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The name of the second author, Inna Biryukova, was published incorrectly. The name is correct as written above.

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An endogenous Su(Hw) insulator separates the *yellow* gene from the *Achaete-scute* gene complex in *Drosophila*

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SUMMARY

The best characterized chromatin insulator in *Drosophila* is the Suppressor of Hairy wing binding region contained within the *gypsy* retrotransposon. Although cellular functions have been suggested, no role has been found yet for the multitude of endogenous Suppressor of Hairy wing binding sites. Here we show that two Suppressor of Hairy wing binding sites in the intergenic region between the *yellow* gene and the *Achaete-scute* gene complex form a functional insulator. Genetic analysis shows that at least two proteins, Suppressor of Hairy wing and Modifier of MDG4, required for the activity of this insulator, are involved in the transcriptional regulation of Achaete-scute.

Key words: *Drosophila melanogaste*, Achaete-scute Complex, Insulator, Su(Hw), Mod(mdg4), Enhancer blocking

INTRODUCTION

Enhancer-mediated activation is a fundamental mechanism of gene regulation in eukaryotes. Enhancers can act over large distances to activate transcription, independent of their orientation and position relative to the promoter and without affecting adjacent genes. Recently, sequences referred to as insulators have been found in different organisms that prevent activation or repression from extending across them to a promoter (Geyer, 1997; Dorsett, 1999; Udvardy, 1999; Gerasimova and Corces, 2001; Bell et al., 2001; West et al., 2002). The *gypsy* insulator of *Drosophila* was first identified within the *gypsy* retrotransposon (Spana et al., 1988; Mazo et al., 1989). It consists of 340 bp containing 12 binding sites for the Su(Hw) protein. Insertion of this sequence between an enhancer and a promoter inhibits the activity of the enhancer (Holdridge and Dorsett, 1991; Geyer and Corces, 1992; Cai and Levine, 1995; Scott and Geyer, 1995). The *gypsy* insulator can also function as a barrier, blocking the silencing activity of Polycomb Response Element (PRE) (Sigrist and Pirrotta, 1997; Mallin et al., 1998) and partially protecting a transgene from silencing when inserted into heterochromatin (Roseman et al., 1993; Roseman et al., 1995).

Genetic and molecular approaches have led to the identification and characterization of two proteins that are required for activity of the *gypsy* insulator. One is Suppressor of Hairy wing [Su(Hw)], a twelve zinc finger protein encoded by the *suppressor of Hairy wing* [su(Hw)] gene, which binds to the repeated sequence motifs in the *gypsy* insulator (Dorsett, 1990; Spana and Corces, 1990). Of the protein domains comprising Su(Hw), 9 out of 12 zinc fingers and a domain of approximately 150 amino acids including the C-terminal leucine zipper, but not the N- and C-terminal acidic regions, are required for enhancer blocking (Harrison et al., 1993; Kim et al., 1996).

Mutations in another gene, *modifier of mdg4*, alter the phenotypes of *gypsy*-induced mutations, indicating that the product of this gene is also involved in the function of the *gypsy* insulator (Georgiev and Gerasimova, 1989; Gerasimova et al., 1995; Georgiev and Kozycina, 1996; Cai and Levine, 1997; Gdula and Corces, 1997; Cai and Shen, 2001). 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 (Georgiev and Kozycina, 1996; Gdula and Corces, 1997; Cai and Chen, 2001). Biochemical studies using purified Su(Hw) and Mod(mdg4) proteins indicate that one protein isoform of the *mod(mdg4*) gene, Mod(mdg4)-67.2, interacts with the enhancer-blocking domain of the Su(Hw) protein (Gause et al., 2001; Ghosh et al., 2001).

The Mod(mdg4)-67.2 protein is present in approximately 500 sites on polytene chromosomes (Gerasimova and Corces, 1998). About 200 of these sites also contain the Su(Hw) protein (Gerasimova and Corces, 1998; Gerasimova et al., 2000). These sites of co-localization do not contain copies of the *gypsy* retrotransposon and are presumed to be endogenous insulators. In spite of these promising observations, no endogenous Su(Hw) insulators have been identified. The viable *mod(mdg4)^{u1}* mutation effects only the isoform of *mod(mdg4)*, Mod(mdg4)-67.2, that directly interacts with the Su(Hw) protein (Gerasimova et al., 1995; Buchner et al., 2000). In contrast to lethal loss of-function alleles of the *mod(mdg4)* gene, *mod(mdg4)^{u1}* flies are viable and have no visible phenotypic defects (Georgiev and Gerasimova, 1989) suggesting that the function of the Mod(mdg4)-67.2 protein can be compensated by other proteins.

Here we describe the identification of the first endogenous functional Su(Hw) insulator, located between the *yellow* gene and *Achaete-scute* gene complex (ASC). The *yellow* gene determines the proper pigmentation of cuticle structures, and its expression in different tissues is controlled by enhancers located in the 5′ region and in the first intron of the gene (Geyer et al., 1986; Geyer and Corces, 1987; Martin et al., 1989). The *achaete* (*ac*), *scute* (*sc*) and *l'sc* genes, members of ASC, are located in the vicinity of the *yellow* gene and differ from *yellow* in their spatial and temporal patterns of expression (Campuzano et al., 1985). The proteins encoded by the *ac* and *sc* genes are essential for the formation of bristle sensory organs (macrochaetae) (Modolell and Campuzano, 1998). A very complex pattern of *ac* and *sc* expression is mediated by the action of site-specific, enhancer-like elements distributed over about 90 kb of the AS-C (Ruiz-Gomez and Modolell, 1987; Gomez-Skarmeta et al., 1995; Modolell and Campuzano, 1998). The new insulator we identified contains two Su(Hw) binding sites that are required for insulator function, blocking the *yellow* and *white* enhancers. Mutations in the *su(Hw)* and *mod(mdg4)* genes strongly affect expression of the AS-C genes in rearrangements that partially disrupt the proper organization of the AS-C regulatory region. Thus, Su(Hw) and Mod(mdg4) proteins participate in proper regulation of the AS-C.

MATERIALS AND METHODS

Drosophila strains, transformation and genetic crosses

All flies were maintained at 25°C 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 is described by Harrison et al. (Harrison et al., 1993). *Df(3R)GC14* is a deletion covering the region where the *mod(mdg4)* gene is located. All other mutant alleles and chromosomes used in this work and all balancer chromosomes are described by Lindsley and Zimm (Lindsley and Zimm, 1992).

The transposon constructs, together with a P element with defective inverted repeats used as a transposase source, P25.7wc (Karess and Rubin, 1984), were injected into *y ac w^{1118}* preblastoderm embryos as described previously (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, *In(3LR)TM3,Sb* for chromosome three. The transformed lines were examined by Southern blot hybridization, to check for transposon integrity and copy number.

