Characterization of Functional Domains of the su(Hw) Protein That Mediate the Silencing Effect of *mod(mdg4)* Mutations

David A. Gdula[1] and Victor G. Corces

Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218

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ABSTRACT

The suppressor of Hairy-wing [su(Hw)] protein represses enhancer function in a unidirectional fashion: enhancers segregated from the promoter by the su(Hw) binding region are rendered inactive, whereas those in the same domain are unaffected. In the case of the *gypsy*-induced y^2 allele, the repressive effect of su(Hw) is rendered bidirectional in *mod(mdg4)* mutant flies, and all enhancers of the affected gene become inactive. This silencing of enhancer elements might be due to exposure of specific domains of su(Hw) when the *mod(mdg4)* protein is absent. Two of three regions of su(Hw) that are located adjacent to the leucine zipper motif and are conserved across Drosophila species are necessary for both the unidirectional and bidirectional repression of transcription by su(Hw). In contrast, two acidic domains that are dispensable for the unidirectional repression of enhancer elements are critical for the bidirectional silencing of enhancer activity observed in mutants lacking functional *mod(mdg4)* protein.

MUTATIONS caused by the insertion of the *gypsy* retrotransposon are suppressed by mutations in the cellular gene *suppressor of Hairy-wing* [*su(Hw)*] (MODOLELL *et al.* 1983; RUTLEDGE *et al.* 1988). The su(Hw) protein binds directly to a sequence within the 5′ transcribed untranslated region of *gypsy*, thereby causing the inactivation of adjacent genes (PARKHURST *et al.* 1988; SPANA *et al.* 1988; MAZO *et al.* 1989; SPANA and CORCES 1990). The su(Hw)-binding region contains 12 copies of a sequence homologous to the octamer motif present in several mammalian enhancers (GEYER *et al.* 1988; PEIFER and BENDER 1988; MAZO *et al.* 1989), and genetic studies have shown that this region alone is necessary and sufficient to elicit the repression of enhancer activity (GEYER and CORCES 1992; SMITH and CORCES 1992). The molecular mechanisms by which su(Hw) represses enhancer function and mediates the mutant effect of the *gypsy* element has been studied extensively in the case of *gypsy*-induced mutations in the *yellow* gene. The y^2 allele is caused by the insertion of the *gypsy* element 700 bp upstream from the start of transcription (GEYER *et al.* 1986; MARTIN *et al.* 1989). Adult flies carrying this mutation display a spatially restricted phenotype: only the wing and body cuticle are mutant in coloration, whereas other tissues of the fly are wild type. Molecular analyses of the *yellow* gene have shown that the enhancers controlling expression of *yellow* in the tissues affected by the insertion of *gypsy* in the y^2 allele are segregated from the promoter by the *gypsy* element itself (GEYER and CORCES 1987; MARTIN *et al.* 1989). Other enhancers are unaffected, indicating that the negative effect of su(Hw) on enhancers is unidirectional and only distal enhancers are repressed (HOLDRIDGE and DORSETT 1991; JACK *et al.* 1991; GEYER and CORCES 1992). In addition to its ability to repress enhancers, the su(Hw) protein has been shown to buffer a transgene from position effects when its binding sites flank the gene (ROSEMAN *et al.* 1993). These two properties of su(Hw) demonstrate a functional similarity between the su(Hw)-binding region and chromatin insulators that prevent the transmission of chromatin structures that establish active or repressed domains of gene activity (KELLUM and SCHEDL 1992; CHUNG *et al.* 1993).

Several domains of the su(Hw) protein are essential for its ability to repress enhancer function. These include a stretch of 12 zinc fingers, which allow su(Hw) to bind to DNA, and a leucine zipper (HARRISON *et al.* 1993). The latter domain of su(Hw) has been implicated in mediating its interaction with other proteins. One such protein is encoded by the *modifier of mdg4* [*mod(mdg4)*] gene (GERASIMOVA *et al.* 1995). Flies bearing the y^2 mutation and carrying the $mod(mdg4)^{u1}$ allele display a complex *yellow* phenotype due to partial inactivation of all enhancers of the *yellow* gene (GERASIMOVA *et al.* 1995). Null mutations in the *mod(mdg4)* gene are lethal, and the $mod(mdg4)^{u1}$ and $mod(mdg4)^{T6}$ alleles used to study effects on enhancer function are hypomorphs that express low levels or a truncated form of the wild-type protein (GERASIMOVA *et al.* 1995; T. GERASIMOVA and V. CORCES, unpublished data). The $mod(mdg4)^{u1}$ mutation affects only *gypsy*-induced alleles and

Corresponding author: Victor G. Corces, Department of Biology, The Johns Hopkins University, 3400 N. Charles St., Baltimore, MD 21218. E-mail: corces_v@jhuvms.hcf.jhu.edu

[1] *Present address:* Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892.

