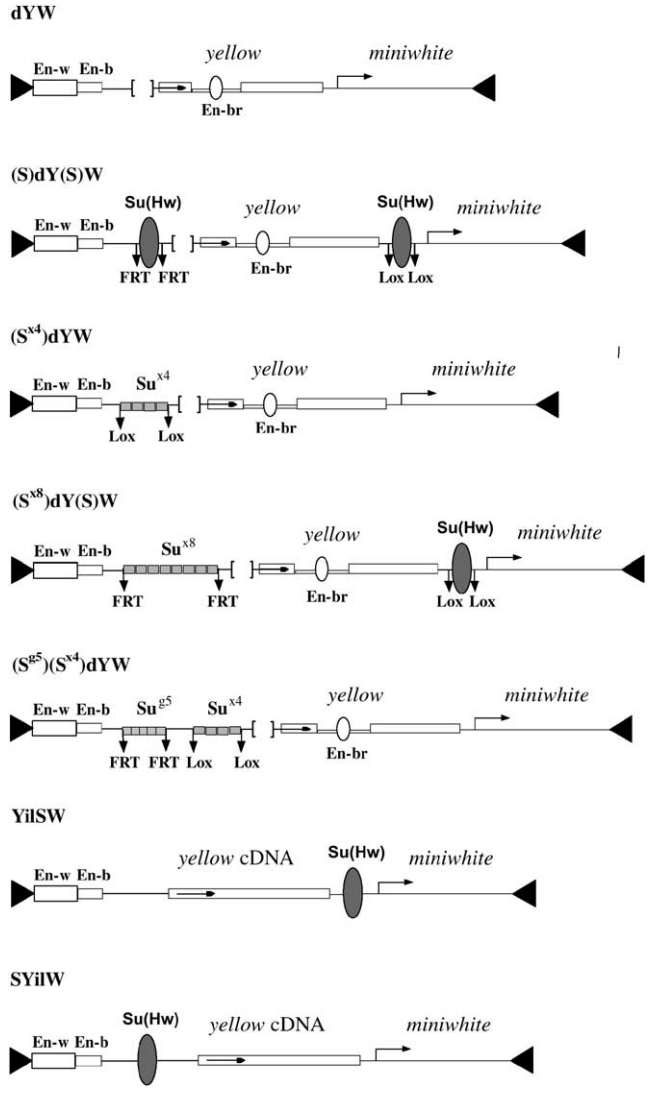

Figure 1.—Schematic of transposon constructs. The maps of the constructs (not to scale) show the *yellow* wing (En-w) and body enhancers (En-b) as partially overlapping open boxes. The bristle enhancer (En-br) is indicated as an open oval in the intron of the *yellow* gene. The arrows indicate the direction of transcription of the *yellow* and *white* genes. Downward arrows labeled FRT or LOX mark the target sites of the Flp or Cre recombinase, respectively. The solid oval represents the Su(Hw) insulator isolated from the *gypsy* retrotransposon. The synthetic Su(Hw)-binding sites are indicated by the open rectangles.

In all dYW lines, flies had yellow-orange eye color, indicating a normal level (euchromatic insertion site) of *mini-white* expression in the absence of the eye enhancer. Thus, in the dYW lines *yellow* transcription is strongly repressed in most of the euchromatic insertions.

To study the assumed stimulatory activity of the Su(Hw) insulator, in (S)dY(S)W (Figure 1) one 340-bp Su(Hw) insulator (S) containing 12 putative Su(Hw)-binding sites (Figure 3A) was inserted at position −525 and another from the 3′ side of the *yellow* gene at +4964

TABLE 1

Summary of phenotypes associated with transgenic dYW, (S)dY(S)W, and (S^{x4})dYW lines

Transgenes	Genotype	*N* of lines[a]	Levels of *yellow* expression in bristles[b]				
			5	w-v	m-v	e-v	1
dYW	P/+	14	—	—	—	2	12
	P/P	14(4)	—	—	2	2	10
(S)dY(S)W	P/+	23	5	3	3	3	9
	P/P	14(12)		2	2	8	2
(S)dY(ΔS)W	P/+	11(6)	3	2	2	2	2
(ΔS)dY(S)W	P/+	11(7)	2	3	1	1	4
(ΔS)dY(ΔS)W	P/+	11(11)	—	—	—	1	10
($S^{\times 4}$)dYW	P/+	12	—	1	3	3	5
	P/P	12(9)	1	3	2	5	1
($\Delta S^{\times 4}$)dYW	P/+	12(7)	—	—	—	1	11
	P/P	12(7)			1	1	10

The phenotypes of transgenic lines (P) were examined in males heterozygous (P/+) or homozygous (P/P) for the construct.

[a] Number of tested transgenic lines. Figures in parentheses show the number of lines in which flies acquired a new y phenotype in comparison with flies from the starting line.

