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The Centrosomal Protein CP190 Is a Component of the *gypsy* Chromatin Insulator

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Summary

Chromatin insulators, or boundary elements, affect promoter-enhancer interactions and buffer transgenes from position effects. The *gypsy* insulator of *Drosophila* is bound by a protein complex with two characterized components, the zinc finger protein Suppressor of Hairy-wing [Su(Hw)] and Mod(mdg4)2.2, which is one of the multiple spliced variants encoded by the *modifier of mdg4* [*mod(mdg4)*] gene. A genetic screen for dominant enhancers of the *mod(mdg4)* phenotype identified the Centrosomal Protein 190 (CP190) as an essential constituent of the *gypsy* insulator. The function of the centrosome is not affected in *CP190* mutants whereas *gypsy* insulator activity is impaired. CP190 associates physically with both Su(Hw) and Mod(mdg4)2.2 and colocalizes with both proteins on polytene chromosomes. CP190 does not interact directly with insulator sequences present in the *gypsy* retrotransposon but binds to a previously characterized endogenous insulator, and it is necessary for the formation of insulator bodies. The results suggest that endogenous *gypsy* insulators contain binding sites for CP190, which is essential for insulator function, and may or may not contain binding sites for Su(Hw) and Mod(mdg4)2.2.

Introduction

Chromatin insulators or boundary elements are DNA sequences that share properties suggestive of a functional role in the organization of higher order chromatin structure. Insulators can protect transgenes from chromosomal position effects (Kellum and Schedl, 1991; Roseman et al., 1995) and can block communication between enhancers and promoters when present between them (Chung et al., 1993; Geyer and Corces, 1992; Holdridge and Dorsett, 1991). Chromatin insulators have been found in a variety of organisms including yeast, *Drosophila*, and vertebrates (Gerasimova and Corces, 2001; West et al., 2002). *Drosophila* sequences with insulator properties include the *gypsy* insulator originally found in the *gypsy* retrotransposon (Geyer and Corces, 1992), the *scs* and *scs'* insulators flanking one of the *hsp70* loci (Kellum and Schedl, 1991), the *Fab7* insulator from the *Bithorax* complex (Zhou et al., 1996), and the *SF1* insulator present in the *Antennapedia* complex (Belozerov et al., 2003). It is currently not known whether these different insulators function independently of each other or whether they share components and have overlapping functions. Insulators exert their function through their interaction with specific DNA binding proteins. For example, the *scs'* insulator interacts with BEAF-32 (Zhao et al., 1995), the *scs* insulator is bound by ZW5 (Gaszner et al., 1999), and the chicken globin insulator interacts with CTCF (Bell et al., 1999).

The insulator element present in the *gypsy* retrotransposon is a 350 bp sequence containing 12 copies of the binding site for Su(Hw). At least four of these binding sites are required for proper insulator function (Scott et al., 1999). A second characterized component of the *gypsy* insulator complex is Mod(mdg4)2.2, (Buchner et al., 2000; Gerasimova et al., 1995; Mongelard et al., 2002). Mod(mdg4)2.2 does not bind DNA directly and is recruited to the *gypsy* insulator complex via interactions between its C-terminal acidic domain and the bHLH-Zip domain of Su(Hw) (Gause et al., 2001; Ghosh et al., 2001). Mod(mdg4)2.2 contains a BTB/POZ domain at the N-terminus that mediates homodimerization or multimerization of this protein (Ghosh et al., 2001).

The Su(Hw) and Mod(mdg4)2.2 proteins colocalize at multiple sites on *Drosophila* polytene chromosomes. These sites do not contain the *gypsy* retrotransposon and are presumed to be endogenous insulator elements. We will refer to them as "*gypsy* endogenous insulators" to reflect the fact that they contain the same proteins as those found in the *gypsy* retrotransposon although their DNA sequence might differ from that of *gypsy*. Several lines of evidence suggest that multiple endogenous *gypsy* insulator elements come together at a single nuclear location, presumably through interactions between their protein components, forming rosette-like structures that organize the chromatin into independent domains of gene expression (Byrd and Corces, 2003; Gerasimova et al., 2000). The same type of interactions between individual insulator sites has been observed in the case of the *scs* and CTCF insulators (Blanton et al., 2003; Yusufzai et al., 2004).

Efforts to characterize endogenous *gypsy* insulators have failed to identify clusters of Su(Hw) binding sites with more than three sites (D.G. and V.G.C., unpublished data). Because at least four of these sites are required for proper insulator activity (Scott et al., 1999), other DNA binding proteins in addition to Su(Hw) may be required to form a functional endogenous *gypsy* insulator. In fact, a recently characterized endogenous *gypsy* insulator contains only two Su(Hw) binding sites but possesses insulator function in the standard transgene assays (Golovnin et al., 2003; Parnell et al., 2003). We have identified CP190, a previously characterized centrosomal protein, as an additional component of the *gypsy* insulator. CP190 contains both a BTB/POZ domain and three C2H2 zinc fingers and might thus combine properties attributed to the two previously characterized *gypsy* insulator components. CP190 interacts with both Su(Hw) and Mod(mdg4)2.2 and appears to be an essential component of endogenous *gypsy* insulator elements.

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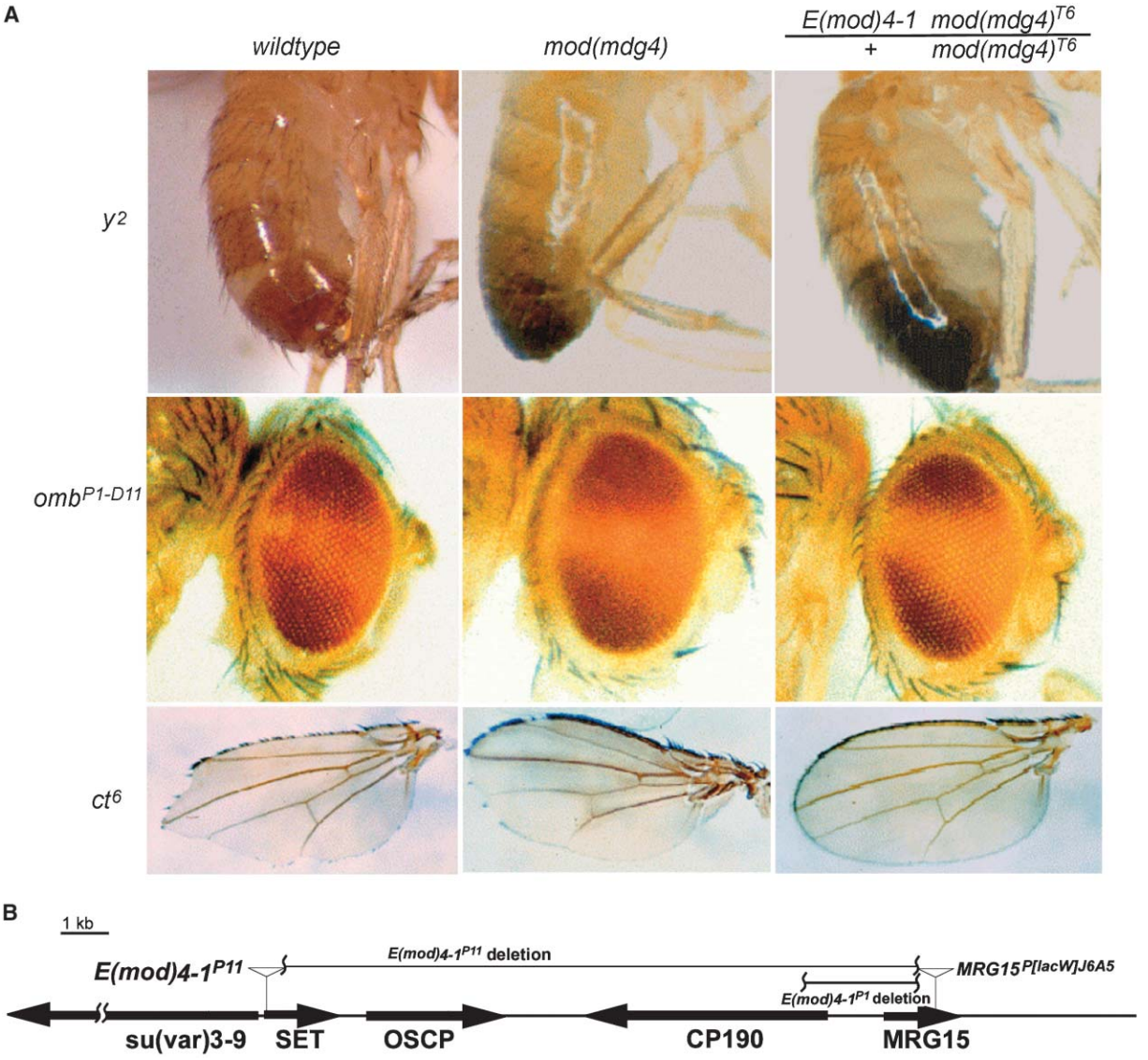

