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Coordinated Control of dCTCF and *gypsy* Chromatin Insulators in *Drosophila*

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SUMMARY

CTCF plays a central role in vertebrate insulators and forms part of the *Fab-8* insulator in *Drosophila*. dCTCF is present at hundreds of sites in the *Drosophila* genome, where it is located at the boundaries between bands and interbands in polytene chromosomes. dCTCF colocalizes with CP190, which is required for proper binding of dCTCF to chromatin, but not with the other *gypsy* insulator proteins Su(Hw) or Mod(mdg4)2.2. Mutations in the *CP190* gene affect *Fab-8* insulator activity, suggesting that CP190 is an essential component of both *gypsy* and dCTCF insulators. dCTCF is present at specific nuclear locations, forming large insulator bodies that overlap with those formed by Su(Hw), Mod(mdg4)2.2, and CP190. The results suggest that Su(Hw) and dCTCF may be the DNA-binding components of two different subsets of insulators that share CP190 and cooperate in the formation of insulator bodies to regulate the organization of the chromatin fiber in the nucleus.

INTRODUCTION

Insulators are DNA regulatory sequences that preclude functionally distinct chromatin domains from improperly interacting with each other. Based on their properties and mechanism of action, these sequences can be classified into barrier insulators, when their role is to stop the spreading of heterochromatin, or enhancer-blocking insulators, when they interfere with enhancer-promoter communication (Gaszner and Felsenfeld, 2006). Enhancer-blocking insulators have been found in a variety of organisms but have been studied in detail in vertebrates and *Drosophila* (Capelson and Corces, 2004).

The best characterized vertebrate insulator is cHS4, a sequence found in the 5′ region of the chicken β-globin locus (Felsenfeld et al., 2004). This insulator displays both barrier and enhancer-blocking activities that reside in different sequence elements. The enhancer-blocking activity is associated with binding sites for the zinc finger protein CTCF (Bell et al., 1999). In fact, most characterized vertebrate enhancer-blocking insulators are composed of sequences that interact with CTCF, and this protein is essential for their function. CTCF insulators play important roles in genomic imprinting at the Igf2/H19 locus (Bell and Felsenfeld, 2000; Hark et al., 2000) as well as in X chromosome inactivation (Chao et al., 2002). In humans, CTCF-binding sites at the DM1 locus form a functional insulator and loss of binding of CTCF may contribute to the phenotype of myotonic dystrophy (Filippova et al., 2001). These observations suggest an important role for enhancer-blocking insulators in the regulation of gene expression.

Contrary to vertebrates, which seem to rely on CTCF as the main source of insulator activity, *Drosophila* has been shown to possess a rich variety of enhancer-blocking insulators utilizing different proteins to accomplish their function (Capelson and Corces, 2004). The *scs* and *scs′* sequences were first found to flank the *hsp70* heat shock puffs; these sequences interact with the ZW5 and BEAF-32 proteins, respectively, which are essential for their insulator activity (Gaszner et al., 1999; Zhao et al., 1995). *Fab-8*, a second insulator element first found in the *Drosophila Abdominal-B* (*Abd-B*) gene of the bithorax complex, has been shown to interact with the *Drosophila* homolog of CTCF, dCTCF (Moon et al., 2005). A third enhancer-blocking insulator named SF1 was first found in the *Scr-ftz* intergenic region and is made up of binding sites for the GAGA protein (Belozerov et al., 2003). The variety of sequences and associated proteins present in *Drosophila* enhancer-blocking insulators in contrast to the widely present CTCF insulator found in vertebrates raises the question of whether vertebrates have lost most insulators except for CTCF or whether other types of insulators remain to be identified in vertebrates.

The *gypsy* enhancer-blocking insulator of *Drosophila*, first found in the *gypsy* retrotransposon, is composed of several proteins that play distinct roles in regulating insulator function (Capelson and Corces, 2004). Su(Hw) is an 11 zinc finger DNA-binding protein that interacts with insulator sequences; mutations in the *su(Hw)* gene cause female sterility but do not result in lethality (Harrison

et al., 1993). Mod(mdg4)2.2 is a BTB domain protein that interacts with Su(Hw). The *mod(mdg4)* gene encodes ~30 different isoforms that arise by alternative *cis*- and *trans*-splicing (Buchner et al., 2000; Labrador and Corces, 2003); null mutations in the gene result in lethality, but mutations affecting the Mod(mdg4)2.2 isoform are viable and show defects in *gypsy* insulator function (Gerasimova et al., 1995). CP190 is a third protein component of the *gypsy* insulator. It contains a BTB domain as well as three zinc fingers, and it interacts with Su(Hw) and Mod(mdg4)2.2. CP190 binds DNA, but it does not interact directly with *gypsy* insulator sequences present in the *gypsy* retrotransposon, where it is recruited through interactions with Su(Hw) and Mod(mdg4)2.2 instead. Mutations in the *CP190* gene are lethal (Pai et al., 2004).

Although enhancer-blocking insulators interfere with enhancer-promoter interactions, this may not be their primary function in nuclear biology. Instead, their primary role may be to establish independent domains of gene expression such that regulatory sequences in one domain cannot affect the expression of genes located in a different domain. Their effect on enhancer-promoter interactions may then be a byproduct of this primary role. This conclusion comes in part from the observation that enhancer-blocking insulators can form chromatin loops. For example, the *scs* and *scs′* insulators have been shown to pair in vivo and, as a consequence, loop out the intervening chromatin fiber (Blanton et al., 2003). The chicken CTCF insulator can also mediate loop formation, and at least some of these loops attach to the nucleolus through the nucleophosmin protein (Yusufzai et al., 2004). In the case of the *gypsy* insulator, interactions between multiple insulator sites form structures called insulator bodies that in turn attach to the nuclear lamina (Byrd and Corces, 2003). The formation of these bodies requires, in addition to the three core proteins Su(Hw), Mod(mdg4)2.2, and CP190, functional RNA silencing machinery. The Rm62 protein is a putative RNA helicase that may interact with small RNAs to regulate insulator function (Lei and Corces, 2006). The *gypsy* insulator can also be regulated by ubiquitination and sumoylation of some of the core proteins by the dTopors ubiquitin E3 ligase (Capelson and Corces, 2005, 2006).

The three core protein components of the *gypsy* insulator colocalize in the insulator bodies, but their distribution in polytene chromosomes, which is presumably a representation of their genomic distribution in the chromatin of interphase cells, does not show an exact overlap. Insulator proteins in *Drosophila* are present at the boundaries between bands (containing condensed chromatin and nontranscribed genes) and interbands (containing decondensed chromatin and actively transcribed genes). Bands and interbands may thus represent chromatin domains defined and maintained by insulators (Labrador and Corces, 2002). CP190 is present at all or most band/interband boundaries in polytene chromosomes, whereas Su(Hw) and Mod(mdg4)2.2 colocalize with each other precisely but are present at only a subset of the CP190 sites (Pai et al., 2004). This distribution suggests that CP190 may be a general component of a widespread insulator present at every band/interband boundary. CP190 may use the Su(Hw) DNA-binding protein as a tether to be recruited to a subset of these insulators but may use other DNA-binding proteins or bind DNA itself to be recruited to the rest of the insulators where Su(Hw) is absent. This hypothesis is also supported by the observation that mutations affecting the Su(Hw) or Mod(mdg4)2.2 proteins are viable, but mutations affecting CP190 are lethal, suggesting a more general function for this protein (Pai et al., 2004).