[b] Number of flies with similar levels of bristle pigmentation. The degree of variegation in bristles of the thorax and head: 1, loss of pigmentation in all bristles at thorax and head; e-v, extreme variegation (only one to three bristles on thorax and head are pigmented); m-v, moderate variegation (about half of bristles are yellow); w-v, weak variegation (only one to three bristles on thorax and head are yellow); 5, pigmentation of all bristles as in wild-type flies.

TABLE 2

Influence of the *su(Hw)* mutations on *yellow* expression in bristles

Transgenes	Genotype	*N* of lines	Levels of *yellow* expression in bristles				
			5	w-v	m-v	e-v	1
dYW	$su(Hw)^+$	4	—	—	—	2	2
	$su(Hw)^-$	4	—	—	—	2	2
(S)dY(S)W	$su(Hw)^+$	7	5	2	—	—	—
	$su(Hw)^-$	7(7)	—	—	—	1	6
(S^{x4})dYW	$su(Hw)^+$	4	—	1	3	—	—
	$su(Hw)^-$	4(4)	—	—	—	—	4
(S^{x8})dY(ΔS)W	$su(Hw)^+$	6	4	2	—	—	—
	$su(Hw)^-$	6(6)	—	—	—	—	6
SYilW	$su(Hw)^+$	4	—	—	2	1	1
	$su(Hw)^-$	4	—	—	2	1	1
YilSW	$su(Hw)^+$	5	—	1	2	1	1
	$su(Hw)^-$	5	—	1	2	1	1

The levels of bristle pigmentation were examined in heterozygotes (P/+) for the transgene males in the $su(Hw)^+$ ($su(Hw)^+/su(Hw)^+$) or $su(Hw)^-$ ($su(Hw)^v/su(Hw)^f$) background. Other designations are as in Table 1.

relative to the *yellow* transcription start site. The Su(Hw) insulators were flanked by FRT or LOX sites to permit their excision from transgenic flies by crossing the latter with flies expressing either Flp (Golic and Lindquist 1989) or Cre recombinase (Siegal and Hartl 2000). In 23 transgenic lines carrying a single (S)dY(S)W insertion, flies had eyes ranging in color from yellow to dark orange. As the Su(Hw) insulator inserted at −525 blocks the wing and body enhancers, in this and the following experiments we examined *yellow* expression only in bristles. The bristle enhancer is located in the *yellow* intron (Geyer and Corces 1987) and thus it is not blocked by the Su(Hw) insulator inserted either upstream or downstream of the *yellow* gene. In contrast to control dYW transgenic lines, flies heterozygous for the (S)dY(S)W construct in 8 of 23 transgenic lines had wild-type or nearly wild-type levels of bristle pigmentation (Table 1), suggesting substantial activation of the *yellow* promoter. In 6 (S)dY(S)W lines, flies had moderate or strong variegation of bristle pigmentation and only 9 lines displayed y^1-like phenotype. In 12 of 14 transgenic lines, flies homozygous for the construct had more pigmented bristles than did heterozygous ones. To test the contribution of the Su(Hw) protein to transcription stimulation, we crossed flies displaying wild-type or nearly wild-type bristle pigmentation from 7 (S)dY(S)W lines into a $su(Hw)^v/su(Hw)^f$ mutant background (Table 2). In all tested lines, the level of bristle pigmentation was decreased to nearly the y^1-like phenotype. These results suggest that the Su(Hw) insulators stimulate transcription from the weakened *yellow* promoter in most of the transgenic lines and that the level of activation strongly depends on the site of construct insertion.

To assess the contribution of each Su(Hw) insulator to transcription stimulation, we deleted either the upstream ((ΔS)dY(S)W) or the downstream [(S)dY(ΔS)W] Su(Hw) insulator or both [(ΔS)dY(ΔS)W] from 11 transgenic lines in which flies had pigmented bristles (Table 1). In 5 transgenic lines, deletion of either Su(Hw) insulator did not significantly change bristle pigmentation, while deletion of both Su(Hw) insulators almost completely abolished it (Figure 2). This finding suggests that the Su(Hw) insulator does not stimulate *yellow* expression just as a neutral boundary that prevents spreading of the negative effects of surrounding chromatin. In contrast, the Su(Hw) insulator appears to be an active stimulator of the weakened *yellow* promoter.