all alleles tested are affected, but the effect on the phenotype varies depending on the gene under consideration. For example, whereas mutations in *mod(mdg4)* enhance the phenotype of *gypsy*-induced *lozenge* and *Hairy-wing*, it suppresses the phenotype of *cut* and *forked* mutations. In addition, mutations in *mod(mdg4)* cause an enhancement of the *scute* phenotype in the head but suppression in the scutellum. The alteration of the *yellow* phenotype of the y^2 allele caused by mutations in *mod(mdg4)* is reminiscent of a variegated phenotype caused by heterochromatic rearrangements (GERASIMOVA *et al.* 1995), and cloning and sequencing of the gene revealed it to be the *Enhancer of variegation 3-93D* [*E(var)3-93D*] (DORN *et al.* 1993). Interactions with a variegated allele of *white, white-mottled-4* (w^{m4}), indicate that *mod(mdg4)* is a classic enhancer of variegation and is dependent on su(Hw) protein for this function (GERASIMOVA *et al.* 1995). This suggests that in the absence of *mod(mdg4)*, su(Hw) causes partial repression of enhancer function in a bidirectional fashion, perhaps by inducing changes in chromatin structure. The effect of *mod(mdg4)* mutations on *yellow* expression in the y^2 mutation could be explained by the following model. The su(Hw) protein binds to *gypsy* DNA situated between the body and wing enhancers and the promoter of the *yellow* gene. The *mod(mdg4)* protein then binds to the su(Hw) protein present on the *gypsy* DNA. In the case where both *su(Hw)* and *mod(mdg4)* are wild type, this complex is functional, the *mod(mdg4)* protein controls the unidirectionality of the complex, and only those enhancers situated distally to the promoter with respect to the *gypsy* insertion site are repressed. These flies appear y^2. In flies carrying a *su(Hw)* null mutation, the su(Hw) protein is not present, the complex fails to form and it cannot interfere with enhancer function. These flies appear y^2. When the *mod(mdg4)* protein is truncated or present at low levels, exposed domains of su(Hw), either directly or through their interaction with other proteins, cause bidirectional repression of enhancers, resulting in a variegated *yellow* phenotype. This silencing effect is likely to occur through changes in chromatin conformation, as variegation is a phenomenon known to involve chromatin structure (HENIKOFF 1992; KARPEN 1994).

Here we describe a thorough analysis of the functional domains of su(Hw) required for its ability to repress enhancers, both unidirectionally and bidirectionally, as well as the identification of domains that may interact directly with the *mod(mdg4)* protein *in vivo*.

MATERIALS AND METHODS

Maintenance of Drosophila strains: Fly stocks were maintained at 22.5° and 65% relative humidity. The *su(Hw)* mutant transformants were maintained as homozygous stocks in a y^2; $su(Hw)^V/TM6B$, *Tb Hu e* background. Phenotypes were scored visually under the dissecting microscope. Ten to 20 individuals were scored and the coloration of cuticular structures was always consistent among individuals.

Isolation and enzymology of nucleic acids: Isolation of plasmid DNA and enzymology of nucleic acids were carried out by standard procedures (SAMBROOK *et al.* 1989). *P* element-mediated transformation was carried out as described by RUBIN and SPRADLING 1982, using the *white* gene as a selectable marker in the CaSpeR vector (PIRROTTA *et al.* 1985).

Site-specific *in vitro* mutagenesis: All *su(Hw)* mutant constructs were synthesized by the Unique Site Elimination method as described by DENG and NICKOLOFF (1991), using the Transformer site-directed mutagenesis kit from Clontech. For these mutants, the CaSpeR 5.2 plasmid (HARRISON *et al.* 1993) was digested with *Pst*I and the resulting 2.8-kb fragment cloned into the pBluescript II SK+ vector such that the *Pst*I site present within the *su(Hw)* coding region was proximal to the *Xba*I site present in the polylinker. Mutagenesis was then performed on this subclone. The sequence of the switch oligo employed is CGGCCGCTCGCGAACTAGTGG. It was used to change the *Xba*I site in the polylinker of pBluescript II SK+ to an *Nru*I site. *Xba*I was therefore used as the selection enzyme in the process, and putative mutant plasmids were left uncut. Sequencing was performed as described above to verify that mutagenesis had occurred as designed. The following mutagenesis oligos were used in the construction of the various deletions of su(Hw): GTTAAACACATCAGCGACACTAGAAAAC-GAAAC for $su(Hw)^{\Delta A}$, GATATGCTAGTGGATCGAGAGTACTG-CAAC for $su(Hw)^{\Delta B}$, CGAGAGTACTGCAAAGAAGGTGGATTG-GAC for $su(Hw)^{\Delta C}$, and CGAATATGTACTGTGAGATGAGGATC for $su(Hw)^{\Delta CTAD}$. Once the content of these mutations was verified, the mutant 2.8-kb *Pst*I fragment was placed back into a pCaSpeR 5.2 plasmid missing the 2.8-kb *Pst*I fragment (designated pCaSpeR 5.2 Δ2.8PP), checked to verify that the fragment was in the proper orientation, and injected into flies as described above. The $su(Hw)^{NoAD2}$ allele was constructed by placing the 2.8-kb *Pst*I fragment containing the point mutation for the $su(Hw)^{NoAD2}$ allele into a plasmid lacking the 2.8-kb *Pst*I fragment and also containing the $su(Hw)^{\Delta 100}$ lesion in the 5′ end called pCaSpeR Δ100 Δ2.8 PP.