Given the widespread role of CTCF in insulator function in vertebrates and its structural similarity to Su(Hw), we decided to investigate the possibility that dCTCF cooperates with the *gypsy* insulator in genome organization and regulation of gene expression. Here we show that this is indeed the case. dCTCF interacts with CP190 at a subset of genomic sites different from those occupied by Su(Hw), but it is present at the same insulator bodies formed by Su(Hw). The results suggest that Su(Hw) and dCTCF are components of two different subsets of insulators that cooperate in the formation of larger insulator bodies to regulate the organization of the chromatin fiber in the nucleus of *Drosophila* cells.

RESULTS

Mutations in *dCTCF* Affect the Function of the *Fab-8* Insulator

The *Fab-8* insulator contains two binding sites for the dCTCF protein. Mutations in the sites that disrupt binding of dCTCF also affect the ability of *Fab-8* to interfere with enhancer-promoter interactions (Moon et al., 2005). To directly test the role of dCTCF in the function of the *Fab-8* insulator, we made mutations in the *dCTCF* gene. A strain carrying an insertion of a P transposable element in the first noncoding exon of the *dCTCF* gene, *P{EPgy2}CTCF*^{EY15833}, produces reduced levels of dCTCF protein (Figure 1A) but is viable and lacks any observable phenotype. In order to produce null mutations in the *dCTCF* gene, we mobilized the P element using the *y*^{1}*w*^{1118}*; P{Δ2-3}Sb/TM6* strain as a source of transposase. The P element in *P{EPgy2}CTCF*^{EY15833} harbors the *yellow* gene reporter located adjacent to the 5′ region of *dCTCF* and the *white* gene reporter adjacent to the 3′ region. In order to obtain P element transpositions that result in deletion of the *dCTCF* gene, we selected for flies displaying a *y*$^{+}$ *w*$^{-}$ phenotype. These deletions extend from the site of the P element insertion in the first exon toward the 3′ end of the *dCTCF* gene and complement mutations in the *quemao* gene; *quemao* is adjacent to the 3′ of *dCTCF*, suggesting that the new mutations only affect *dCTCF*. Figure 1A shows a western analysis of protein extracts from pupae carrying three different *dCTCF* deletion mutations: *dCTCF*$^{y+1}$, *dCTCF*$^{y+2}$, and *dCTCF*$^{y+6}$. All three strains show no detectable levels of dCTCF, which, together with the fact that they carry a deletion of the gene, suggests that the mutations are null. All three strains

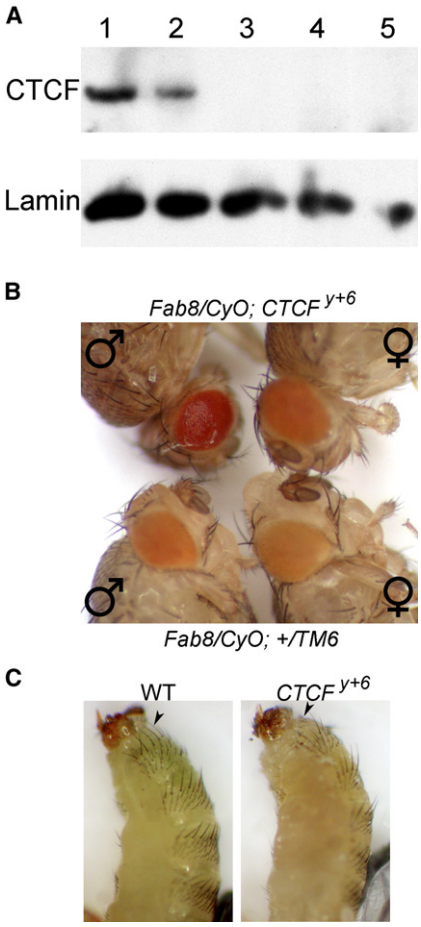

Figure 1. Mutations in the *dCTCF* Gene Affect *Fab-8* Insulator Activity

(A) Protein extracts from larvae of various fly strains were subjected to western analysis. Lane 1, wild-type OreR; lane 2, *P{EPgy2}-CTCF^{EY15833}*; lane 3, *dCTCF^{y+1}*; lane 4, *dCTCF^{y+2}*; lane 5, *dCTCF^{y+6}*.

(B) Eyes of male and female flies of the genotypes *Fab-8$^{60.39.2}$/CyO; dCTCF^{y+6}* (top) and *Fab-8$^{60.39.2}$/CyO; +/TM6* (bottom).

(C) Abdomens of pharate adult flies pulled from the pupal cases. The left panel shows the abdomen of a wild-type fly, whereas the right panel shows the abdomen of a fly homozygous for the *dCTCF^{y+6}* allele; the arrow points to an additional abdominal segment posterior to A6.

display late pupal lethality, although some individuals survive to the pharate adult stage and can be pulled from the pupal case for observation.

In order to test the role of dCTCF in the activity of the *Fab-8* insulator, we examined the phenotype of fly strains *Fab-8$^{60.39.2}$/CyO* or *Fab-8$^{60.71.1}$/CyO* carrying a transgene that includes the *Fab-8* insulator positioned between the eye enhancer and the coding region of the *white* gene. The presence of *Fab-8* at this position results in a nonfunctional enhancer and flies with light yellow eyes (Figure 1B). When the strain is homozygous for a mutation in the *dCTCF* gene, the color of the eye becomes darker. This effect is more pronounced in males, in which the eye color becomes close to wild-type (Figure 1B). This result suggests that dCTCF indeed plays a role in the function of the *Fab-8* insulator. If this is the case, mutations in the *dCTCF* gene should display homeotic transformations affecting the abdominal segments. Results shown in Figure 1C indicate that this is the case. Male flies homozygous for the *dCTCF^{y+6}* mutation display an additional abdominal A7 segment posterior to A6, whereas the A7 and A8 segments are not visible in wild-type males; this phenotype is similar to that observed in Abd-B class I mutants (Estrada et al., 2002).

dCTCF Is Present at a Subset of *gypsy* Insulator Sites

The *Fab-8* insulator is present in the bithorax complex in the 5′ regulatory region of the *Abd-B* gene (Barges et al., 2000). To determine whether dCTCF plays a general role in the *Drosophila* genome rather than a specific role in the regulation of *Abd-B*, we examined the distribution of this protein in polytene chromosomes from third instar larvae. Figure 2A shows that dCTCF is present at many sites and that it is localized at the boundaries between the darkly stained DAPI bands and the light interbands. This distribution correlates with a possible general role of dCTCF in the function of insulators present throughout the *Drosophila* genome.

