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

The authors apologise to readers for this mistake.
An endogenous Su(Hw) insulator separates the yellow gene from the Achaeate-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 Achaeate-scute gene complex form a functional insulator. Genetic analysis shows that at least two proteins, Suppressor of Hairy wing and Modifer of MDG4, required for the activity of this insulator, are involved in the transcriptional regulation of Achaeate-scute.

Key words: Drosophila melanogaste, Achaeate-scute Complex, Insulator, Su(Hw), Mod(mdg4), Enhancer blocking
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)$^\mu/\mu$ 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)$^{\mu/\mu}$ 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 1'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 (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, 1982), were injected into *y ac w$^{118}$* preblastoderm embryos as described previously (Rubin and Spradling, 1982; Spradling and Rubin, 1982). The resulting flies were crossed with *y ac w$^{118}$* 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$^{118}$* balancer stock containing dominant markers: *Int(2LR)CyO* for chromosome two, *Int(3LR)TM3,Stb* for chromosome three. The transformed lines were examined by Southern blot hybridization, to check for transposon integrity and copy number.

The *su(Hw)$/\mu$/*su(Hw)$^\mu$/ *su(Hw)$/\mu$/ *su(Hw)$^\mu$/ *mod(mdg4)$^{\mu/\mu}$/ *mod(mdg4)$^{\mu/\mu}$/ and *mod(mdg4)$^{\mu/\mu}$/ *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 *Su(Hw)-BamHI* fragment containing the *yellow* regulatory region (yr) was subcloned into *BamHI* + *XhoI*-digested pGEM7 (yr plasmid). The 5 kb *BamHI*-BglII 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(so)) and in CaSpeR3 (C3-su). The lox(so) fragment was blunt-ligated to the CaSpeR2 vector restricted with *BglII* (C2-lox(su)).

The *yellow* regulatory region encodes 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' tctaatcttcacctgc 3') and *pr-2* (5' atttctatacacag 3') primers from the *ks133* 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 *ks133* phage DNA restricted with *PstI* between two lox sites (lox(2 kb)). The 454 bp fragment was PCR-amplified with *pr-5* (5' ggaattcactaccgga 3') and *pr-6* (5' caagcttacctgctagt 3') primers from the *ks133* phage and inserted between two 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' gttccgactagttatggtaactatgc 3') and *pr-8* (5' aagcttctcaagtaattaaatgc 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 mutated sequences had been introduced and other PCR-induced mutations did not exist.
Ey(e)(2 kb) YSW and Ey(e)(3x125 bp) YSW

Thelox(2 kb) or lox(3x125 bp) fragment was inserted in the yr-frt(Ee) restricted with Eco47III at -893 from theyellowtranscription start site [yr-frt(Ee)-lox(2 kb) and yr-frt(Ee)-lox(3x125 bp)]. Theyr-frt(Ee)-lox(2 kb) or yr-frt(Ee)-lox(3x125 bp) fragment was ligated intoC3-su restricted withXbaI and BamHI.

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 withEco47III (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 intoC2-lox(su) restricted withXbaI andBamHI.

Ey454 bpYW

The 454 bp fragment was inserted in the yr restricted with Eco47III (yr-454 bp). The yr-454 bp fragment was ligated intoC3-yc restricted withXbaI andBamHI.

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 system. The PCR products 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 fromγ2sc+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 theγ2sc+ mutant in which sc expression is slightly affected which results in the loss of humeral bristles. Additional derivatives, scms1 and scms2, 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 theyellow gene were duplicated between the P3 and P4 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)+ andmod(modg4)+ mutations on the sc phenotype (Fig. 1B). The suppression of the mutation sc phenotype in scms; su(Hw)+/su(Hw)+ or scms; su(Hw)+/su(Hw)+ flies supports the idea that the Su(Hw) protein plays a role in the repression of the sc gene (Fig. 1B). The homozygous mod(modg4)+ mutation had a similar suppressive effect on the sc phenotype (Fig. 1B), implying that Su(Hw) and Mod(modg4) are required for repression of the sc activation in two scms 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 presumpt 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 thegypsy inserter that contains 12 putative binding sites for Su(Hw). These DNA fragments were tested in electrophoretic mobility shift assays (EMSA) 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 thegypsy 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

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**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<sup>ls1</sup>, sc<sup>ls2</sup> and sc<sup>ls3</sup> 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 (Lindsay 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 bristle(s) is (are) 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 the flies, respectively, were present. Number of bristles is the mean of about 100 scored flies. The phenotypes of the flies, respectively, were present. Number of bristles is the mean of about 100 scored flies. The phenotypes of the flies, respectively, were present. Number of bristles is the mean of about 100 scored flies. The phenotypes of the flies, respectively, were present. Number of bristles is the mean of about 100 scored flies.

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**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.
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 (∆Su), of the eye enhancer (∆E), or the tested DNA fragment (∆Fr). Abbreviation: su(Hw)−, su(Hw)+/su(Hw)−.
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)$^-\cdot$ 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)$^-\cdot$ 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(S)ΔS derivatives reduced eye pigmentation and made them insensitive to the additional deletion of the eye enhancer (Fig. 3B, lanes 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 insulator 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$^*$pYW construct. In all 7 transgenic Ey454$^*$pYW 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 duplicated 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)$^{3P}$ 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)$^+/su(Hw)$ and su(Hw)$^+/su(Hw)$2 transheterozygotes strongly affected ac and sc gene expression, but did not influence yellow expression: bristles remained entirely pigmented (Fig. 4B). The homozygous mod(mdg4)$^{µ1}$ mutation and mod(mdg4)$^{µ1/Df(3R)GC14}$ transheterozygotes produced a similar effect on the ac and sc phenotype, although slightly milder than that produced by su(Hw)$^-\cdot$ 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)ss$^{V2}$ and In(1)sc$^{8}$ inversions have breakpoints between ac and sc (Fig. 4A). The breakpoint in In(1)ss$^{V2}$ is located very close to the 3’ end of the ac coding region. Despite the close proximity to centric heterochromatin, both mutations
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: +, 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)\(^{+/+}\) (su(Hw))\(^{+/+}\) or mod(mdg4)\(^{+/+}\) (mod(mdg4))\(^{+/+}\) backgrounds these inversions caused strongly enhanced ac\(^{–}\) and sc\(^{–}\) phenotypes. In the case of In(1)sc\(^{3+}\), 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\(^{–}\)mutations are associated with deletions. The 1.3 kb deletion in the sc\(^{3}\) mutation (Fig. 4A) partially suppresses the formation of scutellar bristles suggesting that the sc enhancer is affected (Campuzano et al., 1985). In the sc\(^{2}\) mutations weakly suppressed ASA, AOR, OC and PV bristle formation (Fig. 4B). sc\(^{2}\), also called ase\(^{l}\), 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)\(^{+/+}\) or with su(Hw)\(^{+/+}\) was lethal. The homozygous mod(mdg4)\(^{+/+}\) mutation or transheterozygous mod(mdg4)\(^{+/+}\) (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)\(^{+/+}\) flies had a very low viability if they were obtained from homozygous mod(mdg4)\(^{+/+}\) females, suggesting that maternally supplied Mod(mdg4) is required for sc\(^{–}\) survival. The proneural gene l\(^{sc}\) is expressed only in early embryos and its inactivation results in embryonic lethality (Campuzano et al., 1985; Carmen 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 heterochromatin 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)\(^{3+}\) 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*- and *In(1)sc-2* 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*- 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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