Western analysis: Protein was prepared by homogenization of six animals, three of each sex, in hot 60 mM Tris hydrochloride pH 6.8, 2% SDS, 10% glycerol, 0.7 M 2-mercaptoethanol followed by boiling for 10 min. Debris was precipitated by centrifugation and the supernatant was subjected to polyacrylamide gel electrophoresis as described by LAEMMLI (1970). Protein was electroblotted to a nitrocellulose membrane (Nitro ME, Micron Separations, Inc.) in 25 mM Tris pH 8.3, 192 mM glycine, 20% methanol (TOWBIN *et al.* 1979). Membranes were blocked and incubated with affinity-purified anti-su(Hw) antibodies (SPANA *et al.* 1988) and the bands visualized by utilizing goat anti-rabbit HRP-conjugated secondary antibodies (Cappel) and the ECL kit from Amersham. Kodak X-ray film was used to develop the images. The amount of su(Hw) protein present in each lane was quantitated using the NIH Image 1.6 software package. Results from five independent Western blots were analyzed to obtain the data presented in RESULTS.

Immunofluorescence analysis: Salivary glands from third instar larvae were dissected in Cohen's buffer (COHEN and GOTCHEL 1971), incubated for 10 min, then fixed in 100 mM NaCl, 2 mM KCl, 2% NP-40, 2% formaldehyde, and 10 mM sodium phosphate pH 7.0 for 15 min. The glands were then transferred to 45% acetic acid for 10–45 min and squashed (HELLER *et al.* 1986). Slides were incubated with affinity-purified polyclonal antibodies to su(Hw) (SPANA *et al.* 1988) at 4° overnight, washed in blocking buffer (COHEN and GOTCHEL 1971), and incubated with FITC-conjugated

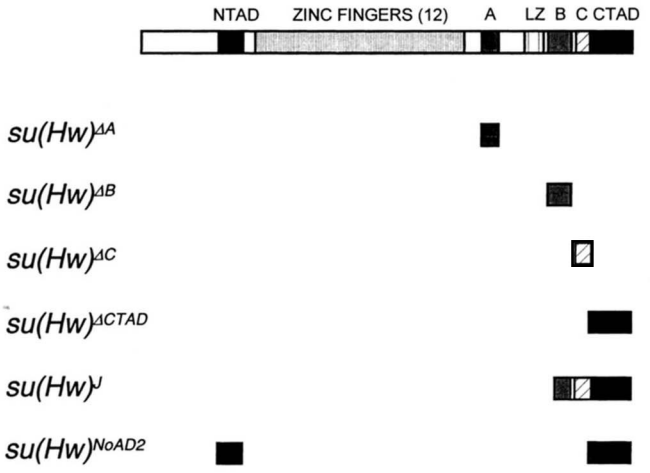

FIGURE 1.—Schematic map of *su(Hw)* mutations. The structure of the su(Hw) protein and the lesions in each of the *su(Hw)* mutations described in the text are presented diagramatically. The various domains are described above the diagram: NTAD, amino-terminal acidic domain; LZ, leucine zipper; CTAD, carboxy-terminal acidic domain. A, B, and C denote regions defined by homology among three different Drosophila species (HARRISON *et al.* 1993). The names of each of the alleles is shown to the left and the regions deleted in each mutant are diagrammed on the right.

goat anti-rabbit secondary antibody (Vector Laboratories). Slides were mounted in Vectashield (Vector Laboratories). The chromosomal DNA was stained with DAPI (0.5 μg/ml) in blocking buffer for 1 min and rinsed before mounting in Vectashield (Vector Laboratories).

Analysis of cuticular phenotypes: Males of various strains were aged to 3 days and their abdomens removed. Soft tissue was expelled as thoroughly as possible, and the abdomens were heated to 98° in 100 μl 10% potassium hydroxide for 2 min. The solution was removed and replaced with 100 μl fresh solution and the flies heated as before, but for 1 min. This solution was removed and replaced with 100 μl distilled and deionized water and the tissue boiled for 5 min. The abdomens were then placed on subbed slides and passed through an ethanol series. Samples were mounted in Permount and photographed under a dissecting microscope with back illumination and Kodak T-160 tungsten color slide film. Other tissues were prepared similarly, except no dissection was performed before treatment with potassium hydroxide. Additionally, they were crushed after the first potassium hydroxide treatment to expel soft tissue. After mounting in Permount, the tissues were scored for pigmentation as described in Table 1.

RESULTS

Deletion of each of three conserved regions in the carboxy-terminal end of su(Hw) has variable effects on the repression of enhancers: Sequence comparisons of su(Hw) proteins from various Drosophila species support the possibility that the conserved regions in the carboxy-terminal end of the protein may be important for the ability of su(Hw) to repress enhancer activity (HARRISON *et al.* 1993). These conserved regions (denoted A, B, and C; see Figure 1) show no recognizable homology to any functional domain found in compre-