The *gypsy* insulator is also present at several hundred sites in polytene chromosomes at the boundaries between bands and interbands (Pai et al., 2004). To test the existence of a possible relationship between the dCTCF and *gypsy* insulators, we examined the distribution in polytene chromosomes of the Su(Hw) *gypsy* insulator protein and dCTCF. The two proteins localize to nonoverlapping sets of sites on the chromosomes (Figure 2B). A similar result was obtained with Mod(mdg4)2.2, a second component of the *gypsy* insulator (Figure 2C). Su(Hw) and Mod(mdg4)2.2 overlap at every chromosome site; a third protein, CP190, overlaps with the other two but is also present at additional sites (Pai et al., 2004). We then tested whether CP190 and dCTCF colocalize in polytene chromosomes. Figure 2D shows that CP190 is present at every dCTCF site in the chromosomes, although not all CP190 sites contain dCTCF. This result suggests that CP190 may be a component of both the *gypsy* and dCTCF insulators.

dCTCF Interacts with the *gypsy* Insulator Protein CP190

To further test the possibility that CP190 is a component of dCTCF-containing insulators, we determined the presence of CP190 at the site of the *Fab-8* insulator in the bithorax complex. To this end, we carried out FISH with a DNA probe containing *Fab-8* insulator sequences simultaneously with immunolocalization using α-dCTCF antibodies. Figures 3A and 3B show that, within the limits of resolution afforded by polytene chromosomes, both dCTCF and CP190 localize to the cytological region where *Fab-8* is present. In order to further test whether dCTCF and CP190 are part of a complex, we carried out affinity chromatography using antibodies to the CP190 protein.

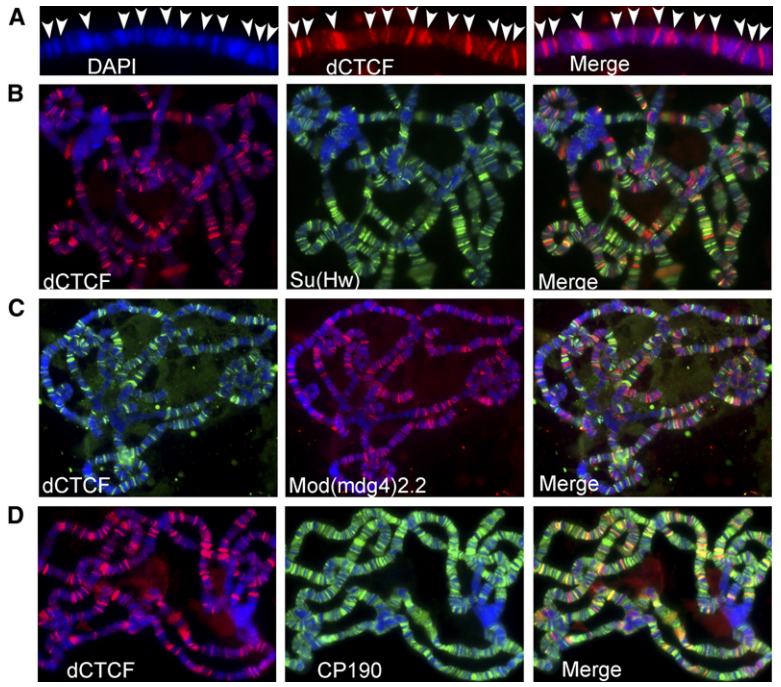

Figure 2. Distribution of dCTCF on Polytene Chromosomes of Wild-Type Third Instar Larvae in Relation to DNA and *gypsy* Insulator Proteins

DNA is stained with DAPI (blue) in all immunofluorescence images. Immunolocalization of the following is shown.

(A) dCTCF (red) on polytene chromosomes. Arrows point to the location of dCTCF sites at the boundary of DAPI band and interbands.

(B) dCTCF (red) and Su(Hw) (green) on polytene chromosomes.

(C) dCTCF (green) and Mod(mdg4)2.2 (red) on polytene chromosomes.

(D) dCTCF (red) and CP190 (green) on polytene chromosomes.

We have previously used this strategy to isolate a protein complex containing CP190 (Lei and Corces, 2006). CP190, Su(Hw), Mod(mdg4)2.2, and dCTCF each associate with the α-CP190, but not preimmune, column (Figure 3C). Because Su(Hw) and Mod(mdg4)2.2 do not overlap with dCTCF in polytene chromosomes, this result suggests that CP190 may form two different complexes, one containing Su(Hw)/Mod(mdg4)2.2 and one including dCTCF. To further test this hypothesis, we carried out chromatin immunoprecipitation (ChIP) analyses in *Drosophila* Kc cells using primers in the Fab-8 insulator as well as adjacent sequences as a control. Figure 3D shows that both dCTCF and CP190 are present at the *Fab-8* insulator but absent from adjacent sequences, whereas Su(Hw) is not present in *Fab-8.* On the other hand, both CP190 and Su(Hw) are present at the *gypsy* insulator whereas dCTCF is absent. These results support the conclusion that dCTCF and CP190 can form an active insulator without Su(Hw) and, therefore, that CP190 is part of two different insulators, *gypsy* and *Fab-8.*

To more directly assess whether CP190 and dCTCF form a complex in the *Fab-8* family of insulators, we tested whether the two proteins interact using the yeast two-hybrid assay. Figure 3E shows the result of this experiment. CP190 and dCTCF appear to interact, and this interaction is similar to that observed between CP190 and Su(Hw). The interaction between dCTCF and CP190 does not take place through the BTB domain (residues 1–140) of this protein; rather, the Glu-rich carboxy-terminal region of CP190, between amino acid residues 605 and 1103, interacts with dCTCF (Figure 3E). If dCTCF and CP190 form part of a complex located at putative dCTCF insulator sites throughout the genome as well as the *Fab-8* insulator, CP190 may be necessary for the proper function of this insulator. To test this possibility, we analyzed the effect of mutations in the *CP190* gene on the activity of the *Fab-8* insulator located between the *white* gene eye enhancer and promoter in the *Fab-8*$^{60.39.2}$ transgene. Figure 3F shows that this is the case. Mutations in *CP190* (*CP190*$^{4-1}$/*CP190*$^{H31-2}$) partially reverse the phenotypic effect of *Fab-8* on the expression of the *white* gene in the *Fab-8*$^{60.39.2}$/*CyO* strain (a similar result was obtained with *Fab-8*$^{60.71.1}$/*CyO*, data not shown), suggesting that CP190 is required for the proper function of *Fab-8*. On the other hand, mutations in the Su(Hw) protein, which is not present at *Fab-8* or other dCTCF sites, have no effect on the expression of the same *white* transgene (Figure 3F). Because CP190 is present at all other genomic sites of dCTCF localization in addition to *Fab-8*, it is possible that these sites also represent dCTCF insulators and that CP190 is required for the function of dCTCF throughout the genome.

dCTCF and CP190 Cooperate with Each Other to Bind to Chromatin

dCTCF and CP190 are zinc finger proteins and, in principle, have the ability to bind DNA. In vertebrates, CTCF has been shown to interact with a GC-rich loosely conserved sequence (Kim et al., 2007), and CP190 binds DNA with low affinity and specificity (Pai et al., 2004). In order to determine the role of dCTCF and CP190 in the dCTCF insulator, we analyzed the ability of each of these proteins to bind to polytene chromosomes in the absence of one another. Mutations in *dCTCF* result in a reduced number of CP190 sites in polytene chromosomes, whereas the binding of Rm62, used as a control, is not affected (Figure 4A). Although the total number of CP190