In the other six transgenic lines, deletion of either Su(Hw) insulator partially reduced or completely eliminated bristle pigmentation (Figure 2). In 4 of 11 cases, deletion of the upstream Su(Hw) insulator had a more

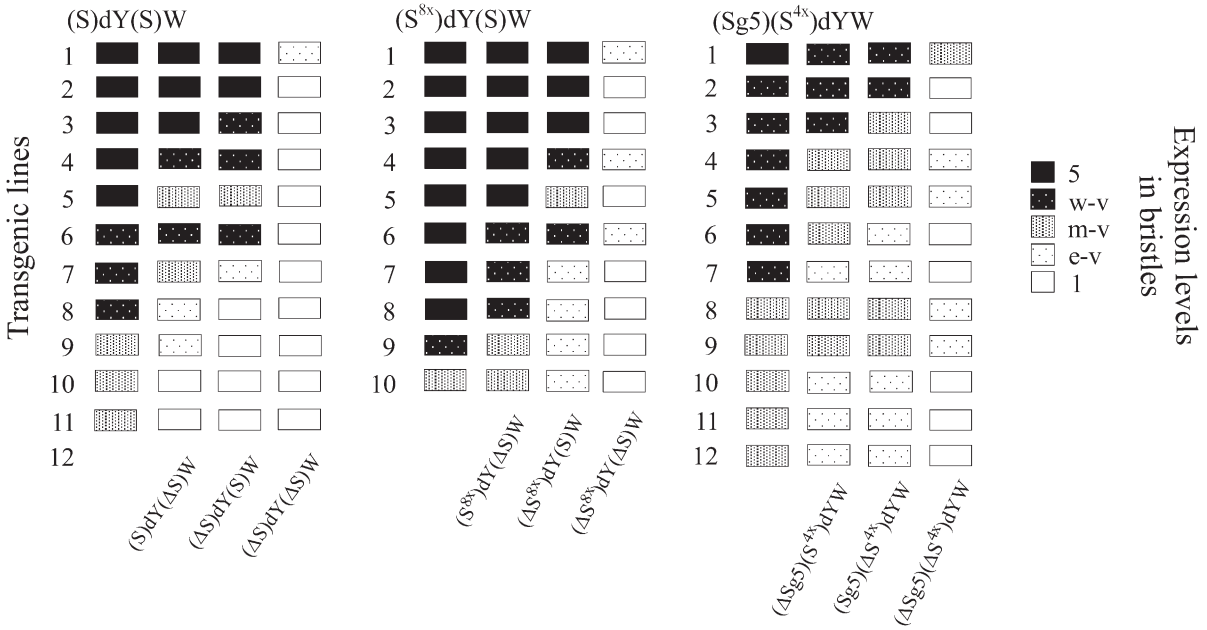

FIGURE 2.—Summary of phenotypes associated with selected transgenic (S)dY(S)W, (S^{g5})(S^{x4})dYW, and (S^{x8})dY(S)W lines and their derivatives. All transgenic lines were numbered. For each line, pigmentation levels reflecting expression of the *yellow* gene in bristles are indicated by boxes using a five-level scale. Open boxes indicate a y^1-like phenotype and solid boxes indicate a wild-type level of bristle pigmentation.

pronounced effect, suggesting that the Su(Hw) insulator located upstream from the *yellow* promoter is more stimulatory.

The level of transcriptional stimulation directly correlates with the number of the Su(Hw)-binding sites: The natural Su(Hw) insulator consists of 12 degenerate Su(Hw)-binding sites (Figure 3A), which have different affinity to the Su(Hw) protein (SPANA and CORCES 1990; KIM *et al.* 1996; SCOTT *et al.* 1999). It is possible that other proteins in addition to Su(Hw) bind with the 12-bp core sequence (consensus, 5′-PyPuTTGCATACCPy-3′) and are also involved in transcription stimulation. To examine this possibility, we used synthetic binding regions with 4 and 8 sites for Su(Hw), generated by concatemerization of a 31-unit oligonucleotide corresponding to the third Su(Hw)-binding site reported as the most effective one (SPANA and CORCES 1990; KIM *et al.* 1996).

In the (S^{x4})dYW construct (Figure 1), four Su(Hw)-binding sites (S^{x4}) were inserted at position −525 relative to the transcription start in the *yellow* gene carrying the 368-bp deletion (dY). The S^{x4} fragment was flanked by LOX sites. In 7 of 12 transgenic lines heterozygous for the (S^{x4})dYW construct and in 11 of 12 lines homozygous for (S^{x4})dYW, flies had partially pigmented bristles (Table 1). Thus, four Su(Hw)-binding sites are able to stimulate *yellow* expression in most of genomic sites of the construct insertion. Deletion of the S^{x4} fragment eliminated bristle pigmentation in most of the lines, confirming the role of the Su(Hw)-binding sites in *yellow* stimulation. To verify the role of the Su(Hw) protein in transcription stimulation, we crossed flies with pigmented bristles from four (S^{x4})dYW lines into a *su(Hw)*^{v}/*su(Hw)*^{f} mutant background (Table 2). Inactivation of Su(Hw) led to a y^1-like phenotype, supporting the significance of Su(Hw) in transcription stimulation in (S^{x4})dYW lines.