The *su(Hw)^{v}/su(Hw)^{f}*, *su(Hw)^{v}/su(Hw)^{2}*, *mod(mdg4)^{u1}/mod(mdg4)^{u1}* and *mod(mdg4)^{u1}/Df(3R)GC14* mutations were combined with *sc* mutations or transposons as previously described (Georgiev and Kozycina, 1996). The lines with a tested DNA fragment, or eye enhancer or Su(Hw) excisions were obtained by crossing flies bearing the transposons with Flp or Cre recombinase-expressing lines. All excisions were confirmed by PCR analysis.

In order to determine the *yellow* and *white* phenotypes, the extent of pigmentation in the abdominal cuticle, as well as eye pigmentation of adult flies was estimated visually in 3- to 5-day-old males developing at 25°C. Wild-type expression in abdominal cuticle and wings was assigned an arbitrary score of 5, while the absence of *y* expression was ranked 1. Flies with the previously characterized *y* allele were used as a reference in order to determine *y* pigmentation levels. Wild-type *w* expression results in bright red eye color (R), while the absence of *w* expression results in white eyes (W). Intermediate levels of pigmentation are defined by eye color ranging through pale yellow (p-Y), yellow (Y), dark yellow (d-Y), orange (Or), dark orange (d-Or), brown (Br), brown-red (Br-R), reflecting, respectively, low, intermediate, and high levels of the *white* expression. The scores were determined independently by two people and based upon at least 30 flies from two independent crosses.

Transgenic constructs and in vitro mutagenesis

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 *Bam*HI + *Xho*I-digested pGEM7 (yr plasmid). The 5 kb *Bam*HI-*Bgl*II fragment containing the coding region (yc) was subcloned into CaSpeR3 (C3-yc).

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 between two loxP sites (lox(su)) and in CaSpeR3 (C3-su). The lox(su) fragment was blunt-ligated to the CaSpeR2 vector restricted with *Bgl*II (C2-lox(su)).

The *yellow* regulatory region includes the body enhancer, located between –1266 bp and –1963 bp, and wing enhancer, located between –1863 bp and –2873 bp relative the transcription start site of the *yellow* gene (Geyer and Corces, 1987). The *white* regulatory sequences from position –1084 to –1465 bp relative to the transcription start site (Ee) were cloned between two frt sites (frt(Ee)). These sequences contain testes and eye enhancers (Qian et al., 1992). After that the frt(Ee) fragment was inserted at position –1868 from the *yellow* transcription start site (yr-frt(Ee)).

The 125 bp sequence containing the Su(Hw) binding region was PCR amplified with pr-1 (5′ tcctaatttccttac 3′) and pr-2 (5′ attcttttaccatgc 3′) primers from the λ*sc*133 phage (donated by J. Modolell). After sequencing to confirm its identity, the product, one copy (125 bp) or three copies (3×125 bp) of the 125 bp fragment were inserted between two lox sites [lox(125 bp) and lox(3×125 bp)]. The 2 kb DNA fragment was cloned from the λ*sc*133 phage DNA restricted with *Pst*I between two lox sites (lox(2 kb)). The 454 bp fragment was PCR-amplified with pr-5 (5′ ggagtactactaccaggc 3′) and pr-6 (5′ caagaacatttccgatatg 3′) primers from the λ*sc*133 phage and inserted between lox sites (lox(454 bp)). To mutate both Su(Hw) binding sites in the 454 bp fragment (454*) oligonucleotides carrying the desired mutated sequences, pr-7 (5′ attggccagtatatattatgtgtttaatac 3′) and pr-8 (5′ agaagtccctcgcaaaaaagtattaaatac 3′) were used to amplify PCR products. Two PCR-amplified DNA fragments with pr-5 and pr-8 primers or pr-6 and pr-7 primers were blunt ligated. The resulting 454* bp DNA fragment was sequenced to verify that the intended mutant sequences had been introduced and other PCR-induced mutations did not exist.

Ey(e)(2 kb)YSW and Ey(e)(3x125 bp)YSW

The lox(2 kb) or lox(3×125 bp) fragment was inserted in the yr-frt(Ee) restricted with *Eco*47III at −893 from the *yellow* transcription start site [yr-frt(Ee)-lox(2 kb) and yr-frt(Ee)-lox(3×125 bp)]. The yr-frt(Ee)-lox(2 kb) or yr-frt(Ee)-lox(3×125 bp) fragment was ligated into C3-su restricted with *Xba*I and *Bam*HI.

Ey(e)125 bpY(S)W and Ey(e)454 bpY(S)W

The 125 bp or 454 bp fragment was inserted in the yr-frt(Ee) restricted with *Eco*47III (yr-frt(Ee)-125 bp and yr-frt(Ee)-454 bp). The yr-frt(Ee)-125 bp and yr-frt(Ee)-454 bp fragments were ligated into C2-lox(su) restricted with *Xba*I and *Bam*HI.

Ey454* bpYW

The 454* bp fragment was inserted in the yr restricted with *Eco*47III (yr-454* bp). The yr-454* bp fragment was ligated into C3-yc restricted with *Xba*I and *Bam*HI.

To alter consensus sequences for the number 1 (#1) Su(Hw) binding site, oligonucleotides carrying the desired mutated sequences (available upon request) were used to amplify PCR products. Both mutant Su(Hw)#1 binding sites were sequenced to verify that the intended mutant sequences had been introduced and other PCR-induced mutations did not exist.

Electrophoretic mobility shift assays

For the purpose of synthesizing Su(Hw) in vitro, the Su(Hw) ORF encoding a 945 amino acid polypeptide was subcloned from the Su(Hw) cDNA (kindly provided by D. Dorsett). Su(Hw) protein was synthesized in vitro in the TNT coupled transcription/translation reticulocyte lysate (Promega) from a T7 promoter-Su(Hw) cDNA template cloned in the pET 30a plasmid (Novagen). In the binding assay, 25 fmole of a radioactively labeled DNA fragment was mixed with 10 μl of the in vitro translation reaction in 25 μl of 15 mM Hepes (pH 7.6), 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 5mM DTT, 10% glycerol and 5 μg of poly[d(I-C)]. After incubation at 4ºC for 10 minutes, the reactions were loaded on 1.5% agarose gel and the complexes fractionated in 1× TBE buffer (89 mM Tris-borate, 89 mM borate and 3 mM EDTA) at 5 V/cm.

PCR was done by standard techniques. The primers used in DNA amplification were derived from the *yellow* and AS-C sequences:

Pr-1 5′ TCCTAATTTCCTTAC 3′
Pr-2 5′ ATTCTTTTACCATGC 3′
Pr-3 5′ GAGGGACTTCTATTG 3′
Pr-4 5′ CACATAATATATACTGGC 3′
Su(Hw)#1* 5′ CTTGTATTGCATACTTTTTTGCG 3′
Su(Hw)#1** 5′ CTTGTATTTAATACTTTTTTGCG 3′

The products of amplification were fractionated by electrophoresis in 1.5% agarose gels in TAE. The successfully amplified products were cloned into a Bluescript plasmid (Stratagene, La Jolla, CA) and sequenced using the Amersham sequence kit (Amersham, Arlington Heights, IL).