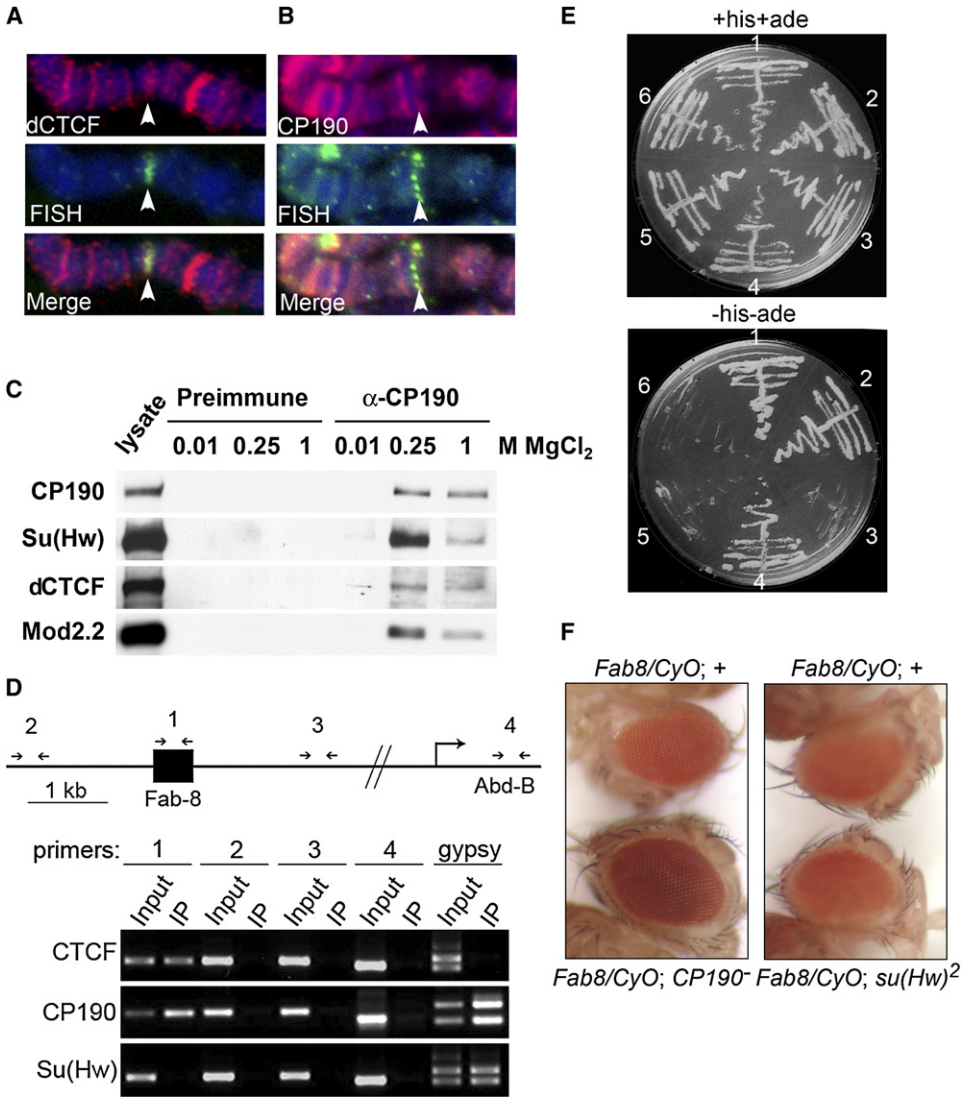

Figure 3. Interaction of dCTCF with *gypsy* Insulator Proteins

(A) dCTCF is present at the cytological location of the *Fab-8* insulator. Red indicates the immunolocalization of dCTCF, green indicates the localization of *Fab-8* sequences by FISH, and DNA is stained with DAPI in blue. Arrow points to the location of *Fab-8*.

(B) CP190 is present at the cytological location of the *Fab-8* insulator. Red indicates the immunolocalization of CP190, green indicates the localization of *Fab-8* sequences by FISH, and DNA is stained with DAPI in blue. Arrow points to the location of *Fab-8*.

(C) Western analysis of control preimmune and α-CP190 column immunoaffinity purifications to verify the presence of CP190, Su(Hw), dCTCF, and Mod(mdg4)2.2 in α-CP190 eluates at the indicated concentrations of $MgCl_2$.

(D) Chromatin immunoprecipitation analysis of the *Fab-8* insulator. The top part of the panel is a diagram of the 5′ region of the *Abd-B* gene, including the *Fab-8* insulator. Numbered arrows above the diagram indicate the location of primers used in the PCR reactions. The lower portion of the panel shows the result of the electrophoretic analysis of the PCR products after immunoprecipitation with antibodies to the proteins indicated on the left. For each primer pair, an input and IP lane are shown. Also shown is a control using primers to the insulator present in the *gypsy* retrotransposon.

(E) Growth of yeast strains expressing dCTCF and Su(Hw), CP190, or control on nonselective (+histidine +adenine) (top) or selective (−histidine −adenine) (bottom) media. Sector 1, Su(Hw)AD-CP190BD. Sector 2, dCTCFAD-CP190BD. Sector 3, dCTCFAD-CP190-BTB domainBD. Sector 4, dCTCFAD-CP190-carboxytermBD. Sector 5, dCTCFAD-emptyBD. Sector 6, CP190BD-emptyAD.

(F) (Left) Eyes of male flies of the genotype *Fab-8*$^{60.39.2}$*/CyO;+/+* (top) and *Fab-8*$^{60.39.2}$*/CyO; CP190*$^{4\text{-}1}$*/CP190*$^{H31\text{-}2}$ (bottom). (Right) Eyes of male flies of the genotype *Fab-8*$^{60.39.2}$*/CyO;+/+* (top) and *Fab-8*$^{60.39.2}$*/CyO; su(Hw)*^{2} (bottom).

sites is lower than in wild-type chromosomes, those sites present in the *dCTCF* mutant appear to be of a similar intensity to those in wild-type, suggesting that lack of dCTCF affects the binding of CP190 at only a subset of the total number of CP190 sites. The sites where CP190 is still present in the chromosomes of *dCTCF* mutants are presumed to be those at which Su(Hw) overlaps with CP190. On the other hand, mutations in *CP190* completely