In the (S^{x8})dY(S)W construct (Figure 1), eight Su(Hw) binding sites (S^{x8}) flanked with FRTs were inserted at −525. The Su(Hw) insulator flanked with LOXs was inserted at the 3′ side of the *yellow* gene. In 14 of 25 lines heterozygous for the (S^{x8})dY(S)W construct, flies had detectable bristle pigmentation (Table 3, Figure 2). Deletion of the Su(Hw) insulator (ΔS) from the 3′ side of *yellow* did not significantly reduce *yellow* expression: flies in 8 transgenic lines heterozygous for (S^{x8})dY(ΔS)W had nearly wild-type levels of bristle pigmentation (Figure 2). Additional deletion of the S^{x8} fragment led to complete repression of *yellow* in 21 of 25 tested (ΔS^{x8})dY(ΔS)W derivative lines. To confirm the role of Su(Hw), *yellow* expression was examined on the *su(Hw)*$^{-}$ background in 6 (S^{x8})dY(ΔS)W lines in which flies had nearly wild-type bristle pigmentation (Table 2). In all cases, inactivation of the Su(Hw) protein led to almost complete *yellow* repression in bristles. Comparison of bristle pigmentation of flies carrying the construct with deletion of either the Su(Hw) insulator [(S^{x8})dY(ΔS)W] or the eight Su(Hw) binding sites [(ΔS^{x8})dY(S)W] demonstrated that the Su(Hw) binding sites inserted at −525 stimulated *yellow* expression more efficiently than the Su(Hw) insulator inserted at the 3′ side of the *yellow* gene (Figure 2). As in the (S)dY(S)W