Figure 1. *CP190* Interacts Genetically with *mod(mdg4)*

(A) Abdomens of males of the genotype y^2 (left), y^2; $mod(mdg4)^{T6}/mod(mdg4)^{T6}$ (center), and y^2; *E(mod)4-1*/+ $mod(mdg4)^{T6}/mod(mdg4)^{T6}$ (right) are shown in the top row. The eyes of males of the genotype $omb^{P1\text{-}D11}$ (left), $omb^{P1\text{-}D11}$; $mod(mdg4)^{T6}/mod(mdg4)^{T6}$ (center), and $omb^{P1\text{-}D11}$; *E(mod)4-1*/+ $mod(mdg4)^{T6}/mod(mdg4)^{T6}$ (right) are shown in the middle row. The wings of ct^6 (left), ct^6; $mod(mdg4)^{T6}/mod(mdg4)^{T6}$ (center), and ct^6; *E(mod)4-1*/+ $mod(mdg4)^{T6}/mod(mdg4)^{T6}$ (right) males are shown in the bottom row.

(B) Organization of genes surrounding *CP190*. Thick lines represent genes, and arrows indicate the direction of transcription. The *P[lacW]* insertion present in the $CP190^{P11}$ allele is located in the *SET* gene, and sequences located between this P element and the one present in the *MRG15* gene are deleted (represented by a thin line). The $CP190^{P1}$ allele is caused by a deletion of part of the *MRG15* and *CP190* genes (thin line).

Results

Identification of Dominant Enhancers of *mod(mdg4)*

In order to identify new components of the *gypsy* insulator, we performed an ethane methyl sulfonate (EMS) mutagenesis screen for dominant enhancers of $mod(mdg4)^{T6}$, a sensitized background with a partially active *gypsy* insulator (Gerasimova et al., 1995). Three different *gypsy*-dependent phenotypes were monitored simultaneously in order to rule out mutations specific to an individual phenotype (Figure 1A). The *gypsy*-dependent phenotypes chosen for this analysis are those caused by the mutations y^2, which affects the coloration of the wings and body cuticle (Parkhurst and Corces, 1986); $omb^{P1\text{-}D11}$, which affects the pigmentation pattern of the eye (Tsai et al., 1997); and ct^6, which affects the shape of the wing (Jack, 1985). Only mutations affecting all three phenotypes were studied further as candidates for *gypsy* insulator components.

Some of the mutations obtained in the screen were alleles of *su(Hw)*, supporting the feasibility of this genetic approach. In addition to *su(Hw)*, we obtained a collection of dominant enhancers of $mod(mdg4)^{T6}$ that are able to complement *su(Hw)* mutations. Five of these belong to the same complementation group and map by recom-

Table 1. Molecular Defects Associated with CP190 EMS-Induced Alleles

Allele	Location (gi:20349357)	Mutation	Effect on mRNA (gi:17737972)	Protein Size	Protein Structure Remaining
$CP190^{4-1}$	11097837	C→T	Stop codon at nucleotide 2483	N-ter 765 aa	BTB zinc-fingers
$CP190^{H53-2}$	11099738	G→A	Stop codon at nucleotide 650	N-ter 154 aa	BTB
$CP190^{H31-2}$	11099854	C→T	Splice junction truncated	N-ter 134 aa	BTB

The first column indicates the name of the allele. Also indicated are the location of the mutation according to the DNA sequence of the *Drosophila* genome available from FlyBase (http://flybase.bio.indiana.edu/), the nature of the mutation and the effect on the mRNA, and the size and structure of the putative protein.

bination to a single locus at 3-56. The gene affected by these five mutations was named *Enhancer of mod(mdg4)4-1* [*E(mod)4-1*]. The effect of mutations in *E(mod)4-1* in combination with $mod(mdg4)^{T6}$ on *gypsy*-induced phenotypes is shown in Figure 1A. To identify the *E(mod)4-1* gene, we performed P-element mutagenesis of the region by mobilizing the P element present in the $MRG15^{P\{lacW\}PJ6A3}$ strain, which is located at 88E close to where the *E(mod)4-1* gene was presumed to map. Several lines containing new P element insertions were isolated and characterized. One of the lines, $E(mod)4\text{-}1^{P1}$, harbors a deletion of DNA sequences from position 11100389 to 11103539 (gi:20349357). This deletion includes the promoter and first exon of *CP190*, likely rendering the gene nonfunctional, and part of the *MRG15* gene (Figure 1B). Because the $MRG15^{P\{lacW\}PJ6A3}$ loss-of-function mutation complements all *E(mod)4-1* EMS alleles and does not enhance the phenotype of $mod(mdg4)^{T6}$ (data not shown), we conclude that the insulator phenotype of *E(mod)4-1* alleles is likely to be caused by mutation of *CP190*. To confirm this, we determined the sequence of *CP190* in three different *E(mod)4-1* EMS alleles. We found that each of them contains a point mutation within the coding sequence of *CP190* (Table 1 and Figure 2A). We will subsequently refer to *E(mod)4-1* as *CP190*.

CP190 Mutations Suppress *gypsy*-Induced Phenotypes

All *CP190* alleles we obtained were identified by their ability to enhance the phenotype of $mod(mdg4)^{T6}$ in a dominant manner, suggesting that the functionality of the *gypsy* insulator decreases when the levels of CP190 protein are reduced in cells with a truncated Mod(mdg4)2.2 protein. CP190 contains a BTB/POZ domain at its N-terminus, three C2H2-type zinc-fingers in its central region, and a Glu-rich C-terminal end (Figure 2A). Interestingly, BTB/POZ and C2H2 zinc-finger domains are also found in the *gypsy* insulator proteins Mod(mdg4)2.2 and Su(Hw), respectively. To establish further the role of CP190 in insulator function, we wished to test the effect of this protein on the *gypsy* insulator in a wild-type (wt) *mod(mdg4)* background. One of the EMS alleles isolated, $CP190^{H4-1}$, appears to be a hypomorph, and it is viable as a homozygote. All other EMS or P induced alleles are lethal. Some transheterozygous combinations of strong alleles, such as $CP190^{H53-2}$/$CP190^{P11}$ (Figures 1B and 2A), are lethal during late pupal stages. When these flies are removed from the pupal cases very late in pupal development, they survive for several hours, enabling us to determine the effect of *CP190* mutants on insulator activity by examining the phenotype of *gypsy*-induced mutations. Flies of the genotype $y^2\ ct^6$; $CP190^{H53-2}$/$CP190^{P11}$ display black cuticle coloration in the abdomen, indicating a complete suppression of y^2. The shape and margin of the wings is normal, indicating a complete suppression of ct^6 (Figure 2B). Flies carrying the viable hypomorphic $CP190^{H4-1}$ allele show similar but much weaker effects on *gypsy*-induced phenotypes (Figure 2C). These results indicate that *CP190* mutations act as recessive suppressors of *gypsy*-induced phenotypes, suggesting that CP190 is an essential component of the *gypsy* insulator.