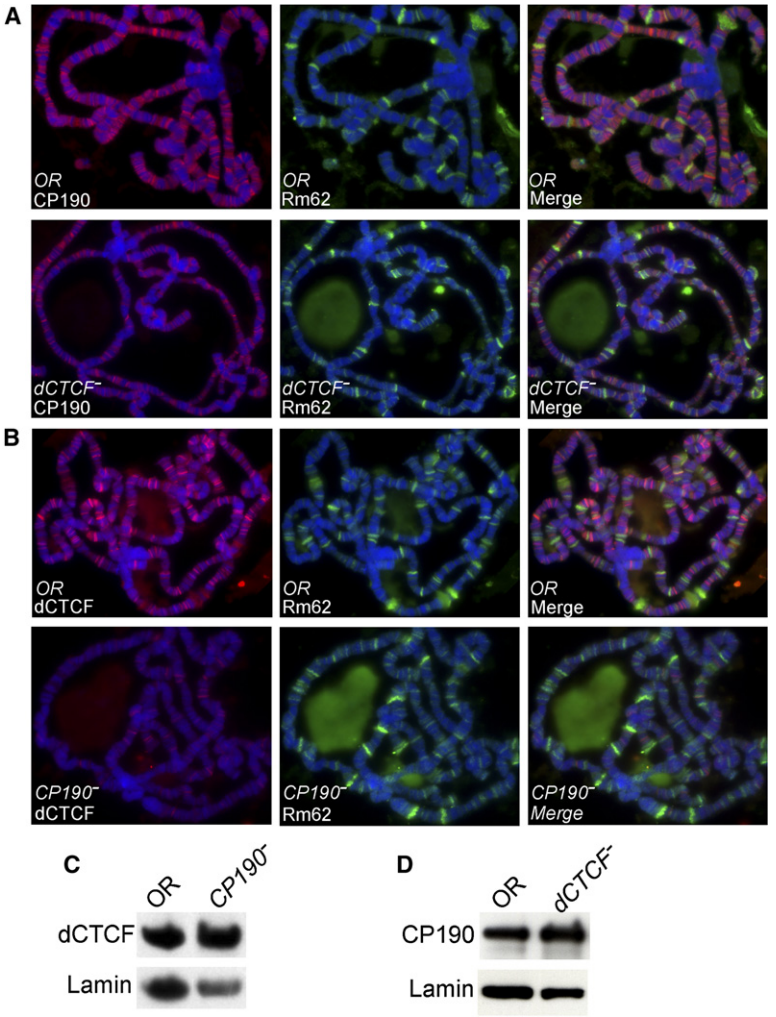

Figure 4. Levels of dCTCF in Wild-Type and Mutant Flies

DNA is stained with DAPI (blue) in all panels.

(A) (Top row) Immunolocalization of CP190 (red) and of Rm62 (control, green) in polytene chromosomes from wild-type OreR larvae. (Bottom row) Immunolocalization of CP190 (red) and of Rm62 (control, green) in polytene chromosomes from $dCTCF^{y+6}/dCTCF^{y+6}$ mutant larvae.

(B) (Top row) Immunolocalization of dCTCF (red) and of Rm62 (control, green) in polytene chromosomes from wild-type larvae. (Bottom row) Immunolocalization of dCTCF (red) and of Rm62 (control, green) in polytene chromosomes from $CP190^{P1}/CP190^{P11}$ mutant larvae.

(C) Western analysis of protein extracts from wild-type (OR) and $CP190^{P1}/CP190^{P11}$ larvae. Lamin was used as a control.

(D) Western analysis of protein extracts from wild-type (OR) and $dCTCF^{y+6}/dCTCF^{y+6}$ larvae. Lamin was used as a control.

abolish binding of dCTCF at all sites in polytene chromosomes, suggesting that CP190 is essential for proper binding of dCTCF to DNA (Figure 4B). The same results were obtained when the JIL-1 kinase or the PEP zinc finger protein was used as control instead of Rm62 (data not shown).

The absence of dCTCF protein in polytene chromosomes of larvae carrying a mutation in the *CP190* gene could be due to an effect on the expression of the *dCTCF* gene. An effect on *CP190* transcription could also be an explanation for the reduced number of CP190 sites in chromosomes of *dCTCF* mutant larvae. To test whether this is the case, we carried out western analyses of protein extracts obtained from larvae bearing each of the two mutations, using lamin as a loading control. Levels of dCTCF appear to be unchanged in larvae carrying a mutation in *CP190* compared to wild-type (Figure 4C). Likewise, larvae mutant in the *dCTCF* gene accumulate similar levels of CP190 than wild-type larvae (Figure 4D). These results suggest that the observed differences in the presence of the dCTCF and CP190 proteins in polytene chromosomes of mutant flies are due to decreased binding to DNA rather than lower levels of the proteins, suggesting that the two proteins cooperate in some fashion in their interaction with DNA.

dCTCF May Be the DNA-Binding Component in a Subset of Insulators that Share the CP190 Protein

The Su(Hw) protein component of the *gypsy* insulator and the dCTCF component of the *Fab-8* insulator are both present at the boundaries between bands and interbands in polytene chromosomes but the two proteins do not overlap, suggesting that their sites of localization are different (Figure 2). Nevertheless, both sets of sites share the CP190 protein (Figures 5A and 5B). To further analyze the pattern of overlap between these proteins, we carried out immunolocalization experiments in which the CP190 protein was detected using FITC-conjugated secondary antibodies whereas Su(Hw) and dCTCF were simultaneously detected using secondary antibodies conjugated to Texas red (Figure 5C). When Su(Hw) and dCTCF are visualized together, the number of sites where CP190 is present alone decreases considerably when compared to the results obtained with each protein separately

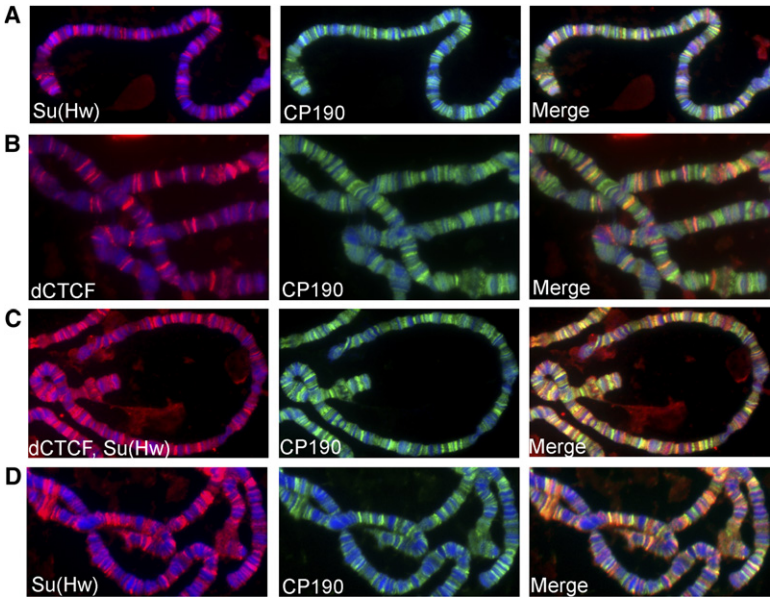

Figure 5. Colocalization of dCTCF and *gypsy* Insulator Proteins on Polytene Chromosomes from Wild-Type Larvae

DNA is stained with DAPI (blue) in all panels.

(A) Su(Hw) (red) and CP190 (green) on polytene chromosomes.

(B) dCTCF (red) and CP190 (green) on polytene chromosomes.

(C) dCTCF plus Su(Hw) (red) and CP190 (green) on polytene chromosomes.

(D) Su(Hw) (red) and CP190 (green) on polytene chromosomes of $dCTCF^{y+6}$ mutants.