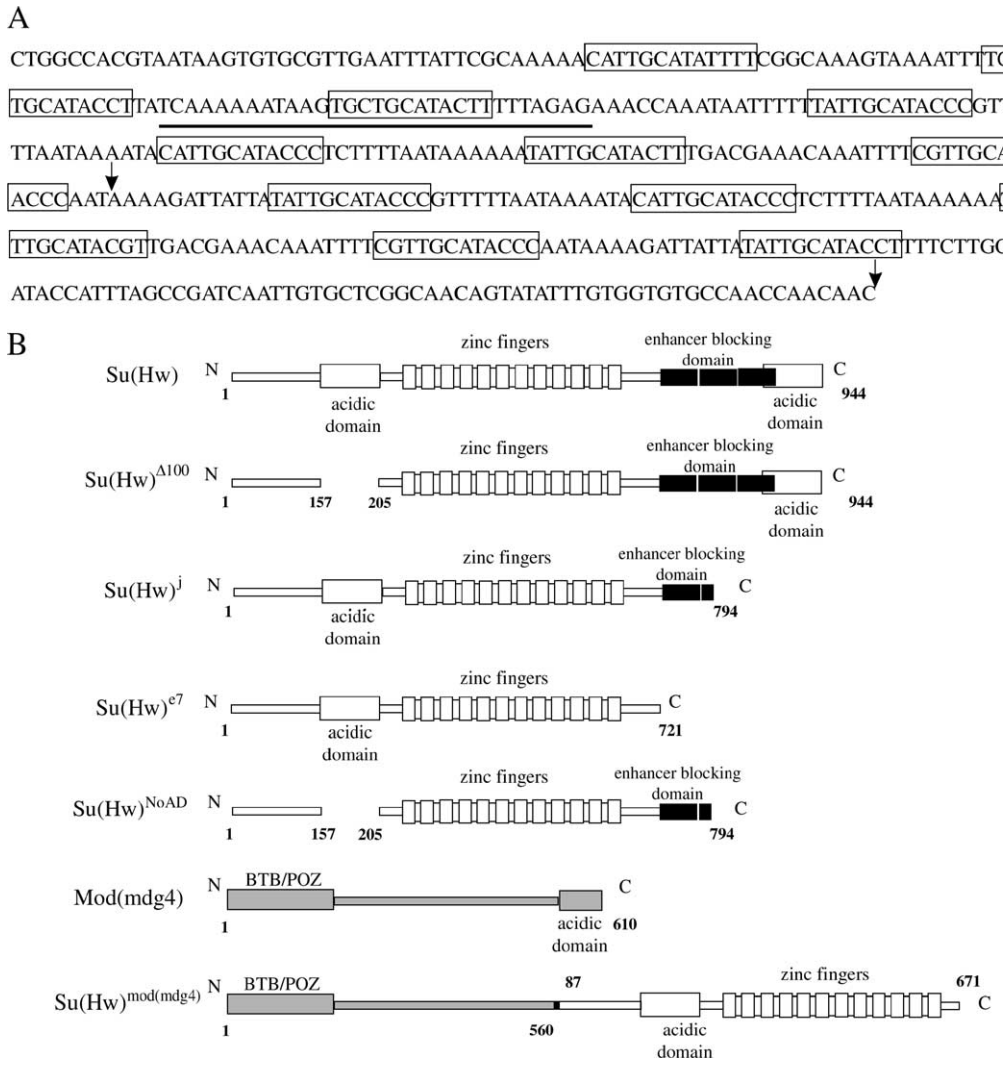

FIGURE 3.—(A) The sequence of the Su(Hw) insulator isolated from the *gypsy* retrotransposon (MARLOR *et al.* 1986). The 12 core binding sites are boxed. The underlining indicates the sequence of the nucleotide used to produce the synthetic Su(Hw)-binding regions. The consensus for the Su(Hw)-binding site was taken from SCOTT *et al.* (1999). The arrows indicate the sequence of the 8–12 Su(Hw)-binding sites. (B) Schematic of the Su(Hw) and Mod(mdg4)-67.2 proteins in mutations and constructions used in this study.

lines, the upstream Su(Hw) insulator had a more pronounced stimulatory effect than the downstream one; we suggest that the Su(Hw) insulator and the eight Su(Hw)-binding sites stimulate transcription with comparable effectiveness.

As flies in the (S^{x8})dY(ΔS)W lines had more pigmented bristles than flies from the (S^{x4})dYW lines, we decided to further examine the correlation between the number of Su(Hw)-binding sites and their ability to stimulate transcription. In the (S^{g5})(S^{x4})dYW construct (Figure 1), a DNA fragment including four Su(Hw)-binding sites flanked with LOXs (S^{x4}) and five Su(Hw)-binding sites (8–12) from the Su(Hw) insulator (S^{g5}) (Figure 3A), flanked with FRTs, was inserted at −525. In 15 of 23 transgenic lines, flies heterozygous for the construct displayed detectable bristle pigmentation (Table 3). Deletion of either four ($S^{\times 4}$) or five (S^{g5}) Su(Hw)-binding sites partially reduced bristle pigmentation, while deletion of all Su(Hw)-binding sites completely eliminated bristle pigmentation in most transgenic lines. These results further confirm that the efficiency of *yellow* stimulation directly correlates with the number of Su(Hw)-binding sites.

The Su(Hw) insulator does not compensate the deletion of the *yellow* enhancer: As the Su(Hw) insulator stimulates the *yellow* expression at a large distance, it is possible that the Su(Hw) insulator acts as an enhancer. To test the ability of the Su(Hw) insulator to activate *yellow* expression in the absence of the bristle enhancer, we made two constructs bearing an intronless *yellow* gene and the Su(Hw) insulator inserted either at −893 bp (ESYilW, Figure 1) or at the 3′-end of the *yellow* gene (YilSW, Figure 1). As shown previously (GEYER and CORCES 1987; MARTIN *et al.* 1989), flies bearing an intronless *yellow* gene produced yellow bristles.

In 11 SYilW lines and 14 YilSW lines, flies had yellow-colored bristles (Table 3). The bristle pigmentation was unchanged in the $su(Hw)^{v}/su(Hw)^{f}$ background in flies from four SYilW lines and five YilSW lines (Table 2). These results indicate that the Su(Hw) insulator is unable to functionally substitute for the bristle enhancer.

Structural and functional analysis of Su(Hw) domains with regard to the insulator activity: Su(Hw) has two acidic domains located at the amino- and carboxy-termini of the protein and an enhancer-blocking region located between 737 and 880 aa that is most important

TABLE 3

Summary of phenotypes associated with the (S^{g5})(S^{x4})dYW, (S^{x8})dY(S)W, SyilW, and YilSW transgenic lines

Transgenes	*N* of lines	Levels of *yellow* expression in bristles				
		5	w-v	m-v	e-v	1
(S^{x8})dY(S)W	25	8	1	1	4	11
(S^{x8})dY(ΔS)W	25(9)	5	3	2	2	13
(ΔS^{x8})dY(S)W	25(12)	3	2	1	4	16
(ΔS^{x8})dY(ΔS)W	25(14)	—	—	—	4	21
(S^{g5})(S^{x4})dYW	23	1	6	5	3	8
(ΔS^{g5})(S^{x4})dYW	23(11)	—	3	5	5	10
(S^{g5})(ΔS^{x4})dYW	23(12)	—	2	5	6	10
(ΔS^{g5})(ΔS^{x4})dYW	23(15)	—	—	1	4	18
SYilW	11	—	—	2	1	8
YilSW	14	—	1	2	2	9

All designations are as in Table 1.

for insulation (Harrison *et al.* 1993; Kim *et al.* 1996; Gdula and Corces 1997). To address the role of individual Su(Hw) protein domains in *yellow* expression, different *su(Hw)* mutations (Figure 3B) were crossed into selected (S)dY(S)W, (S^{x4})dYW, and (S^{x8})dY(S)W lines and their derivatives carrying constructs on the X or second chromosome. In all selected transgenic lines, flies had wild-type or nearly wild-type levels of bristle pigmentation (Table 4).