RESULTS

The *yellow-ac* intergenic region, inserted in the AS-C regulatory region affects *sc* activation

We have previously described the P-element-mediated insertion of fragments of the *yellow* gene into the regulatory region of ASC (Golovnin et al., 1999). Starting from $y^{2s}sc^{+s}$ flies, which have wild-type *sc* expression, mutants were recovered in which *yellow* sequences, including the *yellow* promoter, were inserted between two adjacent P elements (P3 and P4 in Fig. 1A) at the AS-C regulatory region, resulting in the $y^{2s}sc^{ls}$ mutants in which *sc* expression is slightly affected which results in the loss of humeral bristles. Additional derivatives, sc^{ms1} and sc^{ms2}, were isolated with much stronger sc phenotypes in which many bristles regulated by *sc* are affected (Fig. 1B). Southern blot analysis and sequencing of DNA fragments amplified by PCR showed that in these derivatives all coding sequences and 3′ flanking region of the *yellow* gene were duplicated between the *P*3 and *P*4 elements in AS-C (Fig. 1A).

The striking difference in phenotypes suggested that the *yellow* 3′ region, when inserted in the AS-C regulatory region, inhibited the expression of the *sc* function. Since this region contains two consensus binding sequences for the Su(Hw) protein (Fig. 2A), we examined the effect of $su(Hw)^{-}$ and $mod(mdg4)^{u1}$ mutations on the sc^{ms} phenotype (Fig. 1B). The suppression of the mutant sc phenotype in sc^{ms}; $su(Hw)^{v}/su(Hw)^{f}$ or sc^{ms}; $su(Hw)^{v}/su(Hw)^{2}$ flies supports the idea that the Su(Hw) protein plays a role in the repression of the *sc* gene (Fig. 1B). The homozygous $mod(mdg4)^{u1}$ mutation had a similar suppressive effect on the sc phenotype (Fig. 1B), implying that Su(Hw) and Mod(mdg4) are required for repression of the *sc* activation in two sc^{ms} derivatives.

The *yellow-ac* intergenic region contains a functional Su(Hw) insulator

To determine if the effect of Su(Hw) can be attributed to the presumed Su(Hw) binding sites between the *yellow* and *ac* genes, we cloned the 125 bp fragment that contains both Su(Hw) consensus sequences (Fig. 2A) and tested its ability to bind Su(Hw) protein in vitro. As a control we used the *gypsy* insulator that contains 12 putative binding sites for Su(Hw). These DNA fragments were tested in electrophoretic mobility shift assays (EMSAs) using in vitro-synthesized Su(Hw) protein (see Materials and methods). One shifted band (arrow in Fig. 2B) probably corresponds to a complex of the 125 bp DNA fragment with one Su(Hw) protein. The inability of the Su(Hw) protein to simultaneously bind two closely spaced sites was previously described by Kim et al. (Kim et al., 1996) who suggested that the Su(Hw) protein interferes with binding to neighboring sites. To show that both putative binding sites in the 125 bp fragment can interact with Su(Hw), we subcloned smaller fragments that contain only the first or the second Su(Hw) binding site (Fig. 2A) and found that both can be band-shifted by Su(Hw) protein (Fig. 2B) Site #1 contains a C instead of A in the core consensus but this base substitution did not significantly influence the efficiency of Su(Hw) binding (Fig. 2B).

To examine the potential enhancer blocking activity of the new Su(Hw) binding sites, we used the *yellow* gene, required for dark pigmentation of *Drosophila* larval and adult cuticle and its derivatives. Two upstream enhancers, En-b and En-w, activate *yellow* expression in the body cuticle and wing blades, respectively (Geyer and Corces, 1997). The *gypsy* insulator is able to effectively block the wing and body enhancers (Geyer et al., 1986; Geyer and Corces, 1992; Muravyova et al., 2001). To test the insulator activity of the intergenic Su(Hw) sites we made constructs that exploit two properties of the *gypsy* insulator. One is the blocking activity when interposed between enhancer and promoter; the other is the ability of two *gypsy* insulators to neutralize one another (Gause et al., 1998; Cai and Shen, 2001; Muravyova et al., 2001). The constructs depicted in Fig. 3A contain a *gypsy* Su(Hw) insulator inserted between

the *yellow* and *white* gene and the eye enhancer of the *white* gene inserted between the wing and body enhancers of *yellow*. It has been shown that interposition of the Su(Hw) insulator between the eye enhancer and the *white* promoter completely blocked enhancer activity (Roseman et al., 1993; Muravyova et al., 2001).

The eye enhancer was flanked by Flp recognition target sites (FRTs) in order to excise it from transgenic flies by crossing