(Figure 5). An approximate quantification of the sites of overlap suggests that Su(Hw) and dCTCF are each present at one-third of all sites of CP190 localization in the genome and, therefore, approximately one-third of CP190 sites are not occupied by either the Su(Hw) or dCTCF proteins. These results support a model in which each of these two proteins may be part of a distinct insulator and that the dCTCF and *gypsy* insulators may be subclasses of a more general insulator defined by the CP190 protein. A prediction of this model is that, in *dCTCF* mutants, the extent of overlap between Su(Hw) and CP190 should be more extensive than in wild-type flies, as the sites where CP190 and dCTCF are normally present should be absent in these mutants. Figure 5D shows that this is indeed the case, and that Su(Hw) is able to bind normally to the chromatin in the absence of dCTCF, supporting the conclusion that *gypsy* and dCTCF insulators share the CP190 protein but are independent. Figure 5D also shows the existence of sites in which CP190 does not overlap with Su(Hw), suggesting the existence of at least a third protein required for proper binding of CP190 to a third subclass of insulators that lack Su(Hw) and dCTCF. A second prediction of the model is that mutation of the *su(Hw)* and *dCTCF* genes simultaneously will have an additive effect in the phenotype with respect to single mutations in each of the two genes separately. This is indeed the case. Null mutations in *su(Hw)*, such as $su(Hw)^2$, are viable but display female sterility; null mutants in *dCTCF*, such as $dCTCF^{y+6}$, show late pupal lethality. Flies carrying both mutations die during late larvae/early pupae stages, an earlier stage of development (data not shown).

dCTCF Is Present in the Same Insulator Bodies as *gypsy* Insulator Proteins

The observed genetic interaction between mutations in *su(Hw)* and *dCTCF* is suggestive of parallel or related functions for the two subclasses of insulators. In the case of the *gypsy* insulator, it has been shown that insulator activity correlates closely with the formation of insulator bodies (Capelson and Corces, 2005, 2006), which are sites in the nucleus where several insulator sites come together while causing the chromatin fiber to form loops that are presumed to coincide with independent domains of gene expression (Byrd and Corces, 2003; Gerasimova et al., 2000). To test whether the dCTCF protein is present in insulator bodies with a nuclear distribution similar to those of *gypsy* insulator proteins, we examined the pattern of dCTCF localization in nuclei from diploid imaginal disc cells. Figure 6A shows that dCTCF is present in the nucleus in a pattern similar to that of *gypsy* insulator proteins, suggesting that dCTCF may also form insulator bodies. Interestingly, the pattern of dCTCF localization overlaps perfectly with that of CP190 (Figure 6A). Because CP190 forms insulator bodies where other *gypsy* insulator proteins are also located, this result suggests that dCTCF and *gypsy* insulator bodies may overlap. To further test this possibility, we examined the distribution of a second protein component of the *gypsy* insulator, Mod(mdg4)2.2. Although this protein does not overlap with dCTCF in polytene chromosomes, the two proteins coincide perfectly in nuclei of diploid cells (Figure 6B), supporting the idea that dCTCF and *gypsy* insulator bodies are the same or are located closely in the nucleus.

To further determine the relationship between dCTCF and Mod(mdg4)2.2 at insulator bodies, we analyzed the effect of mutations in the genes encoding the two proteins on insulator body integrity. Mutations in *dCTCF* do not affect the presence of either Mod(mdg4)2.2 or CP190 at insulator bodies (Figure 6C). Similarly, the $mod(mdg4)^{T6}$ mutation, which affects the Mod(mdg4)2.2 isoform, does not affect the distribution of dCTCF or its colocalization with CP190 at insulator bodies. These results suggest

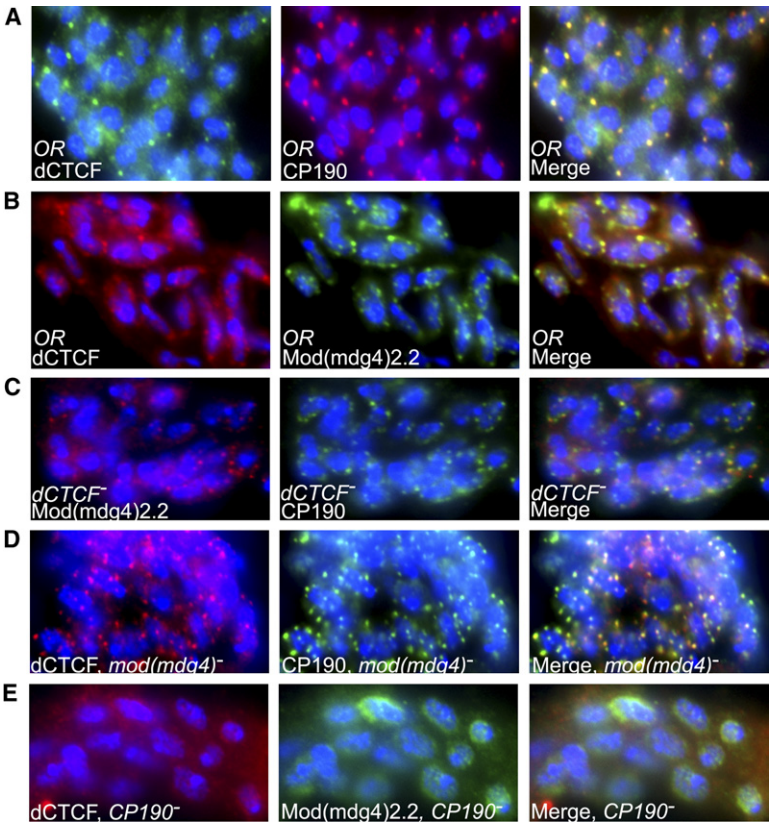

Figure 6. Localization of dCTCF and *gypsy* Insulator Proteins in Diploid Nuclei of Wild-Type and Mutant Larvae

DNA is stained with DAPI (blue) in all images.

(A) Immunolocalization of dCTCF (green) and CP190 (red) in diploid nuclei from imaginal disc cells of wild-type larvae.

(B) Immunolocalization of dCTCF (red) and Mod(mdg4)2.2 (green) in diploid nuclei from imaginal disc cells of wild-type larvae.

(C) Immunolocalization of Mod(mdg4)2.2 (red) and CP190 (green) in diploid nuclei from imaginal disc cells of $dCTCF^{y+6}/dCTCF^{y+6}$ mutant larvae.

(D) Immunolocalization of dCTCF (red) and CP190 (green) in diploid nuclei from imaginal disc cells of $mod(mdg4)^{T6}$ mutant larvae.