The Su(Hw) protein (Figure 3B) contains a large acidic domain in the amino-terminal region and a second minor one in the carboxy terminus (Harrison *et al.* 1993). We have used the $su(Hw)^{\Delta 100}$ allele to address the question whether the amino-terminal acidic domain is involved in *yellow* activation. The $su(Hw)^{\Delta 100}$ mutation has an in-frame deletion of the 48 amino acids that constitute the amino-terminal acidic domain (Harrison *et al.* 1993). Flies heterozygous for the transposon and homozygous for the $su(Hw)^{\Delta 100}$ allele had the same phenotype as those heterozygous for only the *yellow* transposon (Table 4). This result suggests that the N-terminal acidic domain of Su(Hw) is not important for *yellow* stimulation.

To address the effect of the Su(Hw) carboxy-terminal domain on *yellow* expression, the $su(Hw)^{j}$ allele was crossed into flies heterozygous for the *yellow* transposons. The Su(Hw) protein encoded by this allele lacks the 149 terminal residues, including the carboxy-terminal acidic domain and a part of the enhancer-blocking domain (Harrison *et al.* 1993; Kim *et al.* 1996; Gdula and Corces 1997). Similarly to $su(Hw)^{\Delta 100}$, $su(Hw)^{j}$ does not influence *yellow* expression in transgenic lines (Table 4). Thus, the carboxy-terminal portion of the Su(Hw) protein is also not required for *yellow* activation. Because in the $Su(Hw)^{j}$ protein the domain interacting with the Mod(mdg4)-67.2 protein is only partially deleted (Gause *et al.* 2001; Ghosh *et al.* 2001), we examined the role of Mod(mdg4)-67.2 in *yellow* stimulation by the Su(Hw) insulator. The $mod(mdg4)^{u1}$ mutation, which is known to affect the interaction between the Mod(mdg4)-67.2 isoform and Su(Hw) (Gause *et al.* 2001; Ghosh *et al.* 2001), was combined with the $su(Hw)^{j}$ allele (Georgiev and Kozycina 1996). Combination of the $su(Hw)^{j}$ and $mod(mdg4)^{u1}$ mutations did not influence bristle pigmentation in the tested transgenic lines (Table 4). Thus, the Mod(mdg4)-67.2 protein is not required for *yellow* activation by the Su(Hw) insulator.

The $su(Hw)^{e7}$ mutation leads to the loss of 223 amino acids from the carboxy-terminal end of the Su(Hw) protein (Harrison *et al.* 1993). This mutation only slightly affects the *yellow* phenotype in some transgenic lines (Table 4). Thus, the Su(Hw) protein lacking the domain responsible for enhancer blocking and the C-terminal acidic domain is still able to stimulate *yellow* expression when the Su(Hw) insulator is located at either the 5′- or the 3′-end of the *yellow* gene.

The $Su(Hw)^{NoAD}$ protein lacks the amino- and carboxy-terminal acidic domains and the part of the enhancer blocking domain (Harrison *et al.* 1993; Gdula and Corces 1997). The $su(Hw)^{NoAD}$ mutation partially relieved the mutant phenotype of the transgenic lines (Table 4). However, *yellow* repression is considerably less prominent than in the $su(Hw)^{-}$ background. This result might suggest that simultaneous deletion of both acidic domains and the enhancer-blocking domain partially affects the activating capacity of the Su(Hw) protein. Alternatively, the inability of $Su(Hw)^{NoAD}$ to effectively stimulate *yellow* expression could be explained by the instability of the truncated protein or less effective interaction with the Su(Hw) insulator.

Next we obtained two transgenic lines expressing the chimeric protein $Su(Hw)^{Mod(mdg4)}$ under the control of the Su(Hw) promoter as described in Kim *et al.* (1996). $Su(Hw)^{Mod(mdg4)}$ contains only the DNA-binding domain and the amino-terminal acidic domain that is joined to the C-terminal end of the truncated Mod(mdg4)-67.2 protein with deletion of the C-terminal domain required for interaction with Su(Hw) (Figure 3B). In all tested transgenic lines and their derivatives, the $Su(Hw)^{Mod(mdg4)}$ protein efficiently stimulated *yellow* transcription at the level of the $Su(Hw)^{e7}$ protein (Table 4). As Mod(mdg4)-67.2 is not required for *yellow* activation, we suggest that the DNA-binding region and adjacent regions of Su(Hw) are sufficient for the transcriptional stimulation mediated by the Su(Hw) insulator.