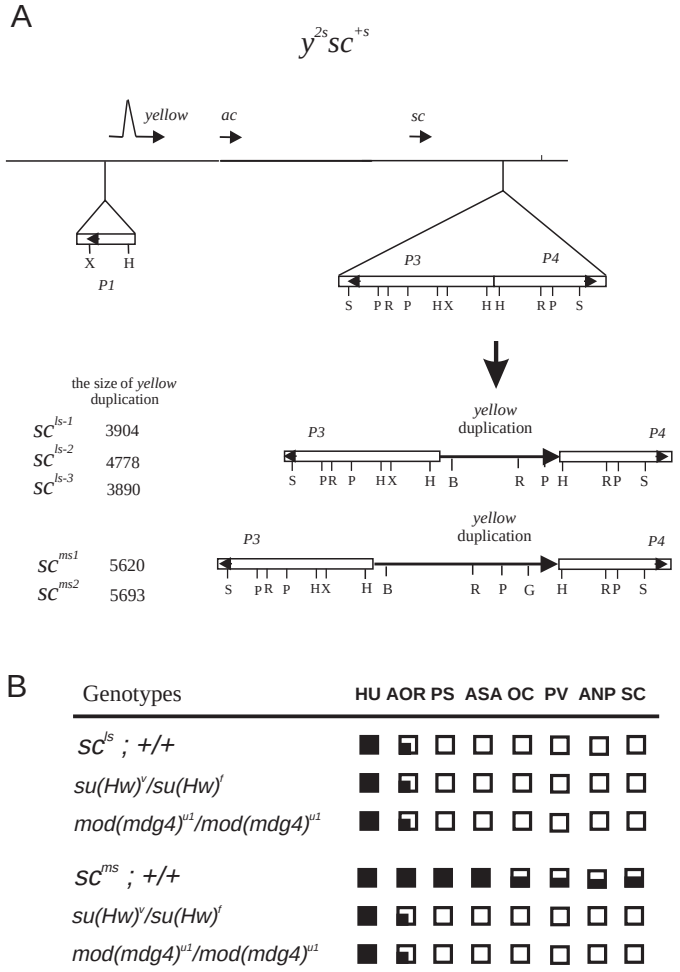


Fig. 1. The nature and properties of original mutations in AS-C. (A) Schematic presentation of the *yellow/ac/sc* region. Small arrowheads show insertions of the P elements associated with certain mutations. Thick horizontal arrows show the direction of transcription of the *yellow*, *ac* and *sc* genes. The arrows in boxes indicate the orientation of the P elements. The structure of the sc^{ls1}, sc^{ls2} and sc^{ls3} alleles was described previously (Golovnin et al., 1999). (B) Phenotypes of the indicated *sc* bristle mutations in males. The standard nomenclature for each bristle is indicated as follows (Lindsley and Zimm, 1992): HU, humeral; AOR, anterior orbital; PS, presutural; ASA, anterior supra-alar; OC, ocellar; PV, postvertical; ANP, anterior notopleural; SC, scutellar. Only the bristles affected in *sc* mutations are shown. Empty boxes indicate that the corresponding bristles are present (wild-type phenotype). In all but the scutellar, one quarter black, half black and fully black boxes mean that the corresponding bristle(s) was (were) absent in over 10%, 50% or 90% of the flies, respectively. For scutellars, quarter black, half black and fully black boxes mean that 3-4, 2-3 or 0-1 scutellar bristles, respectively, were present. Number of bristles is the mean of about 100 scored flies. The phenotypes of sc^{ls1}, sc^{ls2} and sc^{ls3} flies were taken from Golovnin et al. (Golovnin et al., 1999). The $su(Hw)^{v}/su(Hw)^{f}$ and $su(Hw)^{v}/su(Hw)^{2}$ transheterozygous lines had similar effect on the sc^{ms1} and sc^{ms2} mutations.

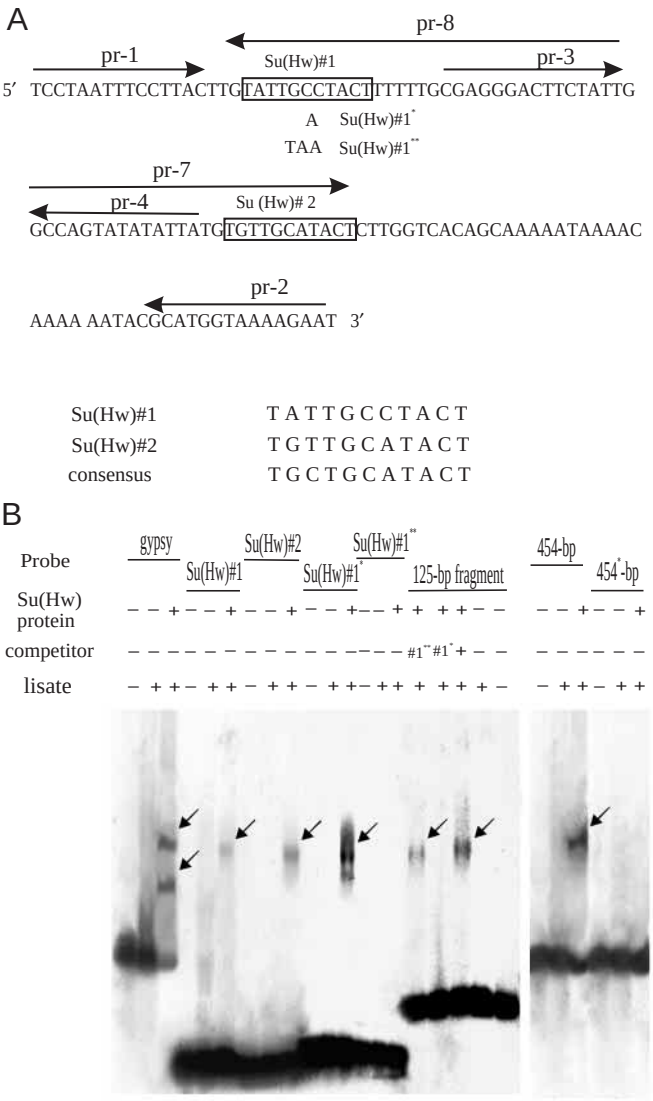


Fig. 2. Binding of in vitro synthesized Su(Hw) to two putative Su(Hw) binding sites in the 125 bp DNA fragment. (A) The sequence of the 125 bp DNA fragment is shown. Putative Su(Hw) binding sites are boxed. The primers used to obtain the DNA fragments are shown as arrows. The mutated residues are indicated below the sequence. The consensus for the Su(Hw) binding site was taken from Scott and Geyer (Scott and Geyer, 1999).
(B) Electrophoretic mobility shift assays. The radioactively labeled *gypsy*, 125 bp fragment, Su(Hw)#1, Su(Hw)#2, Su(Hw)#1*, Su(Hw)#1**, 454 bp and 454* bp fragments were used as probes, incubated with in vitro-synthesized Su(Hw) protein and run on a 1.5% agarose gel (Materials and Methods). One shifted band (indicated by arrows) presumably corresponds to a protein-DNA complex formed by Su(Hw) with only one Su(Hw) binding site. The binding of Su(Hw) to the 125 bp DNA fragment was examined in the presence of competitors. The binding is competed by excess unlabeled Su(Hw)#1* fragment but not by the Su(Hw)#1** fragment with the mutated Su(Hw) binding site.

with flies expressing the Flp recombinase (Golic and Lindquist, 1989). The DNA fragments to be tested were inserted between the *yellow* enhancers and promoter, at position –893 relative to the *yellow* transcription start site (Fig. 3A).

When the *gypsy* insulator is inserted at position –893, the *yellow* enhancer action is completely blocked, resulting in yellow instead of dark pigmentation of body and wing, whereas the eye enhancer was fully active because of neutralization of the enhancer-blocking activity (Muravyova et al., 2001).