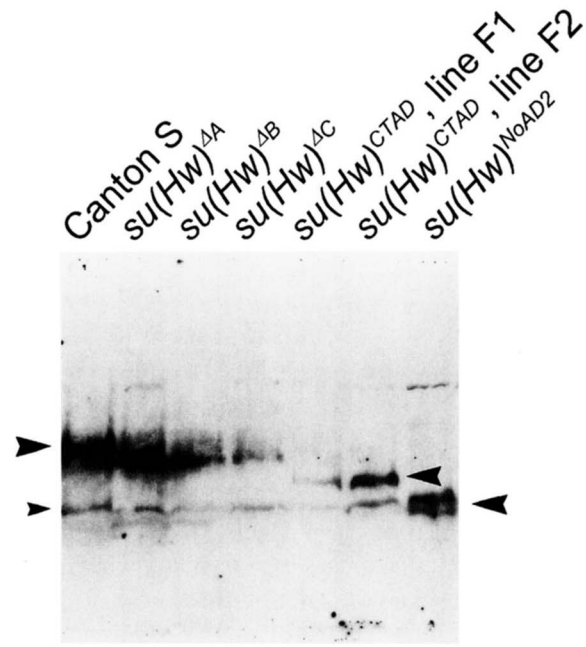

FIGURE 2.—Protein analysis of *su(Hw)* mutants. Protein was isolated from six adult flies (note that all transgenic lines were homozygous for the *P*-element construct), three of each sex, electrophoresed in a 7.5% polyacrylamide-SDS gel and electroblotted to a nitrocellulose membrane. Affinity-purified polyclonal antibodies directed against a su(Hw)-trpE fusion protein (SPANA *et al.* 1988) were used to probe the filter. The name of each strain analyzed is indicated above the figure. A large arrowhead indicates the position of the su(Hw) protein, whereas a small arrowhead denotes the location of a background band probably due to the secondary antibody.

hensive nucleic acid and protein databases. A previously studied allele of *su(Hw)*, *su(Hw)^{J}*, lacks regions B and C in addition to the carboxy-terminal acidic domain (Figure 1), making it difficult to assess the relative contributions of the carboxy-terminal acidic domain and the conserved regions of su(Hw) to the repression of distal enhancers. We have performed a deletion analysis of the region to determine conclusively whether any of these sequences contribute to the repressive effect of su(Hw) and to ascertain what effect, if any, the carboxy-terminal acidic domain has on the inactivation of enhancer function.

We first constructed a mutant su(Hw) protein lacking the 30 amino acids that comprise region A, situated between the last zinc finger and the leucine zipper (Figure 1). This was accomplished by employing an oligonucleotide-directed deletion of bases 2897–2986 to generate the *su(Hw)$^{\Delta A}$* transgene (see PARKHURST *et al.* 1988, for the numbering of nucleotides). Two independent lines were obtained and analyzed. Western blot analysis and quantitation of the amount of su(Hw) protein present in the gel reveals that this mutant produces wild-type levels of protein (Figure 2). To test whether deletion of region A interferes with the ability of su(Hw) to interact with DNA, we analyzed its ability to bind to

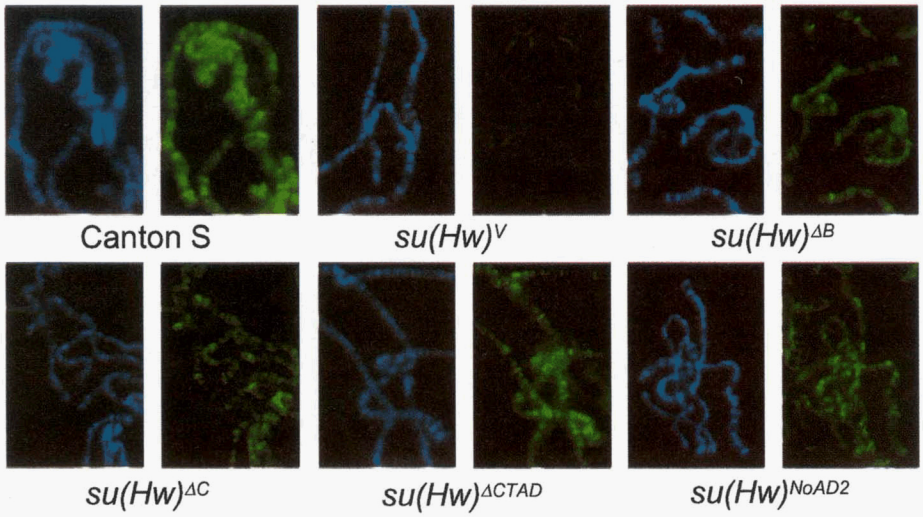

FIGURE 3.—Localization of mutant su(Hw) proteins on polytene chromosomes. Salivary glands were dissected from third instar larvae, carrying the *su(Hw)* mutations indicated in each panel, and fixed. Samples were then incubated with affinity-purified anti-su(Hw) polyclonal antibodies (SPANA *et al.* 1988), followed by FITC-conjugated secondary antibody and detected by epifluorescence. The DNA from each strain is shown to the left stained with DAPI and viewed under UV light.

DNA *in vivo* by immunolocalization of the protein to polytene chromosomes. This method might only give qualitative information on the affinity of altered forms of su(Hw) for their target sequence, but its validity is supported by parallel results recently obtained using *in vitro* approaches (KIM *et al.* 1996). The su(Hw) protein lacking region A shows normal binding to chromosomes (data not shown, but identical to that of Canton S, Figure 3). Both transformed lines failed to suppress the y^2 mutant phenotype when crossed into a $su(Hw)^V$ null background, indicating that a su(Hw) protein lacking region A can still repress distal enhancers (Figure 4 and Table 1).

We then focused our attention on the two conserved regions, B and C, immediately adjacent to the carboxy-terminal acidic domain removed in the $su(Hw)^J$ mutation. First, we eliminated the 45 amino acids comprising region B, by deleting bases 3276–3410. This construct was called $su(Hw)^{\Delta B}$ and was injected into preblastoderm embryos and analyzed as above. Three independent lines all demonstrate a moderate ability to suppress the y^2 phenotype in a $su(Hw)^V$ null background, suggesting that the resulting mutant protein can only partially repress enhancer function (Figure 4 and Table 1). Western analyses indicate that the transformed lines accumulate 95% of the su(Hw) levels present in Canton S flies. Immunolocalization experiments show a normal distribution and intensity of the $su(Hw)^{\Delta B}$ protein on polytene chromosomes. These results suggest that the mutant phenotype is not due to inadequate amounts of protein or the inability of the mutant protein to bind to DNA, thus illustrating that this region is necessary for the repression of enhancer elements, perhaps by acting in concert with the leucine zipper domain (Figures 2 and 3).