DISCUSSION

The Su(Hw) insulator does not notably stimulate *yellow* transcription when the *yellow* promoter is functional (Geyer and Corces 1992). However, the Su(Hw) protein can behave as an activator of the *yellow* promoter if the upstream activator region is deleted. The level of *yellow* activation directly correlates with the number of

TABLE 4

Influence of various *su(Hw)* alleles on *yellow* expression in bristles

		Genotypes								
		+	*v/f*	*v/2*	*j*	*j*	*Δ100*	*e7*	*NoAD*	*Su-M*
Transgene	Levels of *yellow* expression in bristles:	+	+	*m*	+	*m*	+	+	+	+
dYW		ev	ev	ev	ev	ev	ev	ev	ev	ev
(S)dY(S)W-1		5	*ev*	*ev*	5	5	5	5	*wv*	5
(S)dY(ΔS)W-1		5	*ev*	*ev*	5	5	5	5	*mv*	5
(ΔS)dY(S)W-1		5	*ev*	*ev*	5	5	5	5	*ev*	*wv*
(ΔS)dY(ΔS)W-1		ev	ev	ev	ev	ev	ev	ev	ev	ev
(S)dY(S)W-4		5	*1*	*1*	5	5	5	5	*wv*	5
(S)dY(S)W-5		5	*1*	*1*	5	5	5	5	*mv*	5
(S^{x4})dYW		wv	*1*	*1*	wv	wv	wv	*mv*	*1*	*mv*
($ΔS^{x4}$)dYW		1	1	1	1	1	1	1	1	1
(S^{x8})dY(ΔS)W-1		5	*ev*	*ev*	5	5	5	5	*mv*	5
(S^{x8})dY(S)W-2		5	*1*	*1*	5	5	5	5	*wv*	5
(S^{x8})dY(ΔS)W-2		5	*1*	*1*	5	5	5	*wv*	*ev*	5
($ΔS^{x8}$)dY(S)W-2		5	*1*	*1*	5	5	5	*wv*	*ev*	*wv*
($ΔS^{x8}$)dY(ΔS)W-2		1	1	1	1	1	1	1	1	1
(S^{x8})dY(ΔS)W-5		5	*1*	*1*	5	5	5	*wv*	*ev*	*wv*

+, *su(Hw)*$^{+}$ *or mod(mdg4)*$^{+}$; *v/f, su(Hw)*^{v}*/su(Hw)*^{f}; *v/2, su(Hw)*^{v}*/su(Hw)*^{2}; *m, mod(mdg4)*^{u1}*/mod(mdg4)*^{u1}; *j, su(Hw)*^{j}*/su(Hw)*^{j}; *Δ100, su(Hw)*^{Δ100}*/su(Hw)*^{Δ100}; *su(Hw)*^{v}*/su(Hw)*^{2}; *e7, su(Hw)*^{e7}*/su(Hw)*^{v}; *NoAD, su(Hw)*^{v} *su(Hw)*^{NoAD}*/su(Hw)*^{2} *su(Hw)*^{NoAD}; *Su-M, su(Hw)*$^{Mod(mgd4)}$*/su(Hw)*$^{Mod(mdg4)}$; *su(Hw)*^{v}*/su(Hw)*^{f}. Italics indicate cases in which the *su(Hw)* mutations change *yellow* expression. The selected transgenic lines have the same numbers as in Figure 2. Other designations are described in the legend of Table 1.

the Su(Hw)-binding sites. The promoter stimulation activity of the Su(Hw) insulator is not restricted to the *yellow* promoter. Previously it was found that the Su(Hw) insulator stimulates the alcohol dehydrogenase promoter (Wei and Brennan 2001). The Su(Hw) protein also may be an activator of the weak *gypsy* promoter, as levels of *gypsy* RNA considerably decrease in *su(Hw)* mutants (Parkhurst and Corces 1986; Smith and Corces 1995). It seems that the Su(Hw) insulator can strengthen weak promoters but its effect is not visible in the case of a strong promoter.

Like a distance-independent enhancer, the Su(Hw) insulator can stimulate the *yellow* promoter over at least 5 kb. At the same time, the Su(Hw) insulator fails to compensate the deletion of the bristle enhancer, suggesting that the Su(Hw) insulator does not work as an enhancer. The long-distance effect of the Su(Hw) insulator cannot be explained by the boundary activity. As we found in many genomic sites, stimulation of transcription requires only one copy of the Su(Hw) insulator located either upstream or downstream from the *yellow* promoter. If only boundary function is important for the transcriptional stimulation, the location of the Su(Hw) insulator relative to the promoter must be crucial. As the transcriptional stimulation by the Su(Hw) insulator could be observed when the *yellow* promoter was partially inactivated by deletion of UPR, we suggest that Su(Hw) facilitates the assembling of a transcriptional complex at the *yellow* promoter.