To test the intergenic Su(Hw) sites we first made

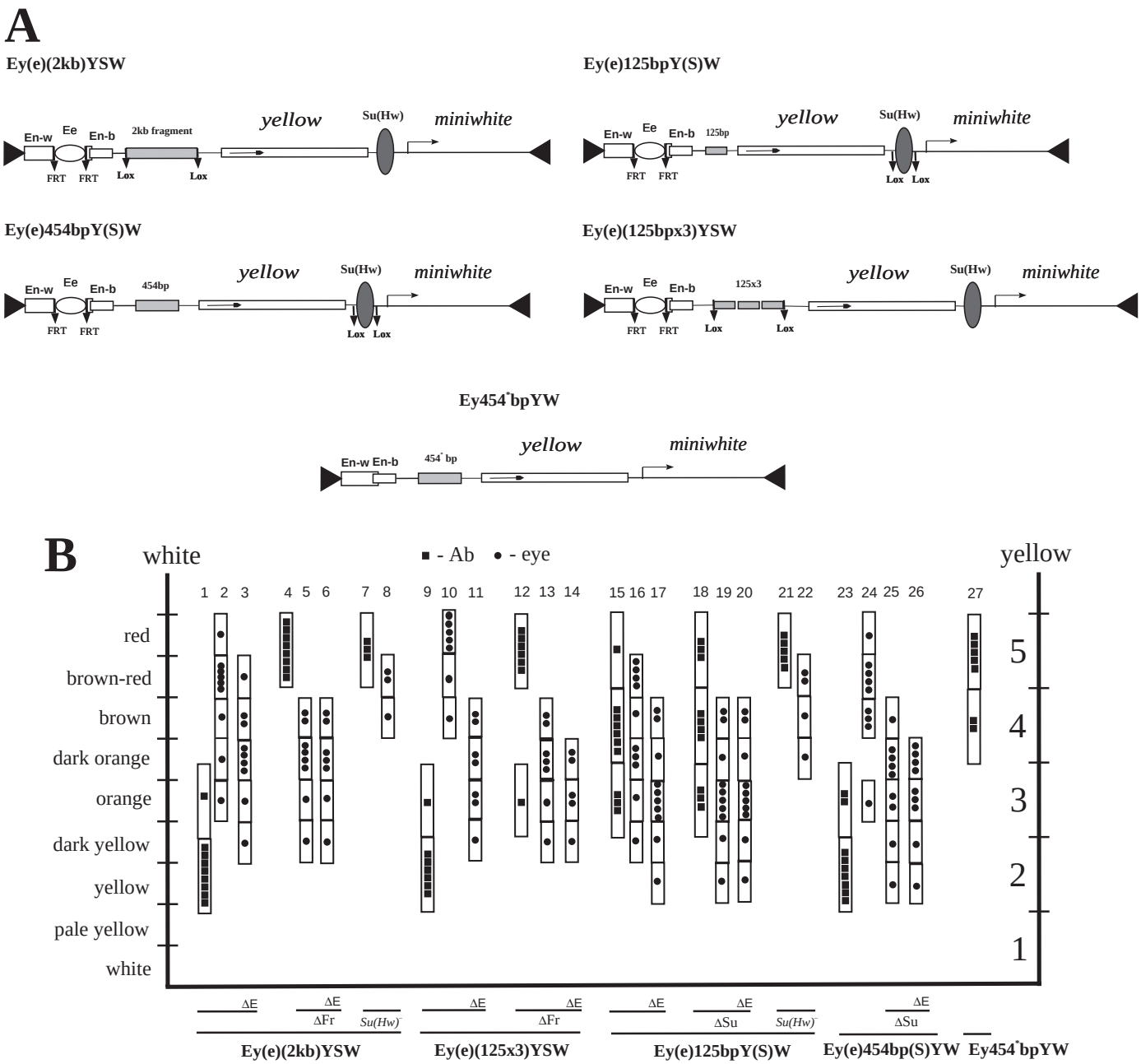


Fig. 3. Study of transgenic lines to test the enhancer-blocking activity. (A) Transposon constructs. The maps of the constructs (not to scale) show the *yellow* wing (En-w) and body enhancers (En-b) as partially overlapping white boxes and the eye enhancer (Eye) as a white oval. Downward pointing arrows labeled FRT or Lox mark the target sites of the Flp or Cre recombinase, respectively. The 125 bp, 454 bp, 454* bp, 125 bp×3 and 2 kb DNA fragments were inserted at –893 bp relative to the *yellow* transcription start site. The Su(Hw) insulator was inserted between *yellow* and *white*. The *yellow* and *white* genes are shown with arrows indicating the direction of transcription. (B) Analysis of *yellow* and *white* expression in males from transgenic lines heterozygous for the construct. Small symbols in the boxes indicate the number of independent transgenic lines displaying similar abdominal (black square) or eye (black circle) pigmentation. To determine the *y* and *w* phenotypes, the extent of pigmentation in the abdominal cuticle (reflecting the activity of the En-b enhancer) as well as the eye pigmentation of adult flies were estimated visually in 3- to 5-day-old males developing at 25°C (see Materials and Methods). Expression levels were determined without excision of functional elements in the wild type and after excision of the Su(Hw) insulator (ΔSu), of the eye enhancer (ΔE), or the tested DNA fragment (ΔFr). Abbreviation: $su(Hw)^{-}$, $su(Hw)^{v}/su(Hw)^{f}$.

Ey(e)(2kb)YSW, in which the 2 kb DNA fragment containing the 3′ part of the *yellow* coding region and the 5′ part of the *ac* regulatory region (Fig. 3A, Fig. 4A) is inserted at the –893 position. The 2 kb DNA fragment was flanked by Cre recognition (Lox) sites to permit its excision from transgenic flies (Siegal and Hartl, 2000). In all 9 transgenic Ey(e)(2kb)YSW lines, wing and body pigmentation was yellow suggesting that the 2 kb DNA fragment is able to completely block the *yellow* enhancers (Fig. 3B, lanes 1, 4). The deletion of the 2 kb DNA fragment in the Ey(e)(Δ2kb)YSW derivatives restored wild-type cuticle pigmentation. When three of the less pigmented lines were tested in the *su(Hw)*$^{-}$ background, wild-type pigmentation was restored (Fig. 3B, lane 7). Thus, the Su(Hw) protein is required to block the *yellow* enhancers.

At the same time, *white* expression was stronger in Ey(e)(2kb)YSW transgenic lines than in Ey(e)(Δ2kb)YSW derivatives bearing only the *gypsy* insulator (Fig. 3B, lanes 2-3, 5-6). The role of the eye enhancer in activation of the *white* promoter was supported by deleting the eye enhancer from the Ey(e)(2kb)YSW lines, which strongly diminished eye pigmentation. Thus, the 2 kb fragment can neutralize the enhancer-blocking activity of the *gypsy* insulator.

We next tested the minimal 125 bp fragment from the intergenic region by inserting it at position –893 to give the Ey(e)125bpY(S)W construct (Fig. 3A). In this construct the *gypsy* insulator between the *yellow* and *white* genes was flanked by lox sites. In 10 Ey(e)125bpY(S)W lines, wing and body pigmentation was between yellow and wild type (Fig. 3B, lane 15), indicating that the *yellow* enhancers were only partially blocked in comparison with the transgenic lines with the 2 kb fragment (Fig. 3B, lane 1). Five Ey(e)125bpY(S)W lines tested in the *su(Hw)*$^{-}$ background showed restored wild-type level of pigmentation, confirming that the binding of the Su(Hw) protein to the 125 bp fragment is required to block the *yellow* enhancers (Fig. 3B, lane 21). The deletion of the eye enhancer diminished eye pigmentation in 8 out of 10 Ey(e)125bpY(S)W transgenic lines, implying that the minimal 125 bp fragment is able to neutralize the *gypsy* insulator (Fig. 3B, lanes 16, 17). The deletion of the *gypsy* insulator (ΔS) in most Ey(e)125bpY(ΔS)W derivatives reduced eye pigmentation and made them insensitive to the additional deletion of the eye enhancer (Fig. 3B, 19, 20). These results suggest that the 125 bp fragment by itself can block the interaction between the eye enhancer and the *white* promoter.

The 2 kb DNA fragment has stronger enhancer-blocking activity than the 125 bp fragment. To exclude a role of the *yellow* coding and the *ac* regulatory regions in the insulation activity, we tested a 454 bp DNA subfragment that contains the 125 bp fragment and surrounding sequences (Fig. 3A). In all 9 transgenic Ey(e)454bpY(S)W lines, wing and body pigmentation was yellow suggesting that the 454 bp DNA fragment blocks the *yellow* enhancer as well as the 2 kb DNA fragment (Fig. 3B, lane 23). Like the 125 bp fragment, the 454 bp fragment also blocks the eye enhancer and efficiently neutralizes the activity of the *gypsy* insulator (Fig. 3B, lanes 24-26).