Finally, we precisely deleted the 35 amino acids that make up region *C*, which begins amino-terminal to the carboxy-terminal acidic domain and extends five amino acids into it. This was accomplished by deleting bases 3426 to 3530 in the same way as for the above two constructs. The mutant transgene was named $su(Hw)^{\Delta C}$ and was subjected to the same analysis as above. Two independent lines show significant suppression of the y^2 phenotype (see Figure 4). Even though these flies accumulate only ~35% of the protein seen in wild-type flies (Figure 2), the phenotype is not due to reduced amount of protein: the $su(Hw)^{\Delta CTAD}$ transgenic line F_1 produces only 20% of protein (Figure 2), yet still shows complete repression of the body and wing enhancers (Figure 4 and see below), whether the transgene is present in one or two copies (data not shown but phenotypically identical to the $su(Hw)^{\Delta CTAD}$ cuticle shown in Figure 4). Binding of the mutant $su(Hw)^{\Delta C}$ protein to DNA, as judged by its presence on polytene chromosomes, was unaffected (Figure 3). Therefore, deletion of region B or C affects the ability of su(Hw) to repress enhancer elements and the suppression seen in $su(Hw)^J$ mutants could be due to the loss of these regions alone, and not due to the loss of the carboxy-terminal acidic domain.

The acidic domains of the su(Hw) protein are dispensable for its ability to repress enhancer function unidirectionally: To definitively test whether the carboxy-terminal acidic domain plays a role in the repression of enhancer function, we first designed a su(Hw) mutant protein that contains a deletion of this region as defined in Figure 1. This deletion was generated *in vitro* by replacing the codon for glycine at position 861 with a stop codon by a *G* to *T* transversion at base 3512. This mutant construct was called $su(Hw)^{\Delta CTAD}$ and was introduced into flies as described above. Three separate transgenic lines were isolated and tested for their ability to suppress the y^2 phenotype. In each case, this mutant protein completely represses distal enhancer function as shown in Figure 4, indicating that the moderate suppression of the abdominal cuticular phenotype ob-

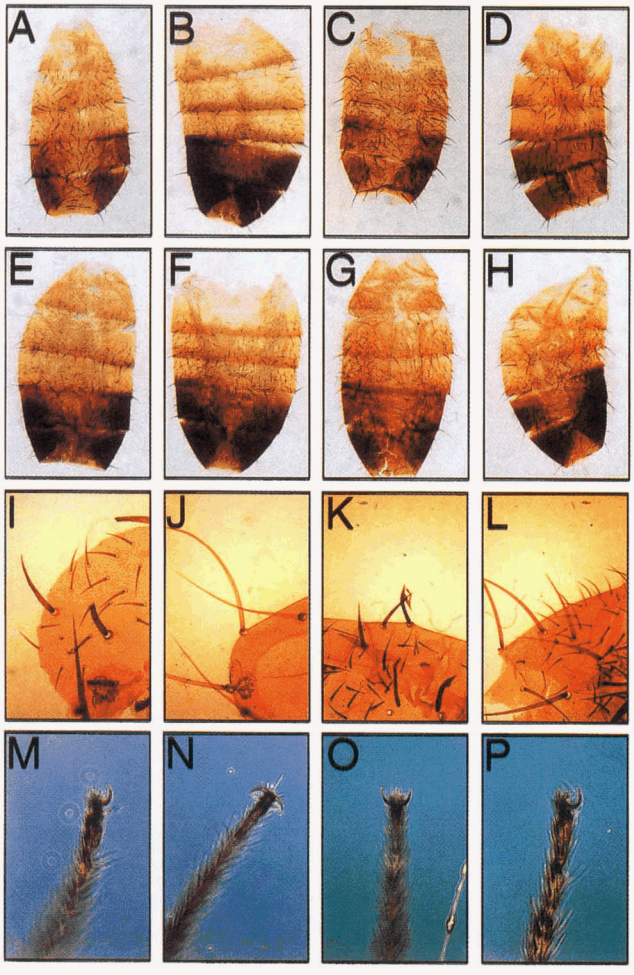

FIGURE 4.—Phenotypic effect of alterations in the structure of the su(Hw) protein. The figure shows light micrographs of the abdomens, scutellar bristles and tarsal claws of 3-day-old males from wild type (Canton S), y^2, and combinations of y^2 with different *su(Hw)* and *mod(mdg4)* mutations. (A) y^2. (B) y^2; $su(Hw)^V$. (C) y^2; $su(Hw)^{\Delta A}$. (D) y^2; $su(Hw)^{\Delta CTAD}$. (E) y^2; $su(Hw)^{\Delta B}$. (F) y^2; $su(Hw)^{\Delta C}$. (G) y^2; $mod(mdg4)^{u1}$. (H) y^2; $su(Hw)^{\Delta CTAD}$, $mod(mdg4)^{u1}$. (I) y^2. (J) y^2; $mod(mdg4)^{u1}$. (K) y^2; $su(Hw)^{\Delta CTAD}$. (L) y^2; $su(Hw)^{\Delta CTAD}$, $mod(mdg4)^{u1}$. (M) y^2. (N) y^2; $mod(mdg4)^{u1}$. (O) y^2; $su(Hw)^{\Delta CTAD}$. (P) y^2; $su(Hw)^{\Delta CTAD}$, $mod(mdg4)^{u1}$.