The Su(Hw) insulator completely lost the ability to stimulate *yellow* transcription on the *Su(Hw)*$^{-}$ background, suggesting the main role of the Su(Hw) protein in this activity. Previous studies showed that the Su(Hw) protein has several different activities in the regulation of transcription. The enhancer-blocking activity mainly depends on the conserved domain located between the DNA-binding and carboxy-terminal acidic domains of the Su(Hw) protein (Harrison *et al.* 1993; Kim *et al.* 1996). The Mod(mdg4)-67.2 protein interacts with the enhancer-blocking domain of the Su(Hw) protein and contributes to the insulator activity (Gause *et al.* 2001; Ghosh *et al.* 2001). Inactivation of the Mod(mdg4)-67.2 protein in the *mod(mdg4)*^{u1} mutant converts the Su(Hw) insulator to a promoter-specific silencer (Gerasimova *et al.* 1995; Georgiev and Kozycina 1996; Cai and Levine 1997; Wei and Brennan 2001). It is likely that

in the absence of the Mod(mdg4)-67.2 protein, Su(Hw) can directly interfere with the transcription complex at a promoter (Georgiev and Kozycina 1996; Cai and Levine 1997). Genetic analysis of the *su(Hw)* mutations involving deletions of particular domains of the Su(Hw) protein showed that the carboxy-terminal acidic domain is responsible for direct repression of the *yellow* promoter in the absence of Mod(mdg4)-67.2 (Georgiev and Kozycina 1996; Gdula and Corces 1997).

Here we found that deletion of either the acidic domain or the enhancer-blocking domain does not affect the ability of Su(Hw) to stimulate the weakened *yellow* promoter. However, deletion of both acidic domains and the enhancer-blocking domain in the $Su(Hw)^{NoAD}$ protein affects the activating capacity of the Su(Hw) insulator. Previously it was found that interaction with Mod(mdg4)-67.2 facilitates the binding of Su(Hw) to insulator sequences *in vivo* (Gerasimova and Corces 1998). As $Su(Hw)^{NoAD}$ fails to interact with Mod(mdg4)-67.2, we suggest that the deletion of the acidic and enhancer-blocking domains decreases DNA-binding affinity of the truncated $Su(Hw)^{NoAD}$ protein. As the level of transcriptional stimulation directly correlates with the number of the Su(Hw)-binding sites, the reducing of DNA-binding affinity of $Su(Hw)^{NoAD}$ might lead to the inability of the Su(Hw) insulator to efficiently stimulate transcription.

The chimeric $Su(Hw)^{Mod(mdg4)}$ protein consisting of the Su(Hw) DNA-binding domain, amino-terminal acidic domain, and Mod(mdg4)-67.2 can effectively stimulate transcription. As the Mod(mgd4)-67.2 protein and the amino-terminal acidic domain of Su(Hw) are not required for transcriptional stimulation by the Su(Hw) insulator, we suggest that the DNA-binding domain of Su(Hw) fulfills the main role in activation of the weak *yellow* promoter. Thus, different domains of Su(Hw) are required for enhancer blocking, promoter repression, and transcriptional stimulation. Interestingly, the enhancer-blocking and acidic domains of Su(Hw) are also not required for the boundary function of the Su(Hw) insulator in preventing gene repression by centric or telomeric heterochromatin (Geyer and Clark 2002).

As only DNA-binding domain and adjacent regions of Su(Hw) are required for long-distance transcriptional stimulation, we suggest that these domains form an entry site for the modification complexes. Recently, it was found (Torigoi *et al.* 2000) that the DNA-binding domain of Su(Hw) interacts with Chip that has been proposed to be a facilitator protein required for communication between an enhancer and a promoter (Morcillo *et al.* 1997; Dorsett 1999). Since only some of the 12 zinc fingers are required for DNA binding (Kim *et al.* 1996), other zinc fingers can be involved in recruiting protein complexes. The long-distance transcriptional stimulation might be explained by spreading of modification complexes to the *yellow* promoter. Consistent with this possibility, Chen and Corces (2001) showed that the Su(Hw) insulator increases the long-distance accessibility of the DNA to nucleases independently of the transcriptional status of the *yellow* gene. As a result of chromatin modifications, general transcription factors would gain access to the promoter region with a higher probability. Alternatively, the Su(Hw) insulator can directly interact with the *yellow* promoter by looping out the intervening DNA. The ability of the Su(Hw) insulator to repress *yellow* transcription in the absence of Mod(mdg4)-67.2 supports the possibility of direct interactions between proteins bound to the *yellow* promoter and the Su(Hw) insulator. Further study is required to understand the mechanism of the long-distance transcriptional stimulation of the *yellow* promoter by the Su(Hw) insulator.

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