CP190 was identified originally by its affinity to β-tubulin. It associates with centrosomes during mitosis and with chromosomes during interphase (Jimenez and Goday, 1993; Oegema et al., 1995; Raff et al., 1993). Therefore, we investigated whether centrosome structure or cell division was affected in diploid *CP190* mutant cells. Diploid larval brain cells were stained with anti-γ-tubulin (Figure 2D) or anticentrosomin antibodies (data not shown). Mitotic chromosomes were labeled by staining with antibodies against phosphorylated histone H3. All mitotic cells at stages ranging from prophase to telophase appeared normal and had nicely formed centrosomes (Figure 2D). These results are similar to a recent finding that CP190 is essential for viability but dispensable for centrosomal function (Butcher et al., 2004).

The Localization of CP190 on Polytene Chromosomes Overlaps with Known *gypsy* Insulator Components

Genetic evidence indicates that *CP190* is essential for *gypsy* insulator function, suggesting that CP190 may be a component of the *gypsy* insulator. To confirm this possibility, we determined whether CP190 localizes to a site of *gypsy* insertion by examining larval polytene chromosomes from a strain carrying the y^2 mutation, which is caused by the insertion of the *gypsy* retrotransposon in the *yellow* gene at the tip of the X chromosome. A strong immunofluorescence signal can be detected at y^2 by using antibodies against the insulator proteins Su(Hw) and Mod(mdg4)2.2 (Gerasimova et al., 1995). The distribution of CP190 on polytene chromosomes overlaps significantly with that of Mod(mdg4)2.2 and Su(Hw) (Figure 3A and data not shown), suggesting that CP190 may interact with these two proteins at many, although not all, endogenous *gypsy* insulator sites. At sites of colocalization, the CP190 immunolocalization signals do not always correlate in intensity with those of Su(Hw) and Mod(mdg4)2.2, whereas the intensities of Mod(mdg4)2.2 and Su(Hw) are generally very similar. In some cases, CP190 appears to be present at loci

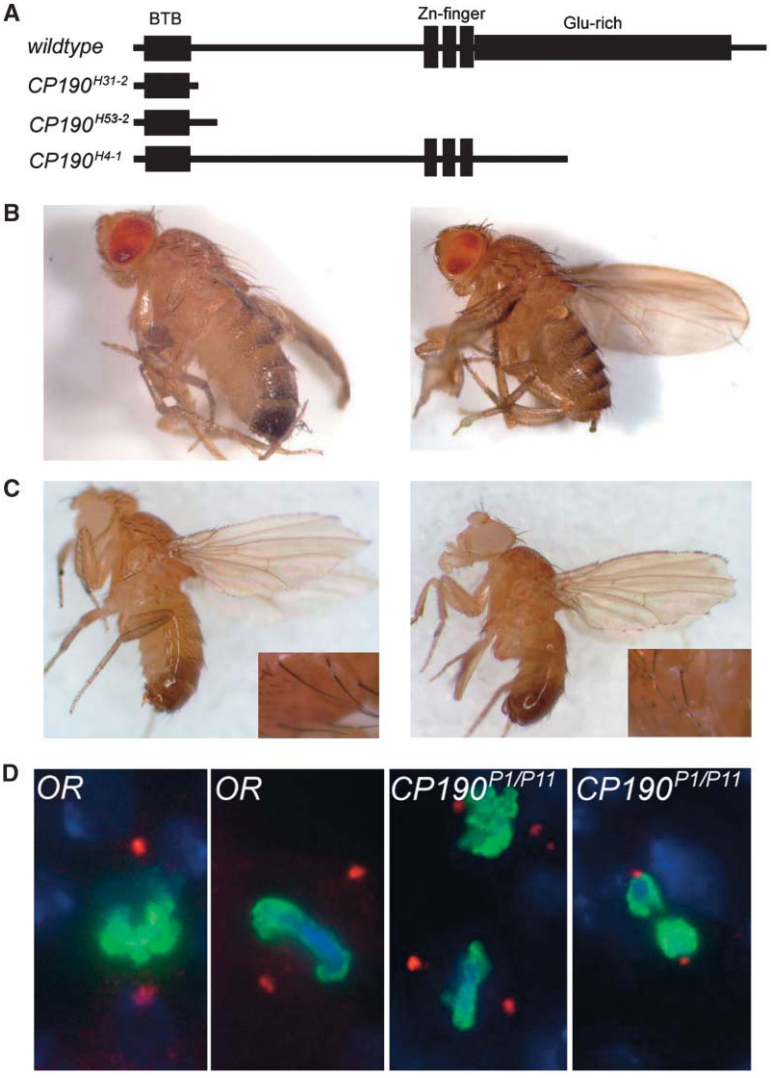

Figure 2. Protein Structure and Phenotypes of *CP190* Alleles

(A) Structure of the CP190 protein in wt and mutant alleles. The wt CP190 protein contains a BTB domain, three zinc-fingers, and a Glu-rich domain (black boxes). The two homozygous lethal alleles, $CP190^{H31-2}$ and $CP190^{H53-2}$, contain only the BTB domain and some adjacent sequences, whereas the viable $CP190^{H4-1}$ allele contains the BTB domain, all three zinc-fingers, and part of the Glu-rich region.

(B) Effect of strong *CP190* allele combinations on *gypsy*-induced phenotypes. Male (left) and female adult escapers of the genotype $y^2\ ct^6$; $CP190^{H53-2}/CP190^{P11}$ show a dark coloration of the abdomen and normal wing shape, suggesting a complete suppression of *gypsy*-induced phenotypes (compare to the male in [C], left).

(C) Effect of weak *CP190* alleles on gypsy-induced phenotypes. On the left is a male of the genotype $y^2\ ct^6$, and on the right is a $y^2\ ct^6$; $CP190^{H4-1}$ male. The wings of the $CP190^{H4-1}$ male are rounder in shape, suggesting that ct^6 is partially suppressed, whereas the bristles are yellow (lower right corner in both), indicating that y^2 phenotype is altered. The body cuticle color is only slightly darker in the background of the $CP190^{H4-1}$ mutation.

(D) Cells from larval brains of wt Oregon R (left), and $CP190^{P1}/CP190^{P11}$ (right), were stained with mouse-anti-γ-tubulin to reveal the centrosomes (red) and rabbit anti-Ser10 phospho-histone H3 to mark the chromosomes of mitotic cells (green). The appearance of centrosomes and mitotic figures is the same in wt and mutant cells.

where Su(Hw) and Mod(mdg4)2.2 are not (Figure 3A, green arrows). An intense signal of CP190 can be seen also at the *gypsy*-containing *yellow* locus in the y^2 allele, and this signal overlaps with that of Su(Hw) and Mod(mdg4)2.2 (Figure 3B). These results support the hypothesis that CP190 is a component of the insulator present in the *gypsy* retrotransposon. Signals corresponding to both possible types of complexes, those containing all three proteins and those containing CP190 but lacking Su(Hw) and Mod(mdg4)2.2, are present at the boundary between bands and interbands (Figure 3C). Because Su(Hw) is the DNA binding protein that tethers Mod(mdg4)2.2 to insulator DNA (Ghosh et al., 2001), the discovery of sites containing CP190 but lacking other *gypsy* insulator proteins suggests that either CP190 is able to bind DNA on its own or can interact with other DNA binding protein(s) distinct from Su(Hw).