served in a y^2; $su(Hw)^J$ mutant must be due to deletion of the *B*- and *C*-conserved regions between the leucine zipper and the carboxy-terminal acidic domain. Surprisingly, Western analysis (Figure 2) showed that one transgenic line, line F_2 accumulates ~45% of the amount of protein as wild-type flies, whereas another line, line F_1 accumulates as little as 20% of the protein as wild-type flies. Yet, these mutants still repress enhancer function as well as the wild-type su(Hw) protein (Figure 4 and Table 1), even when the transgene is present in only one copy (data not shown; phenotypically identical to the $su(Hw)^{\Delta CTAD}$ cuticle shown in Figure 4). These data indicate that even a very low level of protein is sufficient to repress the wing and body enhancers of *yellow*. Immunolocalization to polytene chromosomes shows that the $su(Hw)^{\Delta CTAD}$ protein accumulates on chromosomes similarly to the wild-type protein (Figure 3), suggesting that the DNA-binding activity of this mutant protein is not diminished.

Deletion of the amino-terminal acidic domain of su(Hw), as in the $su(Hw)^{\Delta 100}$ mutant, results in a protein that is fully capable of repressing the activity of distal enhancers (HARRISON *et al.* 1993). To assess the consequences of deleting both amino- and carboxy-terminal acidic domains, we designed a mutant construct carrying a precise deletion of both acidic domains but retaining all other sequences. To do this, we introduced DNA carrying the point mutation for $su(Hw)^{\Delta CTAD}$ into the $su(Hw)^{\Delta 100}$ transformation vector (HARRISON *et al.* 1993), which encodes a mutant protein lacking the amino-terminal acidic domain (Figure 1). This construct was named $su(Hw)^{NoAD2}$ and was used to transform flies as above. The $su(Hw)^{NoAD2}$ construct differs from the previously reported $su(Hw)^{NoAD}$ (HARRISON *et al.* 1993) in that the latter is also missing domains *B* and *C*. Three independent transgenic lines carrying the $su(Hw)^{NoAD2}$ construct failed to suppress y^2 in a *su(Hw)* null background, indicating that neither acidic domain plays a direct role in the unidirectional repression of enhancers (Table 1). Western analysis of these lines shows accumulation of the altered protein at levels 70% of those present in wild-type flies, and immunolocalization experiments suggest normal accumulation and DNA-binding of the $su(Hw)^{NoAD2}$ mutant protein on polytene chromosomes (Figures 2 and 3). The difference in the phenotypes observed in strains carrying the $su(Hw)^{NoAD}$ *vs.* the $su(Hw)^{NoAD2}$ constructs can be attributed to the absence of the *B* and *C* domains in the former (Table 1).

Conserved regions in the carboxy terminal end of the su(Hw) protein are required for the bidirectional repression of enhancer activity: Mutations in *mod(mdg4)* cause an enhancement of the y^2 phenotype due to the inactivation of all enhancers of the *yellow* gene, presumably due to the presence of the su(Hw) protein by itself or in combination with other proteins in the absence of *mod(mdg4)* (GERASIMOVA *et al.* 1995). Previous analysis have shown that the leucine zipper domain of su(Hw) is essential to repress enhancer activity either unidirectionally or bidirectionally (HARRISON *et al.* 1993; GERASIMOVA *et al.* 1995). Because regions A, B, and C are highly conserved across Drosophila species (HARRISON *et al.* 1993), we decided to test whether these regions were important in the bidirectional silencing of enhancer activity in flies lacking *mod(mdg4)* protein. Deletion of region A alone had no effect on the ability of the mutant protein to repress distal enhancers (Figure 4 and Table 1). When the $su(Hw)^{\Delta A}$ mutant was crossed into a *mod(mdg4)* background, the flies display a slight enhancement of the y^2 phenotype in the bristles and the tarsal claws, while the pigmentation of the wing

TABLE 1

Interactions between *mod(mdg4)* mutations and various alleles of *su(Hw)*

Strain	*yellow* phenotype			
	Wing	Abdomen	Bristles	Tarsal claws
Canton S	+++	+++	+++	+++
y^2	−	−	+++	+++
y^2; *mod(mdg4)*^{ul}	−	−	−	−
y^2; *su(Hw)*^{V}	+++	+++	+++	+++
y^2; *su(Hw)*^{V} *mod(mdg4)*^{ul}	+++	+++	+++	+++
y^2; *su(Hw)*$^{\Delta CTAD}$	−	−	+++	+++
y^2; *su(Hw)*$^{\Delta CTAD}$ *mod(mdg4)*^{ul}	+	++	++	++
y^2; *su(Hw)*^{f}	−	++	+++	+++
y^2; *su(Hw)*^{f} *mod(mdg4)*^{ul}	+	+	+++	+++
y^2; *su(Hw)*^{NoAD}	+++	+++	+++	+++
y^2; *su(Hw)*^{NoAD} *mod(mdg4)*^{ul}	+++	+++	+++	+++
y^2; *su(Hw)*^{NoAD2}	−	−	+++	+++
y^2; *su(Hw)*^{NoAD2} *mod(mdg4)*^{ul}	−	−	+++	+++
y^2; *su(Hw)*$^{\Delta A}$	−	−	+++	+++
y^2; *su(Hw)*$^{\Delta A}$ *mod(mdg4)*^{ul}	+	++	++	++
y^2; *su(Hw)*$^{\Delta B}$	+	++	+++	+++
y^2; *su(Hw)*$^{\Delta B}$ *mod(mdg4)*^{ul}	+	++	+++	+++
y^2; *su(Hw)*$^{\Delta C}$	+	++	+++	+++
y^2; *su(Hw)*$^{\Delta C}$ *mod(mdg4)*^{ul}	+	++	+++	+++