(E) Immunolocalization of dCTCF (red) and Mod(mdg4)2.2 (green) in diploid nuclei from imaginal disc cells of $CP190^{P1}/CP190^{P11}$ mutant larvae.

that dCTCF and *gypsy* insulators colocalize in the nucleus but the integrity of dCTCF insulators is not necessary for *gypsy* insulator body formation and vice versa. To the contrary, mutations in *CP190* affect the integrity of the insulator bodies formed by dCTCF and Mod(mdg4)2.2; in the absence of CP190 protein, both dCTCF and Mod(mdg4)2.2 fail to form insulator bodies and have an aberrant cellular localization and dCTCF appears distributed throughout the cytoplasm (Figure 6E). These results suggest that, although dCTCF and *gypsy* insulator bodies are present at the same nuclear location and share the CP190 protein, they can form independently of each other. This observation on the integrity of insulator bodies formed by dCTCF and Mod(mdg4)2.2 in the presence of mutations in either gene correlates well with analyses of these mutations on the function of each insulator. Mutations in Mod(mdg4)2.2 or Su(Hw) affect the function of the *gypsy* insulator, as determined by the phenotypic reversion of *gypsy*-induced mutations such as y^2, but they do not affect the phenotypic effect of the *Fab-8* insulator on the expression of the *white* gene (data not shown). Mutations in *dCTCF* do not affect the function of *gypsy* insulator in *gypsy*-induced mutations (data not shown), but they affect the *Fab-8* insulator. On the other hand, mutations in *CP190* impair the function of both the *gypsy* and dCTCF insulators. These results suggest that Su(Hw)/Mod(mdg4)2.2 and dCTCF form two subfamilies of insulators that share the CP190 protein. Both subfamilies form insulator bodies that colocalize in the nucleus but can form and function independently of each other.

DISCUSSION

Insulators are thought to compartmentalize the genome into independent domains of gene expression by creating chromatin loops between individual insulator sites and physically tethering the chromatin fiber to a fixed nuclear substrate such as the nuclear matrix (Capelson and Corces, 2004). In the case of the *gypsy* insulator, the formation of loops is manifested in the formation of insulator bodies, structures in which multiple individual insulators coalesce in specific nuclear locations (Gerasimova et al., 2000). Formation of large insulator bodies has not been shown for other *Drosophila* or vertebrate insulators. However, the chicken CTCF insulator interacts with the nucleolus through the nucleophosmin protein and CTCF is present in the nuclear matrix fraction, suggesting the need for a nuclear scaffold for its function (Yusufzai and Felsenfeld, 2004; Yusufzai et al., 2004).

In order to address this issue, we analyzed the properties and functional characteristics of the *Drosophila* dCTCF protein. dCTCF in *Drosophila* has been shown to interact with the *Fab-8* insulator in the *Abd-B* gene of the bithorax complex; mutations in the binding site that abolish dCTCF binding also affect *Fab-8* insulator function (Moon et al., 2005). Using antibodies to *Drosophila*

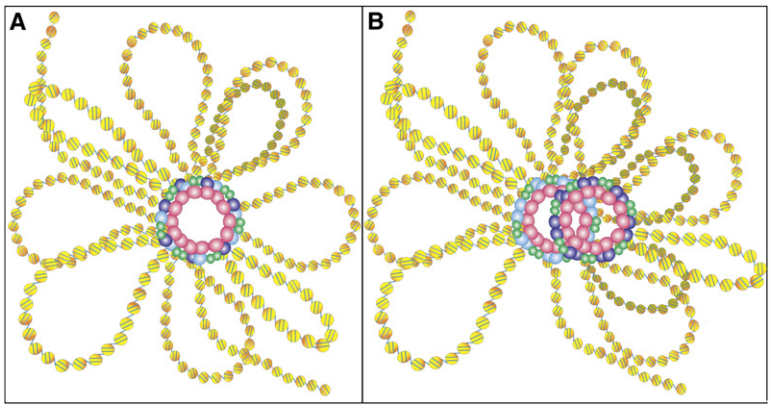

Figure 7. Possible Models to Explain the Formation of Insulator Bodies by dCTCF
In both panels insulator proteins are represented by differently colored ovals: Su(Hw) is shown in blue, Mod(mdg4)2.2 in green, dCTCF in purple, and CP190 in pink.
(A) Su(Hw) and dCTCF bind different insulator sequences but coalesce in the same insulator bodies through interactions between the CP190 protein present in both insulators.
(B) Su(Hw) and dCTCF form independent insulator bodies, but both colocalize in the same region of the nucleus. This may imply the existence of special nuclear compartments where insulator bodies are located.

dCTCF, we find that this protein is located at several hundred sites in polytene chromosomes from third instar larvae, suggesting a general role for dCTCF in nuclear biology rather than a specific function in the regulation of *Abd-B* expression. In addition, we find that dCTCF is located at the borders of bands and interbands in polytene chromosomes. This pattern of localization appears to be a hallmark of insulator proteins in *Drosophila*, suggesting that dCTCF may be part of an insulator with broad function similar to that of CTCF in vertebrates, where CTCF is the main insulator found thus far.

gypsy insulator proteins such as Su(Hw) or Mod(mdg4)2.2 are also present at the boundaries between bands and interbands, but only in a subset of all possible boundaries. This observation prompts the question of whether boundaries that lack Su(Hw)/Mod(mdg4)2.2 contain a different insulator. If bands and interbands correspond to domains of gene expression established or maintained by insulators, and the creation of these domains requires the formation of chromatin loops between adjacent insulators, the proteins present at each insulator site must be able to interact in order to form these loops. Therefore, insulator proteins located at non-*gypsy* insulators should then interact with Su(Hw), Mod(mdg4)2.2, or CP190. A solution to this issue is the finding that, although dCTCF does not colocalize with Su(Hw) or Mod(mdg4)2.2 in polytene chromosomes, sites of dCTCF also contain CP190. Therefore, CP190 may be a component of both *gypsy* and dCTCF insulators that can mediate interactions between the two. Support for the conclusion that CP190 is a component of the dCTCF insulator comes from the observation that dCTCF copurifies with CP190 and that the two proteins are present at the *Fab-8* insulator and interact in the yeast two-hybrid assay. These interactions have in vivo significance, as mutations in CP190 affect the function of the *Fab-8* insulator. These results suggest that, although dCTCF and Su(Hw)/Mod(mdg4)2.2 are not present at the same chromosomal locations, they share the CP190 insulator protein. Su(Hw) and dCTCF are very similar proteins, containing 11 zinc fingers that can mediate DNA binding, and they both interact with CP190. It is possible that the zinc fingers in CP190 mediate protein interactions rather than binding to DNA, and that this protein is tethered to DNA through interactions with Su(Hw) or dCTCF. This is supported by the fact that CP190 is absent in two different subsets of chromosomal locations in larvae carrying mutations in either the *su(Hw)* or *dCTCF* genes, and suggests that the Su(Hw) (*gypsy*) and dCTCF insulators are two subfamilies of a much larger class of insulators whose defining characteristic is the presence of CP190. In agreement with this model, mutations in *su(Hw)* affect the function of the *gypsy* insulator but not *Fab-8*, whereas mutations in *dCTCF* affect *Fab-8* but not *gypsy* and mutations in *CP190* affect both. Nevertheless, the role of CP190 in the dCTCF insulator appears more complex than to passively be recruited to DNA by this protein. In a *CP190* mutant, Su(Hw) binds normally to polytene chromosomes (Pai et al., 2004) but dCTCF is absent, suggesting that CP190 is necessary for proper binding of dCTCF. It would be interesting to examine whether CTCF in vertebrates also requires a CP190-like protein for proper DNA binding.