CP190 Interacts Directly with Other Insulator Components

To test whether CP190 associates physically with *gypsy* insulator components, we determined whether Su(Hw) and Mod(mdg4)2.2 can be coimmunoprecipated with antibodies against CP190 from protein extract obtained from *Drosophila* embryos. Both Su(Hw) and Mod(mdg4)2.2 proteins coprecipitate with CP190 by using anti-CP190 antibodies, but not preimmune serum (Figure 4A), suggesting that all three proteins form a complex in vivo. To test further the possible interactions between CP190 and other *gypsy* insulator components, we used the yeast two-hybrid system as an assay to detect protein-protein interactions. A plasmid expressing a fusion protein of full-length CP190 and the DNA binding domain of GAL4 (GAL4BD) was introduced into yeast. The ability of CP190-GAL4BD to interact with Su(Hw)-GAL4AD (GAL4 activation domain) and Mod(mdg4)2.2-GAL4AD was determined based on growth on media lacking histidine and adenine. CP190-GAL4BD interacts with Mod(mdg4)2.2-GAL4AD and Su(Hw)-GAL4AD (Figure 4B, quadrants 2 and 3). In reciprocal two-hybrid assays, full-length CP190-GAL4AD interacts strongly with both Mod(mdg4)2.2-GAL4BD (Figure 4B, quadrant 5) and Su(Hw)-GAL4BD (data not shown), and the strength of this interaction is comparable to that observed between Su(Hw)-GAL4BD and Mod(mdg4)2.2-GAL4AD (Figure

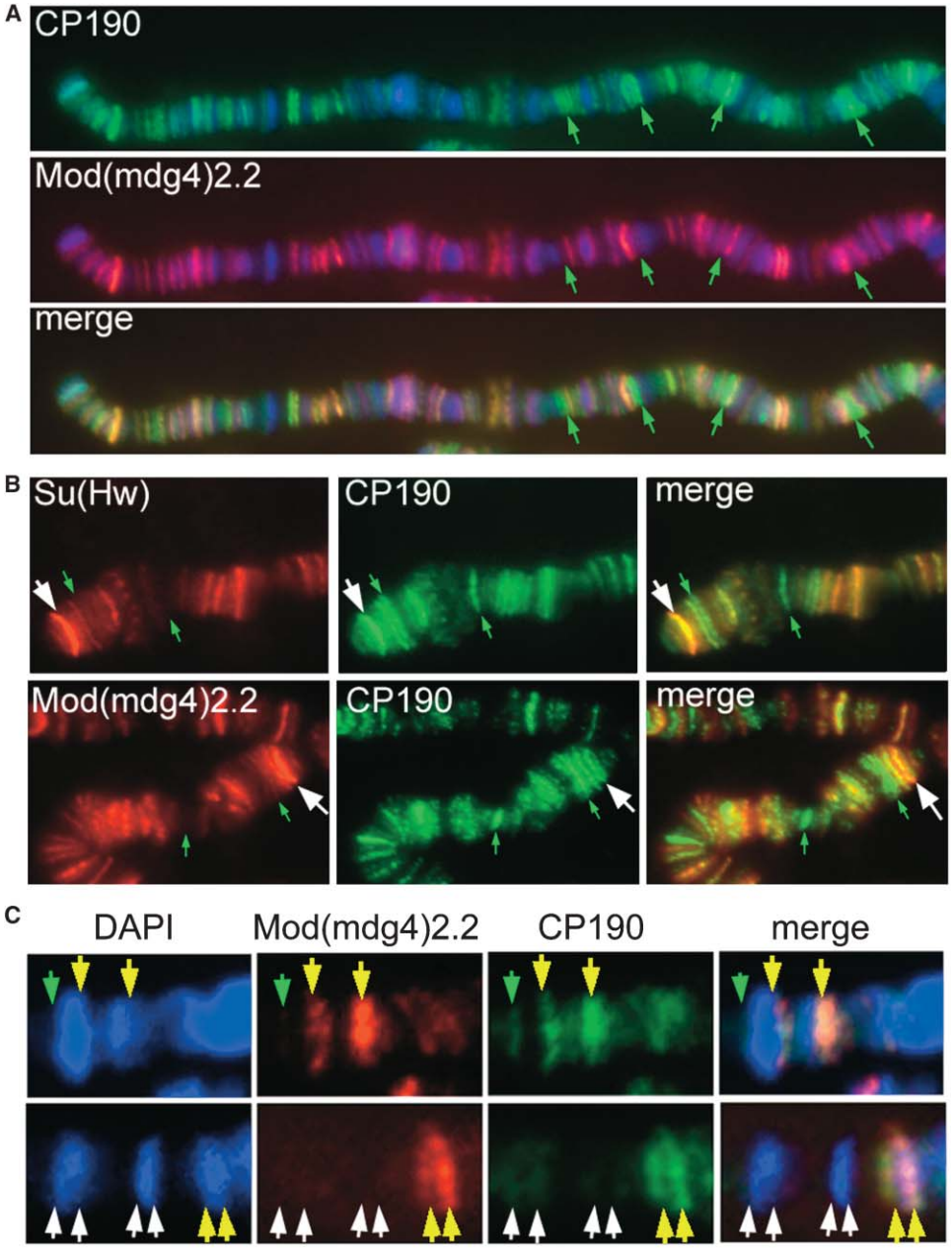

Figure 3. Distribution of CP190 and Other *gypsy* Insulator Components on Polytene Chromosomes

(A) Immunolocalization of CP190 (green) and Mod(mdg4)2.2 (red) on polytene chromosomes. DNA stained with DAPI (blue). Green arrows point to locations where CP190 is present in the absence of Mod(mdg4)2.2.

(B) Detailed view of the tip of the X chromosome from salivary glands of a y^2 larva. The top row shows immunolocalization of Su(Hw) (red) and CP190 (green). The bottom row shows immunolocalization of Mod(mdg4)2.2 (red) and CP190 (green). White arrows indicate the location of the *y* locus at the tip of the X chromosome. The green arrows mark CP190 bands that do not contain detectable Su(Hw) or Mod(mdg4)2.2 signals.

(C) Detailed views of polytene chromosomes stained with anti-CP190 (green), anti-Mod(mdg4)2.2 (red), and DAPI (blue). Yellow arrows point to junctions between DAPI bands and interbands that contain both CP190 and Mod(mdg4)2.2 proteins. Green arrows point to junctions that contain only CP190. White arrows point to junctions that contain neither of the two proteins.

4B, quadrant 6). No growth was detected when yeast was transformed with any of the single plasmids (data not shown).

The CP190 protein contains a BTB/POZ domain in its N-terminus, which has been shown to mediate homodimerization of some proteins. However, upon transformation of yeast with CP190-GAL4BD and CP190-GAL4AD, no growth was observed on plates lacking histidine and adenine, suggesting that the CP190 protein may be unable to interact with itself (Figure 4B, quadrant 1). We also tested whether CP190 is able to interact with GAGA, another BTB/POZ domain-containing protein. Yeast transformed with CP190-GAL4BD and GAGA-GAL4AD is unable to grow in selective conditions, suggesting