The strong blocking of the *yellow* enhancer by the 454 bp fragment, compared with 125 bp fragment, may be explained either by existence of additional Su(Hw) binding sites in the 454 bp fragment or by the possible involvement of one or more other proteins binding to neighboring sequences. To test these possibilities, we mutated both Su(Hw) binding sites in the 454 bp fragment (454*). The 454 bp and 454* bp DNA fragments were tested in electrophoretic mobility shift assays (EMSAs) using in vitro-synthesized Su(Hw) protein (Fig. 2B). The binding of Su(Hw) to the 454 bp fragment but not to 454* argues against additional Su(Hw) binding sites in the 454 bp fragment. To examine the ability of 454* to block the *yellow* enhancer, we inserted the 454* bp fragment at position –893 to give the Ey454*bpYW construct. In all 7 transgenic Ey454*bpYW lines, flies had nearly wild-type levels of wing and body pigmentation suggesting that the 454* bp fragment has lost the insulator activity (Fig. 3B, lane 27). Thus, these results confirm that the Su(Hw) protein is required but not sufficient for the blocking activity of the 454 bp fragment.

The multiplication of binding sites for the Su(Hw) protein has been shown to increase insulator activity (Scott et al., 1999). To test this rule, we inserted three copies of the 125 bp fragment between lox sites at –893 in the *yellow* regulatory region (Fig. 3A). All seven transgenic Ey(e)(125bp×3)YSW lines obtained had yellow wing and body cuticle indicating strong blocking of the wing and body enhancers (Fig. 3B, lane 9). At the same time, these lines had high levels of eye pigmentation that were strongly reduced after deletion of the eye enhancer (Fig. 3B, lanes 10, 11), indicating mutual neutralization of the triplicated 125 bp fragment and the *gypsy* insulator.

These results suggest that the intergenic region contains binding sites for other protein(s) in addition to Su(Hw) that is (are) required for efficient blocking of the *yellow* enhancers.

The *su(Hw)* and *mod(mdg4)* mutations influence expression of ASC alleles

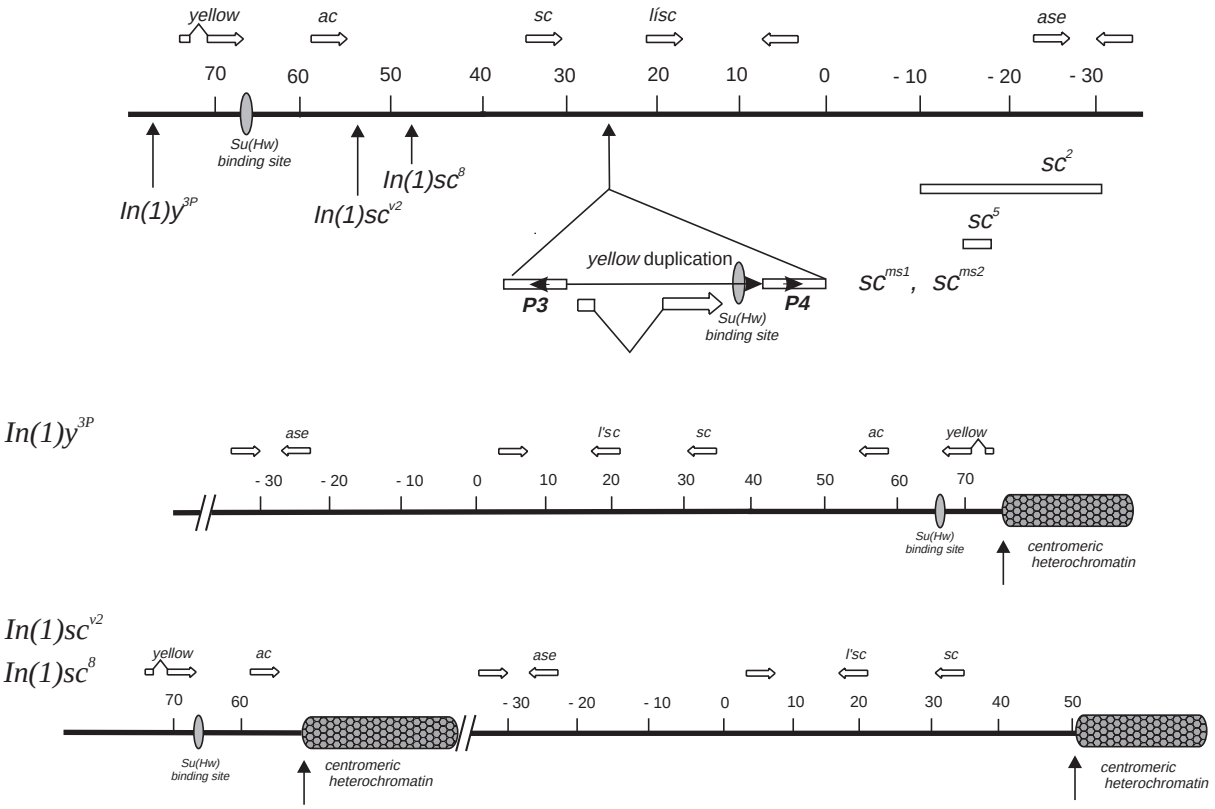
Mutations in the *su(Hw)* and *mod(mdg4)* genes have no visible effect on the *ac* or *sc* phenotype. To determine the potential role of these genes in the regulation of AS-C, we examined the influence of the *su(Hw)* and *mod(mdg4)* mutations on the mutant phenotype of the AS-C alleles.

First, we examined several inversions with breakpoints in the regulatory region of the *yellow* and AS-C and the centric heterochromatin.

The breakpoint in the *In(1)y*^{3P} mutation is located in the regulatory region of the *yellow* gene (Fig. 4A) (Campuzano et al., 1985). The centric heterochromatin in the *In(1)y*^{3P} mutation does not influence *yellow* expression in bristles or expression of the ASC genes, but the loss of the upstream body and wing enhancers causes a yellow wing and body phenotype. The *su(Hw)*^{v}*/su(Hw)*^{f} and *su(Hw)*^{v}*/su(Hw)*^{2} transheterozygotes strongly affected *ac* and *sc* gene expression, but did not influence *yellow* expression: bristles remained entirely pigmented (Fig. 4B). The homozygous *mod(mdg4)*^{u1} mutation and *mod(mdg4)*^{u1}*/Df(3R)GC14* transheterozygotes produced a similar effect on the *ac* and *sc* phenotype, although slightly milder than that produced by *su(Hw)*$^{-}$. These results suggest an involvement of Su(Hw) and Mod(mdg4) proteins in protecting the AS-C genes from heterochromatic silencing.