Phenotypes were quantitated by visual inspection of cuticle preparations under a dissecting microscope. Levels of coloration were assigned values between null (−) and wild type (+++).

and abdomen darkens somewhat (Table 1). Flies carrying the *su(Hw)*$^{\Delta B}$ or the *su(Hw)*$^{\Delta C}$ mutations partially suppress the y^2 phenotype in a *mod(mdg4)*$^{+}$ background (Table 1), suggesting that these regions are necessary for the repression of distal enhancers. When these same mutant constructs are crossed into flies lacking *mod(mdg4)* protein, the phenotype is the same (Table 1). The fact that these two mutants are unresponsive to the allelic state of *mod(mdg4),* and therefore cannot mediate an enhancement of the y^2 phenotype, suggests that these regions are important for the bidirectional silencing effect of su(Hw).

The acidic domains of su(Hw) play an important role in the bidirectional repression of *yellow* enhancers in *mod(mdg4)* mutants: We have shown above that when a mutant su(Hw) protein is missing either or both acidic domains, it can still completely repress the function of distal enhancers (Figure 4 and Table 1). Therefore, these domains are dispensable for the insulating effect on distal enhancers. Additionally, these results suggest that the *mod(mdg4)* protein does not directly interact with the acidic domains of su(Hw) because flies producing a mutant su(Hw) protein lacking both acidic domains, such as *su(Hw)*^{NoAD2}, still display a unidirectional repression of enhancer activity (Table 1). Since the *mod(mdg4)* protein controls this directionality, it cannot interact with the acidic domains of su(Hw).

When the *su(Hw)*$^{\Delta CTAD}$ allele, which encodes a protein lacking the carboxy-terminal acidic domain (Figure 1), is crossed into a mutant *mod(mdg4)* background, the flies display bristle, tarsal claws, abdomen and wings with a coloration intermediate between null mutant and wild type (Figure 4 and Table 1). A similar effect is observed when the *su(Hw)*$^{\Delta 100}$ allele, which produces a protein missing only the amino-terminal acidic domain, is crossed into flies bearing a mutation in *mod(mdg4)* (GERASIMOVA *et al.* 1995). These results suggest that the acidic domains are important to mediate the bidirectional silencing effect of su(Hw), and deleting each domain independently results in a su(Hw) protein that can only partially repress enhancer function in a bidirectional fashion. Furthermore, when the *su(Hw)*^{NoAD2} allele is examined in the background of a *mod(mdg4)* mutation, the results observed agree with this interpretation. In this case, flies producing a mutant su(Hw) protein missing both acidic domains and also lacking *mod(mdg4)* demonstrate a completely suppressed y^2 phenotype (Table 1), suggesting that both acidic domains together are the cause of the bidirectional silencing of enhancers observed in y^2; *mod(mdg4)* mutants. The intermediate phenotypes observed with *mod(mdg4)* mutants producing a su(Hw) protein lacking only one acidic domain may be due to a decreased ability to interfere with enhancer elements in these mutants and the full effect is seen only when both acidic domains are missing.

DISCUSSION

Insertion of the *gypsy* element into the 5′ region of the *yellow* gene causes the inactivation of transcriptional

enhancers located distally from the *gypsy* insertion site with respect to the *yellow* promoter. This results in a tissue-specific mutant phenotype. Only those tissues in which *yellow* expression is controlled by enhancers inactivated by the presence of the *gypsy* element are mutant (reviewed by CORCES and GEYER 1991). This effect is not caused by *gypsy* itself but rather by the su(Hw) protein bound to sequences located in the transcribed untranslated region of *gypsy* (GEYER and CORCES 1992). The unusual polar effect of su(Hw) can be explained by a model in which the su(Hw) and *mod(mdg4)* proteins form a chromatin insulator or boundary element that prevents the transmission of a chromatin conformation associated with active chromosomal domains of gene expression (ROSEMAN *et al.* 1993; WOLFFE 1994; CORCES 1995). This role for su(Hw)-binding sequences is supported by their ability to insulate the expression of a *white* transgene from chromosomal position effects by neighboring sequences, independent of their location in the genome (ROSEMAN *et al.* 1993). The su(Hw)-binding region is thus functionally similar to the specialized chromosome structures (scs and scs′) located at the junctions between active and inactive chromatin in the Drosophila 87A7 *hsp70* locus (KELLUM and SCHEDL 1991, 1992) and to the insulator element located in the 5′ region of the chicken β-globin gene cluster (CHUNG *et al.* 1993).