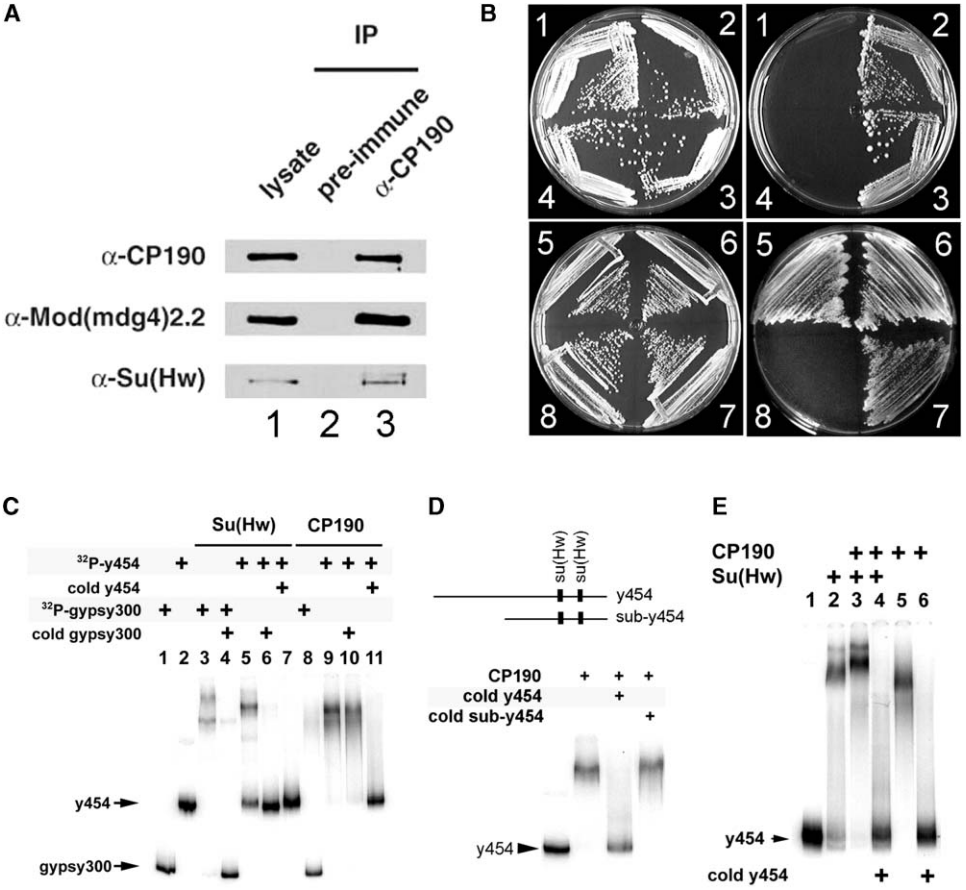

Figure 4. Interactions of CP190 with Other *gypsy* Insulator Components and DNA

(A) Su(Hw) and Mod(mdg4)2.2 proteins coimmunoprecipitate with CP190. Lysates (lane 1), material immunoprecipitated with preimmune serum (lane 2), and rabbit-anti-CP190 (lane 3) were run on an SDS-PAGE gel, transferred to nitrocellulose, and Western blotted with anti-CP190 (top), anti-Mod(mdg4)2.2 (middle), and anti-Su(Hw) (bottom). Approximately 6%, 3%, and 1% of total CP190, Mod(mdg4)2.2, and Su(Hw), respectively, were immunoprecipitated.

(B) Growth of yeast strain *pJ694A* expressing Su(Hw), Mod(mdg4)2.2, GAGA, and CP190 proteins in various combinations. Plates on the left are nonselective (+ade +his) for the reporter genes, whereas plates on the right are selective (−ade −his) for the reporter genes used in the yeast two-hybrid assays. (1) Yeast expressing CP190-GAL4BD and CP190 GAL4AD. (2) CP190-GAL4BD and Mod(mdg4)2.2-GAL4AD. (3) CP190-GAL4BD and Su(Hw)-GAL4AD. (4) CP190-GAL4BD and GAGA-GAL4AD. (5) Yeast expressing Mod(mdg4)2.2-GAL4BD and CP190-GAL4AD. (6) Su(Hw)-GAL4BD and Mod(mdg4)2.2-GAL4AD. (7) Mod(mdg4)2.2-GAL4BD and GAGA-GAL4AD. (8) Su(Hw)-GAL4BD and GAGA-GAL4AD.

(C) Su(Hw) (lanes 3–7) and CP190 (lanes 8–11) protein synthesized with a rabbit reticulocyte extract were incubated with a ^{32}P labeled DNA fragment of the *gypsy* retrotransposon (gypsy300) containing 8 Su(Hw)-binding sites (lanes 3, 4, and 8), or a ^{32}P labeled y454 fragment containing an endogenous insulator from the *yellow-achaete* region with 2 Su(Hw)-binding sites (lanes 5, 6, 7, 9, 10, and 11). Unlabelled gypsy300 (lanes 4, 6, and 10) or unlabelled y454 (lanes 7 and 11) DNAs were added as competitor. The protein-DNA complex was analyzed by electrophoresis on a 4% native gel and visualized by autoradiography.

(D) CP190 protein was incubated with a ^{32}P labeled y454 fragment containing 2 Su(Hw)-binding sites. Unlabelled y454 or sub-y454 DNAs were added as competitor. The protein-DNA complex was analyzed by electrophoresis on a 4% native gel and visualized by autoradiography.

(E) Su(Hw) (lane 2), CP190 (lanes 5 and 6), or both (lanes 3 and 4) proteins synthesized as above were incubated with ^{32}P labeled y454 fragment with (lanes 4 and 6) or without cold competitor y454 DNA. Protein-DNA complex formation was analyzed as above.

that the two proteins are unable to interact with each other (Figure 4B, quadrant 4). Mod(mdg4)2.2-GAL4BD is able to interact with GAGA-GAL4AD whereas Su(Hw)-GAL4BD is not (Figure 4B, quadrants 7 and 8). These results suggest that CP190 can distinguish between the BTB/POZ domains of Mod(mdg4)2.2 and GAGA.

Association of CP190 with the *gypsy* Retrotransposon Insulator Requires Su(Hw), but Not Mod(mdg4)2.2

To determine the requirements of each insulator component for the stability of the whole complex in vivo, we analyzed the effect of mutations in each insulator protein on the ability of the other components to associate with polytene chromosomes. Because CP190 has an essential role in insulator function, we tested first whether this protein is required for the formation of the complex between Su(Hw) and Mod(mdg4)2.2 and their association with chromosomes. We thus examined the distribution of Su(Hw) and Mod(mdg4)2.2 proteins on polytene chromosomes of y^2; $CP190^{P1}/CP190^{P11}$ larvae. Su(Hw) and Mod(mdg4)2.2 are still present on these chromosomes in the absence of CP190 at both the *gypsy* ele-