Similar results were obtained with two other inversions tested. The *In(1)sc*^{V2} and *In(1)sc*^{8} inversions have breakpoints between *ac* and *sc* (Fig. 4A). The breakpoint in *In(1)sc*^{V2} is located very close to the 3′ end of the *ac* coding region. Despite the close proximity to centric heterochromatin, both mutations

A



B

Genotypes	SC: HU	ASA	PS	AOR	OC	PV	ANP	PNP	SC	AC: PSA	MC	A/P	DC	AVT	viability	bristle pigmentation
y^{3P}; +/+	□	□	□	□	□	□	□	□	□	□	□	□	□	□	+	+
$su(Hw)^f/su(Hw)^v$	◒	◒	◒	◒	◒	◒	◒	◒	◒	◒	◒	◒	◒	◒	+	+
$mod(mdg)^{u1}/mod(mdg4)^{u1}$	◒	◒	◒	◒	□	□	□	□	◒	◒	◒	□	◒		+	w-v
$mod(mdg)^{u1}/Df(3R)GC14$	◒	◒	◒	◒	◒	◒	□	□	◒	◒	◒	□	◒		+	w-v
sc^{v2}; +/+	□	□	□	□	□	□	□	□	◒	□	□	□	□		+	+
$su(Hw)^f/su(Hw)^v$	◒	◒	◒	◒	◒	◒	◒	◒	◒	◒	■	◒	◒		+	m-v
$mod(mdg)^{u1}/mod(mdg4)^{u1}$	◒	◒	◒	◒	◒	◒	□	□	◒	◒	■	■	◒		+	m-v
$mod(mdg)^{u1}/df(3r)gc14$	◒	◒	◒	◒	◒	◒	◒	□	◒	◒	■	◒	◒		+	m-v
sc^8; +/+	□	□	□	□	□	□	□	◒	◒	□	□	□	□		+	+
$su(Hw)^f/su(Hw)^v$	◒	■	◒	□	□	□	□	◒	■	◒	◒	◒	◒		+	+
$mod(mdg)^{u1}/mod(mdg4)^{u1}$	◒	◒	◒	□	□	□	□	◒	■	□	□	□	□		+	+
$mod(mdg)^{u1}/df(3r)gc14$	◒	◒	◒	□	□	□	□	◒	■	□	□	□	□		+	+
sc^2; +/+	□	□	□	□	□	◒	□	□	◒	□	□	□	□		+	+
$su(Hw)^f/Su(Hw)^v$															lethal	-
$mod(mdg)^{u1}/mod(mdg4)^{u1}$	□	□	□	□	◒	◒	□	□	■	□	□	□	□		0.02(590)	+
$mod(mdg)^{u1}/df(3r)gc14$															lethal	-
sc^5; +/+	□	□	□	□	□	□	□	□	◒	□	□	□	□		+	+
$su(Hw)^f/su(Hw)^v$	□	◒	◒	◒	◒	◒	□	□	■	□	□	□	□		+	+
$mod(mdg)^{u1}/mod(mdg4)^{u1}$	□	□	□	□	□	□	□	□	■	□	□	□	□		+	+

Fig. 4. Role of Su(Hw) and Mod(mdg4) in the regulation of ASC. (A) Schematic presentation of the *yellow/ac/sc* region in the previously described *y*, *ac* and *sc* mutants (Campuzano et al., 1985). The coordinates of the ASC region are as defined in Campuzano et al. Vertical arrows indicate the positions of chromosomal breakpoints associated with the y^{3P}, sc^{v2} and sc^8 mutations. The localization of the sc^2 and sc^5 deletions is indicated by an elongated open box (Campuzano et al., 1985). Arrows with a triangle show insertions of P elements associated with duplication of the *yellow* sequences. Relative orientations of P elements are indicated by arrows in boxes. Thick horizontal white arrows show the positions and direction of *yellow* and ASC genes transcripts. The gray oval indicates the putative Su(Hw) binding sites in the 125 bp DNA fragment. (B) The effect of the *su(Hw)* ($su(Hw)^v/su(Hw)^f$ and $su(Hw)^v/su(Hw)^2$) and *mod(mdg4)* ($mod(mdg4)^{u1}/mod(mdg4)^{u1}$ and $mod(mdg4)^{u1}/Df(3R)GC14$) mutations on the phenotype of the mutations in ASC. The $su(Hw)^v/su(Hw)^f$ and $su(Hw)^v/su(Hw)^2$ transheterozygous lines had similar effects on the mutations in ASC. Phenotypes of the indicated *sc* mutations were examined in males. The standard nomenclature for bristles whose formation is regulated by *ac* are as follows (Lindsley and Zimm, 1992): ADC, anterior dorsocentral; PDC, posterior dorsocentral; PSA, posterior supraalar; AVT, anterior vertical; MC, the rows of microchaetae on the notum. Other designations as in Fig. 1. Only affected bristles in *ac* and *sc* mutations are shown. Bristle pigmentation: w-v, weak variegation indicates that 1-3 bristles in thorax and head are yellow; m-v, mild variegation shows that about half of bristles are yellow; +, wild-type pigmentation of all bristles. Viability: +, about normal viability; –, lethal. The figures indicate viability for combination of $mod(mdg4)^{u1}/mod(mdg4)^{u1}$ with sc^2, i.e. ratio of sc^2 males to *yw* males obtained in the progeny of heterozygous yw/sc^2; $mod(mdg4)^{u1}/mod(mdg4)^{u1}$ females. The total number of sc^2 and *yw* males scored is shown in brackets.

cause only a weak mutant phenotype (Fig. 4B). However, in *su(Hw)^{v}/su(Hw)^{f}* (*su(Hw)^{v}/su(Hw)^{2}*) or *mod(mdg4)^{u1}/mod(mdg4)^{u1}* (*mod(mdg4)^{u1}/Df(3R)GC14*) backgrounds these inversions caused strongly enhanced *ac*$^{-}$ and *sc*$^{-}$ phenotypes. In the case of *In(1)sc^{V2}*, in particular, the *mod(mdg4)* and *su(Hw)* mutations induced strong variegation of bristle pigmentation (Fig. 4B) suggesting that the Su(Hw)-Mod(mdg4) complex blocks the spread of heterochromatin in the *yellow* region.

The *sc^{2}* and *sc^{5}* mutations are associated with deletions. The 1.3 kb deletion in the *sc^{5}* mutation (Fig. 4A) partially suppresses the formation of scutellar bristles suggesting that the *sc* enhancer is affected (Campuzano et al., 1985). The *su(Hw)* mutations weakly suppressed ASA, AOR, OC and PV bristle formation (Fig. 4B). *sc^{2}*, also called *ase^{1}*, is an intercalary 17-18 kb deletion that removes the regulatory sequences for the SC bristles and also the coding sequence of the *ase* gene (Gonzalez et al., 1989). The *sc^{2}* mutation has a weak sc phenotype associated with partial suppression of SC bristle formation (Fig. 4B). Unexpectedly the combination of *sc^{2}* with *su(Hw)^{v}/su(Hw)^{f}* or with *su(Hw)^{v}/su(Hw)^{2}* was lethal. The homozygous *mod(mdg4)^{u1}* mutation or transheterozygous *mod(mdg4)^{u1}/Df(3R)GC14* also strongly decreased the survival of *sc^{2}* mutants and completely blocked the formation of SC bristles. Even *sc^{2}; mod(mdg4)^{u1}/+* flies had a very low viability if they were obtained from homozygous *mod(mdg4)^{u1}* females, suggesting that maternally supplied Mod(mdg4) is required for *sc^{2}* survival. The proneural gene *l'sc* is expressed only in early embryos and its inactivation results in embryonic lethality (Campuzano et al., 1985; Carmena et al., 1995), suggesting that loss of Mod(mdg4) or Su(Hw) causes repression of *l'sc* in the *sc^{2}* mutant. We hypothesize that an additional Su(Hw) insulator might normally protect the *l'sc* gene and might become essential when enhancer elements in the *sc^{2}* region are deleted.