As a first step in understanding how su(Hw) negatively affects enhancer function, we performed an extensive mutagenesis of this protein. Several domains were apparent by inspection of the sequence of the *su(Hw)* gene from *D. melanogaster.* su(Hw) contains two acidic domains, one at either end of the protein, a stretch of 12 zinc fingers in the central region, and a leucine zipper near the carboxy-terminal end. By comparing the sequence of the *su(Hw)* gene from several Drosophila species (HARRISON *et al.* 1993), we were able to identify three additional regions of homology, all in the carboxy-terminal end of the protein, none of which shows any similarity to a functionally defined protein domain. Analysis of the $su(Hw)^{f}$ mutant suggested that the carboxy-terminal acidic domain of the su(Hw) protein was important in its ability to repress enhancer function (HARRISON *et al.* 1993). However, because this mutant also lacks regions B and C, which were highly conserved across Drosophila species, it was necessary to perform a more thorough analysis to determine the respective contributions of these sequences in the repression of enhancer function.

In *D. melanogaster,* deletion of the amino-terminal acidic domain, the carboxy-terminal acidic domain, or both has no consequence on the mutagenic effect of the su(Hw) protein. Deletion of either region B or C results in a phenotype indistinguishable from $su(Hw)^{f}$. A third conserved region, region A, has no role in the repression of distal enhancers as flies producing a mutant protein in which this region is deleted appear y^{2}. The results presented here suggest a nonessential role for the acidic domains in the unidirectional repression of enhancer activity, but these domains appear to be essential for the bidirectional effect seen in a *mod(mdg4)* mutant background. Therefore, the region of su(Hw) necessary for repressing the function of distal enhancers lies in the carboxy-terminal end of the protein, between amino acids 685 and 866. This region contains the leucine zipper domain, a structure known to mediate protein-protein interactions, and two regions highly conserved among Drosophila species but with no known homologies.

Analysis of the interaction between mutations in *mod(mdg4)* and *su(Hw)* alleles affecting various structural domains of the protein indicate that regions of su(Hw) typically known to mediate protein-protein interactions are essential for the effect of *mod(mdg4)* on *yellow* expression. Regions of the two proteins participating in their interaction have not yet been defined biochemically. If *mod(mdg4)* interacts directly with su(Hw), the absence of *mod(mdg4)* protein in a *mod(mdg4)* mutant background might result in the exposure of particular domains of the su(Hw) protein that normally interact with and/or are occluded by the *mod(mdg4)* protein. Conceivably, in flies lacking *mod(mdg4)* protein, these domains of su(Hw) could then be free to interact with enhancer-bound transcription factors, other proteins present in the chromatin fiber, or other components of the nuclear framework. Therefore, the bidirectional repressive effect on *yellow* transcription observed in *mod(mdg4)* mutants might be due to the display of su(Hw) domains functional in protein-protein interactions that can now interact with other nuclear components. In support of this model, mutations of *su(Hw)* that affect these domains are consequential to the phenotypic effects of *mod(mdg4)* mutations.

Genetic studies have demonstrated that a su(Hw) protein lacking a functional leucine zipper fails to repress enhancer function, either in a $mod(mdg4)^{+}$ or mutant *mod(mdg4)* background (GERASIMOVA *et al.* 1995). This suggests that this domain is fundamental to the ability of the su(Hw) protein to negatively affect enhancers both unidirectionally and bidirectionally. Similarly, when regions B or C have been deleted, the resulting mutant proteins do not respond to mutations in *mod(mdg4),* implying that they are also necessary for su(Hw) to repress enhancer function in either fashion. However, the acidic domains of su(Hw), while dispensable for the unidirectional repression of enhancer activity, appear to be directly responsible for the bidirectional repression observed in *mod(mdg4)* mutants. When flies carrying the $su(Hw)^{NoAD2}$ mutation, which encodes a protein lacking both acidic domains, are wild type for *mod(mdg4)* the distal enhancers of *yellow* are repressed indistinguishably from flies producing wild-type su(Hw)

protein. In the absence of *mod(mdg4)*, the su(Hw)^{NoAD2} protein fails to repress *yellow* enhancers bidirectionally, indicating that the acidic domains are responsible for the silencing of enhancer elements seen in these flies. Additionally, these observations suggest that the acidic domains of su(Hw) do not directly interact with the *mod(mdg4)* protein. Since the unidirectionality of enhancer repression is preserved in the *su(Hw)*^{NoAD2} mutant and this directionality of repression is known to be controlled by the *mod(mdg4)* protein (GERASIMOVA *et al.* 1995), the interaction between the two proteins must be intact in the mutant. The intermediate phenotypes observed in flies that are mutant for *mod(mdg4)* and express a su(Hw) protein lacking either acidic domain may be due to a partial ability of these proteins to negatively affect enhancer function. The full effect (*i.e.*, complete repression of all enhancer elements of *yellow*) occurs only when both acidic domains are present.

In light of the genetic data, we propose that su(Hw) forms discrete domains of gene activity by segregating promoters from enhancer elements through a change in chromatin organization. The extensive localization of su(Hw) protein throughout the genome (SPANA *et al.* 1988) might have a role in establishing different domains of gene activity and this function was fortuitously discovered by studies of the mutagenic effect of the *gypsy* element. If this is so, this function must be a redundant one as *su(Hw)* null mutants are viable (HARRISON *et al.* 1992). The importance of regulating chromatin structure cannot be overlooked, however, so it is quite possible that multiple methods for accomplishing this task may exist in the fly. While it remains to be seen whether su(Hw) has a normal role in establishing domains of differential gene expression, the interaction between the su(Hw) and *mod(mdg4)* proteins and the *gypsy* element provides a well characterized model with which to study the effects of changes in chromatin structure on gene activity.

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