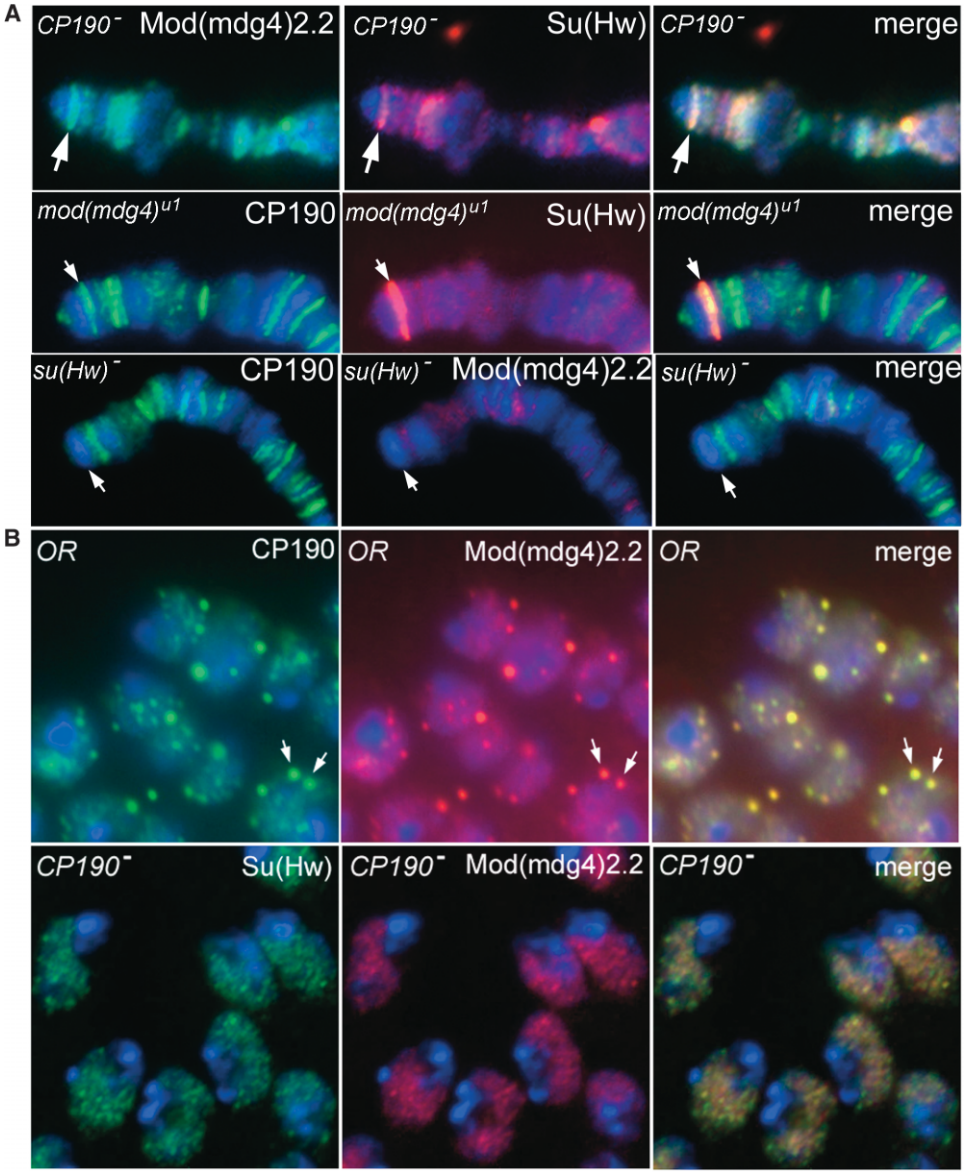

Figure 5. Interactions between *gypsy* Insulator Components on Polytene Chromosomes

(A) Localization of insulator proteins on polytene chromosomes from various mutant strains. Arrows point to the location of the *y* locus containing a copy of the *gypsy* retrotransposon in the y^2 allele. Polytene chromosomes from y^2 *w* ct^6; $CP190^{P1}/CP190^{P11}$ were stained with rabbit-anti-Mod(mdg4)2.2 (green) and rat-anti-Su(Hw) (red) (top). Polytene chromosomes from y^2 *w* ct^6; $mod(mdg4)^{u1}$ larvae were stained with anti-CP190 (green) and anti-Su(Hw) (red) (middle). Polytene chromosomes from y^2 *w* ct^6; $su(Hw)^v/ su(Hw)^{v\,P[CasX/K]}$ larvae were stained with anti-CP190 (green) and anti-Mod(mdg4)2.2 (red) (bottom).

(B) Immunolocalization of CP190 (green) and Mod(mdg4)2.2 in wt (*OR*) brain cells. Arrows point to some of the insulator bodies seen in the nuclei of these cells (top). Brain cells from y^2 *w* ct^6; $CP190^{P1}/CP190^{P11}$ mutant larvae were stained with anti-Su(Hw) (green) and anti-Mod(mdg4) (red). The signals overlap but mislocalize to small diffuse dots instead of large insulator bodies (bottom).

ment in the *y* gene and endogenous insulator sites (Figure 5A, top). This result suggests that the presence of Su(Hw) and Mod(mdg4)2.2 is not sufficient for the insulator function of the *gypsy* retrotransposon because y^2; $CP190^{P1}/CP190^{P11}$ flies show a complete lack of *gypsy* insulator activity.

Su(Hw) is able to bind DNA in vitro in the absence of Mod(mdg4)2.2 (Spana and Corces, 1990), but the distribution of Su(Hw) on the polytene chromosome is altered in *mod(mdg4)* mutants, suggesting that the association of Su(Hw) with DNA in vivo is stabilized by the Mod(mdg4)2.2 protein (Gerasimova and Corces, 1998). In flies lacking Mod(mdg4)2.2, Su(Hw) is present at normal levels in the *gypsy* element present in the *y* locus in polytene chromosomes of y^2; $mod(mdg4)^{u1}$ larvae but is considerably reduced at endogenous insulator sites (Figure 5A, middle). An interpretation of these results is that Su(Hw) requires Mod(mdg4)2.2 in order to bind to endogenous *gypsy* insulators, but not to the insulator present in the *gypsy* retrotransposon. CP190 remains

colocalized with Su(Hw) at the *y* locus of y^2; $mod(mdg4)^{u1}$ larvae (Figure 5A, middle). Furthermore, CP190 appears to be present at normal levels at endogenous insulator sites in $mod(mdg4)^{u1}$ mutants (compare Figures 3B and 5A), indicating that CP190 does not require Mod(mdg4)2.2 in order to localize to endogenous insulator sites or the *gypsy* retrotransposon. Next, we examined the distribution of CP190 on polytene chromosomes of y^2; $su(Hw)^v$ larvae, which lack Su(Hw) protein. The levels of Mod(mdg4)2.2 on polytene chromosomes in the $su(Hw)^v$ mutant are dramatically reduced (Figure 5A, bottom). Although the levels of CP190 appear normal at endogenous insulator sites, CP190 is no longer present at the *gypsy* element inserted in the *y* locus (Figure 5A, bottom). These results indicate that CP190 requires Su(Hw) in order to bind to the insulator present in the *gypsy* retrotransposon, but Su(Hw) is not necessary for the interaction of CP190 with endogenous insulator sites.

CP190 Binds DNA at Sequences Different from Su(Hw)

The distinct behavior of CP190 with respect to *gypsy* versus endogenous insulators could be explained on the basis of their different structure. The insulator present in *gypsy* contains 12 tightly clustered Su(Hw) binding sites, whereas putative endogenous insulators contain at most 2–3 Su(Hw) recognition sequences (Golovnin et al., 2003; Parnell et al., 2003). CP190 may be unable to bind to the insulator of the *gypsy* retrotransposon on its own and may need to be tethered by Su(Hw). On the other hand, CP190 may be able to bind directly to DNA at endogenous insulator sites independently of Su(Hw) by using its three C2H2 type zinc-fingers, which are often involved in DNA recognition. To test this hypothesis, we compared the affinity of CP190 and Su(Hw) for two different DNA fragments that possess insulator activity. One of these fragments is a truncated version of the insulator present in the *gypsy* retrotransposon and contains eight copies of the Su(Hw) binding site (gypsy300); the second fragment (y454) includes the endogenous insulator from the *yellow-achaete* region, which contains only two Su(Hw) binding sites (Golovnin et al., 2003; Parnell et al., 2003). Electrophoretic mobility shift assays (EMSA) show that Su(Hw) binds strongly to the gypsy300 fragment, whereas CP190 interacts very weakly, if at all, with this DNA (Figure 4C). In addition, Su(Hw) interacts with the y454 fragment, and this binding can be competed with cold gypsy300 DNA. CP190 interacts strongly with y454, and, more importantly, the interaction of CP190 with y454 cannot be competed with cold gypsy300 but is competed with cold y454 DNA (Figure 4C). These results suggest that CP190 is able to bind DNA but does not compete with Su(Hw) for the same binding sites, and that the y454 fragment may contain recognition sites for CP190 distinct from those bound by Su(Hw). To further test this possibility, we performed EMSA experiments with y454 DNA by using a subfragment (sub-y454) containing the two Su(Hw) binding sites as a competitor. The complete y454 DNA fragment can compete fully the binding of CP190 to labeled y454, but the subfragment containing the two Su(Hw) binding sites fails to compete. This result supports the conclusion that the two proteins bind to separate sites in the y454 DNA. In addition, EMSA experiments with the y454 DNA fragment and combinations of the Su(Hw) and CP190 proteins further support the idea that the two proteins bind to the same fragment (Figure 4E). The addition of CP190 causes a supershift, decreasing the mobility of the Su(Hw)-y454 DNA complex. This result is in agreement with the hypothesis that CP190 binds to DNA independently of Su(Hw) and/or it interacts directly with this protein.