DISCUSSION

To explain how the long-range activation potential of eukaryotic enhancers could be restricted to the relevant target promoter, it was proposed that eukaryotic chromatin is organized into functionally independent domains that prevent illegitimate enhancer-promoter communication (West et al., 2002). Recent publications (Gerasimova and Corces, 1998; Gerasimova et al., 2000; Gerasimova and Corces, 2001; Labrador and Corces, 2002) suggest a model in which distant chromosomal binding sites of Su(Hw) are brought together by Mod(mdg4) into a small number of insulator bodies located at the nuclear periphery. It was suggested that in this way Su(Hw) marks the base of topologically independent looped chromatin domains. However, despite the presence of many endogenous Su(Hw) binding sites in polytene chromosomes, no specific function has been attributed to any site in a particular gene.

Using in vivo and in vitro assays, we have shown that there exists a functional Su(Hw) insulator between the *yellow* gene and AS-C. Previously it was found that at least four Su(Hw) binding sites are required for effective enhancer blocking (Scott et al., 1999). Here we found that the 125 bp fragment including only two Su(Hw) binding sites can partially block the strong *yellow* enhancer, while the larger 454 bp fragment including the same Su(Hw) sites completely blocks *yellow* enhancers. Thus, additional proteins binding to neighboring sequences are required for strong insulator action of the element between *yellow* and AS-C. The sequencing of the *Drosophila* genome shows the absence of large clusters of endogenous Su(Hw) binding sites, such as are found in the *gypsy* retrotransposon. It seems possible that in endogenous insulators, Su(Hw) cooperates with additional DNA-binding proteins to produce insulator activity. This assumption may also explain the absence of lethal phenotypes in the *su(Hw)*$^{-}$ background since other proteins would partly compensate for the loss of Su(Hw) function.

Our results further confirm the initial observation of the interaction between two *gypsy* insulators (Gause et al., 1998; Cai and Shen, 2001; Muravyova et al., 2001). The two Su(Hw) binding sites in the 125 bp fragment and the *gypsy* insulator mutually neutralize each other's enhancer-blocking activity. Thus, the difference in the number of Su(Hw) binding sites between interacting insulators is not critical for the effective neutralization of the enhancer blocking activity.

As has been observed previously (Scott et al., 1999; Smith and Corces, 1992; Hagstrom et al., 1996; Hoover et al., 1992), increasing the number of Su(Hw) binding sites increases insulator strength, and three copies of the 125 bp insulator block better than a single copy. How can this be reconciled with the observation that two Su(Hw) insulators neutralize one another? We suppose that, as proposed earlier (Cai and Shen, 2001; Muravyova et al., 2001), the neutralization requires the pairing between two insulators. Interaction between neighboring insulators would pre-empt their interaction with larger assemblies of Su(Hw) binding sites that have been proposed to associate together at the nuclear periphery through the Mod(mdg4) protein (Mongelard and Corces, 2001; West et al., 2002; Labrador and Corces, 2002). Thus, for neutralization, we suppose that the Su(Hw) binding sites must adopt a paired configuration, therefore requiring a sufficient distance between them for DNA to form a loop. In contrast, putting more Su(Hw) binding sites very close together merely ensures that enough Su(Hw) protein will be bound at any one time to produce insulator action.

The role of the Su(Hw) and Mod(mdg4) proteins in the expression of ASC genes becomes obvious when the normal architecture of the ASC regulatory region is altered by chromosome rearrangements. Many previously described inversions with breakpoints in the AS-C regulatory region and centric heterochromatin (Campuzano et al., 1985) have weak mutant phenotypes, suggesting the presence of sequences that effectively impede the spread of heterochromatic silencing. The appearance of strong variegating repression of the *ac* and *sc* genes when the inversions are combined with loss of *su(Hw)* or *mod(mdg4)* function suggests that the Su(Hw) and Mod(mdg4) proteins are involved in the stability of the *ac* and *sc* expression.

In the *In(1)y^{3p}* mutation, a heterochromatic breakpoint in the upstream regulatory region does not effect *yellow* expression suggesting that the *yellow* promoter is relatively resistant to heterochromatin proximity at this breakpoint. At the same time, *ac* and *sc* expression is strongly affected by *su(Hw)* or *mod(mdg4)* mutations, supporting the idea that Su(Hw) binding sites between *yellow* and *ac* block heterochromatin spreading.

The *In(1)sc*^{8} and *In(1)sc*^{v2} inversions separate the *ac* and *sc* genes. The requirement of the Su(Hw) and Mod(mdg4) proteins for normal *sc* expression suggests the existence of additional Su(Hw) binding sites in the AS-C regulatory region. The strong genetic interaction between *sc*^{2} and mutations in *mod(mdg4)* or *su(Hw)* also supports the presence of additional Su(Hw) binding sites in ASC. The expression of ASC genes is regulated by a large number of enhancer-like elements (Ruiz-Gomez and Modolell, 1987; Gomez-Skarmeta et al., 1995; Modolell and Campuzano, 1998). It seems reasonable that these ASC enhancers should be separated by boundary elements as was found for the 3′ cis-regulatory region of *Abdominal B* (*Abd-B*), which is subdivided into a series of *iab* domains (Mihaly et al., 1998). Boundary elements like MCP, Fab-7 and Fab-8 separate the *iab* domains and protect each against positive and negative chromatin modifications induced by neighboring *iab* domains (Barges et al., 2000; Hagstrom et al., 1996; Mihaly et al., 1998; Zhou et al., 1996; Zhou et al., 1999). Our genetic results might be explained by the assumption that the Su(Hw)-Mod(mdg4) protein complex participates in formation of boundary elements between certain AS-C enhancers. The absence of noticeable changes in the wild-type AS-C gene expression on the *su(Hw)* or *mod(mdg4)* mutant background might be the consequence of the functional redundancy of the Su(Hw)-Mod(mdg4) protein complex. We did not find clusters of potential endogenous Su(Hw) binding sites inside the AS-C sequence. Thus, it seems possible that Su(Hw)-Mod(mdg4) cooperates with other non-identified proteins in formation of the functional boundaries in the regulatory region of AS-C. The identification and characterization of new Su(Hw) binding sites may help in understanding the role of Su(Hw)/Mod(mdg4) in transcriptional regulation of AS-C genes and provide new insights into the mechanisms of the insulator action.

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