The CP190 Protein Is Essential for the Formation of Insulator Bodies

Previous results have shown that the function of the insulator present in the *gypsy* retrotransposon requires the formation of chromatin loops via interactions with endogenous *gypsy* insulators present throughout the genome (Byrd and Corces, 2003; Cai and Shen, 2001; Gerasimova et al., 2000; Muravyova et al., 2001). Interactions among multiple insulators can be visualized in the form of insulator bodies, which are large aggregates of individual insulators present mostly in the nuclear periphery of cells during interphase. To better ascertain the role of CP190 in *gypsy* insulator function, we examined its distribution in diploid nuclei of imaginal disc or brain cells from third instar larvae. CP190 is distributed in a nonuniform pattern in nuclei of diploid cells, where it overlaps with Mod(mdg4)2.2 at all insulator body sites (Figure 5B, top). To explore the role of CP190 in the formation of insulator bodies, we examined the distribution of Su(Hw) and Mod(mdg4)2.2 in diploid cells of y^2; $CP190^{P1}/CP190^{P11}$ larvae. In wt cells, Su(Hw) and Mod(mdg4)2.2 overlap extensively in large, well-defined insulator bodies (Figure 5B, top); however, in nuclei of *CP190* mutant cells these two proteins localize in small, semidiffuse dots (Figure 5B, bottom). This result indicates that CP190 is important for the formation or stability of the large aggregates of individual insulator sites represented by the insulator bodies. Because the formation of these bodies correlates with a functional *gypsy* retrotransposon insulator, this observation suggests that CP190 is also essential for the function of endogenous *gypsy* insulators.

Discussion

A genetic screen for dominant enhancers of *mod(mdg4)* has resulted in the identification of CP190 as a third component of the *gypsy* insulator. CP190 is present at *gypsy* retrotransposon insulator sites and overlaps extensively with Su(Hw) and Mod(mdg4)2.2 at presumed endogenous insulators. CP190 displays a specific distribution pattern on polytene chromosomes, showing significant overlap with Su(Hw) and Mod(mdg4)2.2 at the junctions between transcriptionally inert bands and transcriptionally active interbands. Similar localization patterns have been reported for other insulators. For example, the fa^{swb} insulator at the *notch* locus and the BEAF-32 protein of the *scs'* insulator are also present at the boundaries between bands and interbands (Vazquez and Schedl, 2000; Zhao et al., 1995). Results suggest that CP190 can bind DNA on its own or can be tethered to the chromosome through interactions with Su(Hw).

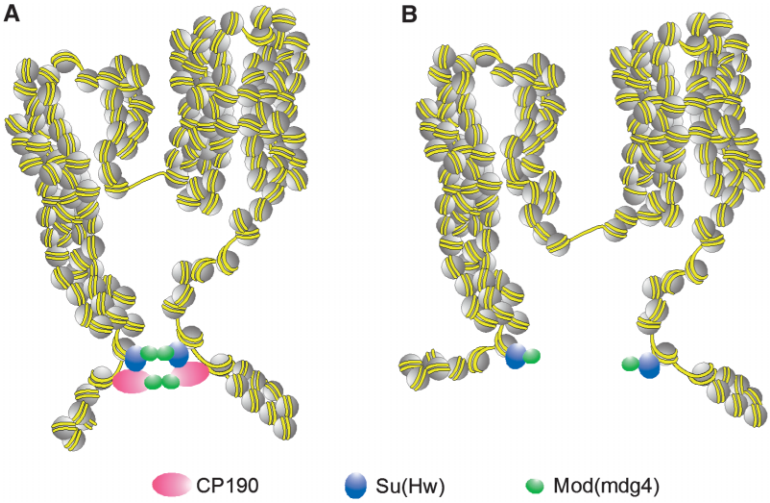

Figure 6. Model Showing the Role of Insulator Components in the Establishment of Chromatin Domains

(A) In a wt cell, endogenous *gypsy* insulators contain binding sites for both Su(Hw) and CP190. Both proteins interact with Mod(mdg4)2.2, which is able to form chromatin loops through interactions with the Mod(mdg4)2.2 protein present at other endogenous insulator sites.

(B) In a *CP190* mutant cell, the Su(Hw) protein is not sufficient to maintain interactions between individual insulator sites, and the insulator body-induced organization falls apart.

Mutations in the *CP190* gene impair the function of the insulator present in the *gypsy* retrotransposon without affecting the presence of Su(Hw) and Mod(mdg4)2.2, suggesting an essential task for CP190 in the activity of this insulator. In addition, the lethality of *CP190* mutants suggests a critical role for the CP190 protein in the function of *gypsy* endogenous insulators. This essential role may be a consequence of the requirement of CP190 for the formation of insulator bodies in the nuclei of diploid cells.

The insulator present in the *gypsy* retrotransposon contains only Su(Hw) binding sites, and CP190 is present in this insulator through direct interactions with Su(Hw). The *gypsy* insulator contains 12 Su(Hw) binding sites, and at least four are needed for insulator activity (Scott et al., 1999). However, clusters of three or more Su(Hw) binding sites are rare in the genome (D.G. and V.G.C., unpublished data). Therefore, a critical question is whether the sites of Su(Hw) and Mod(mdg4)2.2 localization present throughout the genome truly function as insulators. The presence of CP190 at these sites and its ability to bind DNA might explain this apparent paradox. For example, the endogenous insulator present in the *yellow-achaete* region has only two binding sites for Su(Hw) (Golovnin et al., 2003; Parnell et al., 2003). Nevertheless, the y454 fragment containing this insulator is able to bind CP190, suggesting that this protein might act in concert with Su(Hw) to confer insulator activity. It is therefore possible that endogenous *gypsy* insulators are composed of binding sites for Su(Hw) and/or for CP190 and, together with Mod(mdg4)2.2, form a complex. Endogenous *gypsy* insulators may have few or no Su(Hw) binding sites, and they may rely on CP190 to bind DNA and tether other insulator components such as Mod(mdg4)2.2 via protein-protein interactions (Figure 6).

Previous studies have suggested that *gypsy* insulators separated at a distance in the genome may come together and form large insulator bodies in the nucleus during interphase. These aggregates represent higher order structures of chromatin and are implicated in the regulation of gene expression by compartmentalizing the genome into transcriptionally independent domains (Gerasimova et al., 2000). The formation of these aggregates appears to require Mod(mdg4) function because the large aggregates are missing in *mod(mdg4)* mutants (Gerasimova and Corces, 1998). The formation of *gypsy* insulator bodies is severely impaired also in *CP190* mutants, suggesting that CP190 plays an essential role in the formation of these bodies and in the establishment of the chromatin domain organization mediated by *gypsy* endogenous insulators (Figure 6). It is possible that the BTB/POZ protein-protein interaction domains of both CP190 and Mod(mdg4)2.2 are required for and contribute to the stability of the interactions among insulator sites (Figure 6). In vitro-expressed CP190 lacking the BTB/POZ domain is soluble, whereas the wt protein is not (Oegema et al., 1995), further suggesting that CP190 might exist as a complex with itself or other proteins in vivo, and the formation of this complex is likely mediated by the BTB/POZ domain. However, because CP190 is present at the *gypsy* insulator in the absence of Mod(mdg4)2.2 protein, the interaction between these two proteins may not be crucial for CP190 recruitment to the insulator.

Previous studies have identified CP190 as a centrosome-specific protein during mitosis that also associates with chromatin during interphase. Although many of these studies have focused on the possible role of CP190 during cell division (Callaini et al., 1997; Moritz et al., 1998; Oegema et al., 1995, 1997; Riparbelli et al., 1997), our results suggest that centrosomal function and cell division are not affected in *CP190* mutants. This conclusion is supported by independent studies of CP190 function during the cell cycle (Butcher et al., 2004). The main function of CP190 might then be to regulate chromosome-related processes during interphase. Several lines of evidence suggest that this role is related to the function of the *gypsy* insulator: mutations in *CP190* alter *gypsy*-induced phenotypes, CP190 colocalizes with Su(Hw) and Mod(mdg4)2.2 on polytene chromosomes and in diploid cell nuclei, and CP190 associates physically with *gypsy* insulator components in vitro and in vivo. However, the centrosomal localization of CP190 might also be important for its role in the *gypsy* insulator despite being unnecessary for cell

cycle progression. The centrosome could either be a temporary storage site for CP190 during mitosis, or a site for a mitosis-specific modification that could be important for CP190 reassociation with chromosomes later in the cell cycle. The presence of CP190 in the centrosome could also be related to the regulation of the level of this protein in the cell. In fact, it has been shown that some chromatin-binding proteins are targeted to the centrosome for degradation (Chadwick and Willard, 2002). Alternatively, the presence of CP190 at the centrosome might be related to a possible role in the ubiquitin modification pathway. Recent findings have linked BTB/POZ domain proteins to ubiquitin E3 ligase function (Geyer et al., 2003; Pintard et al., 2003), some of which are known to be present at the centrosome (Freed et al., 1999). CP190 may be involved in similar types of interactions as an adaptor for ubiquitin E3 ligases and might target associated insulator proteins to the centrosome during mitosis for ubiquitination and/or degradation, which in turn may be required for properly reestablishing chromosome domain boundaries after mitosis.

Experimental Procedures

Fly Culture and Genetic Screens

For the chemical mutagenesis screen, three-day-old males of the genotype *y^2 w $omb^{P1\text{-}D11}$ ct^6; mod(mdg4)^{T6} e* were starved for 30 min and fed with an EMS-sucrose solution (2.5 mM ethane methyl sulfonate, 10% sucrose) overnight. Treated males were crossed to females of the same genotype and grown at 29°C. Approximately 20,000 F1 offspring were examined for alterations of the *gypsy*-induced phenotypes caused by the *y^2*, *$omb^{P1\text{-}D11}$*, and *ct^6* mutations (Jack, 1985; Parkhurst and Corces, 1986; Tsai et al., 1997), and those with altered phenotypes were backcrossed for amplification. Chromosome-carrying mutations were identified by segregation tests against dominant balancer markers. Established lines were kept at 25°C. The isolated mutations could be placed in one of three different complementation groups. The first group consisted of four new alleles of *su(Hw)*. The second group consisted of three alleles of a gene named *En(mod)11-5*; this gene maps to the third chromosome and has not been characterized further. The third group consisted of five different alleles of *En(mod)4-1*, three of which are discussed in this manuscript. The genetic location of *En(mod)4-1* was determined by recombination and mapped to 3-56.2. P element-tagged *En(mod)4-1* alleles were generated by mobilizing the P element present in the *$MRG15^{P[lacWJ6A3}$* mutation. *En(mod)4-1* mutations affect the coding region of the previously characterized *CP190* gene as indicated in the Results section. *$CP190^{H4\text{-}1}$* is a hypomorphic allele with no obvious morphological defects. Flies carrying this allele are semilethal, move much slower than wt, and cannot fly. The *$CP190^{H53\text{-}2}$* and *$CP190^{H31\text{-}2}$* alleles are late pupal lethal. Some escapers develop into adults that move extremely slow, cannot fly, and die soon after eclosing. Their wings extend slightly outward to about 15 degrees and very often fail to extend and/or contain fluid between the two wing layers. The combination of alleles *$CP190^{P1}$/$CP190^{P11}$* behaves as a null mutation with early pupal lethality. Those flies never develop into pharate adults and show no obvious morphological defects.

Antibody Preparation and Immunocytochemistry

In order to generate CP190 protein for antibody production, the EST clone LD02352 (Research Genetics) containing the coding region of CP190 was amplified with 5′-CGCACCCTCGAGAACGTTAATCG CCAG-3′ and 5′-ATTCGCGCCGAGCTCGAGTGCGTTTATCTGCTG ACC-3′ primers. The resulting fragment was cloned into the pET15B vector (Novagen). The encoded His-CP190dBTB fusion protein, lacking the BTB/POZ domain, was expressed in BL21 cells, purified by His-Bind chromatography, and used to immunize rabbits and rats by standard procedures. Rat anti-Mod(mdg4)2.2 antiserum, rabbit anti-Mod(mdg4) antiserum, and rat anti-Su(Hw) antiserum were reported previously (Gerasimova et al., 1995; Mongelard et al., 2002). Monoclonal mouse anti-γ-tubulin was purchased from Sigma, rabbit anti-phospho-histone H3 antiserum was purchased from Upstate, and rabbit anti-centrosomin antibody was a gift of Dr. T.C. Kaufman. Immunolocalization of proteins on polytene chromosomes and imaginal disc cells were performed as described previously (Gerasimova et al., 2000). All antibodies were used at 1:200 dilution.

Immunoprecipitation and Yeast Two-Hybrid Assays

All steps were performed at 4°C. Mixed stage embryos (0.6 g) were lysed by sonication in 6 ml of ice-cold PBSMT (2.5 mM $MgCl_2$, 3 mM KCl, and 0.3% Triton X-100 in PBS) plus protease inhibitors (1 mM PMSF and Complete protease inhibitor tablet cocktail [Roche]). Lysates were clarified by centrifugation at 16,000 × g for 10 min. Packed Protein A Sepharose (20 μl) was washed three times in PBSMT, added to 6 mg lysate, and raised to 1 ml with PBSMT. Rabbit polyclonal anti-CP190 serum (9 μl) or preimmune serum was added and incubated overnight with agitation. Beads were washed three times with 1 ml PBSMT and once with 1 ml PBS. Sample buffer (20 μl 1 × SDS) was added to samples and boiled for 5 min. Proteins in the precipitate and total lysate (20 μg) were resolved by 7.5% SDS-PAGE and transferred to nitrocellulose in 10 mM CAPS (pH 11) and 1% methanol for 45 min at 400 mA. Blots were probed with rat-anti-CP190 at 1:10,000, rat-anti-Su(Hw) at 1:5000, and rat-anti-Mod(mdg4)2.2 at 1:5000. Plasmids and procedures for the yeast two-hybrid assay were described previously (Ghosh et al., 2001).

Electrophoretic Mobility Shift Assay

For in vitro protein expression, full-length *CP190* cDNA from EST clone LD02352, full-length *su(Hw)* cDNA, and *mod(mdg4)2.2* cDNAs (Ghosh et al., 2001) were inserted into the pCS2+ vector (Turner and Weintraub, 1994) under the control of the SP6 promoter. The gypsy300 fragment containing eight copies of the Su(Hw) consensus binding site was described previously (Spana and Corces, 1990). The y454 fragment was amplified from adult fly genomic DNA by PCR by using the primer pair pr5 and pr6 (Golovnin et al., 2003). The sub-454 fragment was amplified by PCR by using primers pr6 and 5′-TCACTTTAGTTTTCTGCAAG-3′. Purified DNA fragments were labeled with T4 polynucleotide kinase and γ-^{32}P-ATP as described (Spana and Corces, 1990). EMSAs were performed in a 10 μl volume. CP190, Su(Hw), and Mod(mdg4)2.2 proteins were expressed in the TNT-reticulolysate system (Promega). For each binding reaction, 1.5 μl of TNT-expressed protein mixture was incubated with ^{32}P-labelled gypsy300 or y454 fragments (about 100,000 cpm) and unlabelled competitor in BS buffer (10 mM DTT, 100 ng poly(dIdC), 15 mM HEPES [pH7.6], 100 mM KCl, 5 mM $MgCl_2$, 25 μM $ZnCl_2$, and 5% glycerol) at room temperature for 15 min and then on ice for an additional 15 min. The binding reactions were separated by 5% polyacrylamide gel in 0.5× TBE buffer. The gel was run at 15 V/cm at 4°C for 4–6 hours and dried before autoradiography.

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