The formation of chromatin loops representing independent domains of gene expression can be visualized in the case of the *gypsy* insulator by the formation of insulator bodies that attach to the nuclear matrix. These structures represent nuclear sites where multiple individual insulators coalesce. Insulator bodies have not been detected for the CTCF protein in vertebrate cells, although CTCF is present in the nuclear matrix fraction and may attach the chromatin fiber to this structure (Yusufzai and Felsenfeld, 2004). We find that in *Drosophila* the dCTCF protein forms insulator bodies that appear similar to those formed by *gypsy* insulator proteins in number and distribution, suggesting that the dCTCF insulator may function by mechanisms similar to that of the *gypsy* insulator. The reason for the apparent discrepancy between the formation of insulator bodies by the *Drosophila* and vertebrate CTCF proteins is not clear. It is possible that CTCF insulator bodies in vertebrates are made of a lower number of individual insulators, making the insulator bodies difficult to resolve by light microscopy. Alternatively, or in addition, the increased complexity in DNA content and number of chromosomes in chicken or mouse cells may result in

a large number of insulator bodies that make their detection challenging. Finally, it is possible that the different appearance of insulator bodies in *Drosophila* versus vertebrate cells is due to the quality of available antibodies to the CTCF protein, as we have observed a punctate pattern of very small dots with poor quality antibodies to *gypsy* insulator proteins.

dCTCF insulator bodies in *Drosophila* coincide with those formed by CP190, as one would expect from their pattern of colocalization in polytene chromosomes. Surprisingly, dCTCF insulator bodies also colocalize with those formed by Su(Hw) and Mod(mdg4)2.2 in spite of their presence at different genomic sites, and each can be formed in the absence of the other. CP190 is nevertheless essential for the formation of both types of insulator bodies; in its absence, dCTCF and Mod(mdg4)2.2 are mislocalized. These observations can be explained by two different models that have different implications for the role of nuclear architecture in insulator function (Figure 7). One possibility is that Su(Hw) and dCTCF insulators form independent insulator bodies but both colocalize in the same regions of the nucleus (Figure 7B). A corollary of this model is the possible existence of special nuclear compartments where insulator bodies are located. A second model would suggest that Su(Hw) and dCTCF insulators form the same insulator bodies through interactions between the CP190 protein present in both insulators (Figure 7A). This model would not require the existence of a specific nuclear compartment where insulator bodies form, as the colocalization is due to the presence of CP190 in both types of insulators. In either model, although the Su(Hw) and dCTCF insulators interact at insulator bodies through CP190, each type of insulator can still form nuclear bodies in the absence of the other.

Su(Hw) and dCTCF colocalize with CP190 at approximately two-thirds of all the sites where this protein is present in polytene chromosomes, suggesting that at least one additional DNA-binding protein may tether CP190 to chromosomes. This would suggest the existence of a third subclass of CP190 insulators similar to Su(Hw) and dCTCF. If CP190 is the critical protein in the establishment of insulator bodies, why have three different proteins to recruit CP190 to the DNA? One explanation could be that the use of three different DNA-binding proteins affords additional possibilities in the regulation of insulator activity during cell differentiation and development. For example, lack of expression of one of the DNA-binding proteins, or covalent modifications that would interfere with its ability to bind DNA or interact with CP190, would render one subclass of insulators inactive while allowing normal function of the other two subclasses. The flexibility in the regulation of insulator activity provided by the use of three different DNA-binding proteins raises the question of why vertebrates appear to use mostly CTCF. Although a vertebrate homolog of Su(Hw) does not appear to exist, it is possible that vertebrates do have a homolog of the third DNA-binding protein. Identification of this protein in *Drosophila* may provide additional insights into possible mechanisms of regulating insulator function and the role of insulators in the control of nuclear organization during vertebrate development.

EXPERIMENTAL PROCEDURES

Fly Strains and Yeast Two-Hybrid Assays

Fly stocks were maintained in standard medium at 25°C. A P element insertion into the 5′UTR of the *dCTCF* gene, *P{EPgy2}CTCF^{EY15833}*, was obtained from the Indiana Stock Center. Flies carrying this insertion are viable and produce reduced but detectable levels of dCTCF (see Figure 1). In order to produce null mutations in the *dCTCF* gene, we mobilized the P element using the *y^{1}w^{1118}; P{Δ2-3}Sb/TM6* strain as a source of transposase. Three different alleles named *dCTCF^{y+1}*, *dCTCF^{y+2}*, and *dCTCF^{y+6}* were obtained and analyzed further. The *Fab-8$^{60.39.2}$/CyO* and *Fab-8$^{60.71.1}$/CyO* strains carrying a *white* transgene with the *Fab-8* insulator inserted between the eye enhancer and the promoter were obtained from Dr. Paul Schedl.

Two hybrid assays were performed using yeast strain pJ694A and plasmids and protocols obtained from Clontech. Bait and target fusion proteins were produced constitutively under the control of the ADH1 promoter. Cotransformants were plated on media lacking tryptophan and leucine (nonselective plates for the reporter gene) or on plates lacking tryptophan, leucine, histidine, and adenine (selective plates for the reporter gene). Single colonies were streaked out on selective plates to obtain the plates showed in Figure 3.

Protein Analyses

In order to obtain dCTCF protein for antibody production, the carboxyterminus of the dCTCF gene was amplified from cDNA GH14774 (Research Genetics) by PCR using primers 5′-GATGATTCGGC GAATGCTCGCGAA-3′ and 5′-CTAAGAGTCCTGCTCAATCATATCC AT-3′ and cloned into the pET100/D-TOPO vector (Invitrogen) in frame with the N-terminal His_6 tag. Purified His-dCTCF was used to immunize rats and rabbits, and antibodies were affinity purified by standard procedures.

Immunoaffinity purification of CP190 complexes was carried out as previously described (Lei and Corces, 2006). For western analysis, larvae were homogenized in either HEMGN buffer (25 mM HEPES-KOH [pH 7.6], 0.1 mM EDTA, 12.5 mM $MgCl_2$, 10% glycerol, 0.1% NP40, 1 mM DTT, 0.1 mM PMSF, and 0.3 M KCl) or in cold sodium dodecyl sulfate (SDS) sample buffer (20 mM sodium phosphate, 2% SDS, 0.001% bromophenol blue, 0.2 M DTT, and 2% glycerol). In the former case, an equal volume of 2× SDS reducing buffer was added and then boiled for 5 min. The extract was spun, and the supernatant was run on an SDS-10% polyacrylamide gel and transferred onto a polyvinylidene difluoride (PVDF) membrane. The presence of dCTCF and CP190 was detected using the Super Signal West Pico chemiluminescent substrate from Pierce.

Immunocytochemistry and In Situ Hybridization of Polytene Chromosomes

Salivary glands were fixed in 3.7% formaldehyde, 45% acetic acid mix for 2 min and squashed in 45% acetic acid on subbed slides. The slides were incubated overnight in dilution buffer containing primary antibodies at 1:1000 dilution for α-CP190, 1:100 for α-Mod(mdg4)2.2, and 1:50 for α-CTCF and α-Su(Hw). Slides were then washed in antibody dilution buffer three times and incubated with FITC- or Texas red-conjugated goat anti-rabbit IgG (Jackson Laboratories) at 1:250 dilution for 1 hr at room temperature or 30 min at 37°C. Slides were stained for 2 min with 4′,6-diamidino-2-phenylindole (DAPI; 0.5 μg/ml) and mounted in Vectashield antifade mounting medium (Vector Laboratories).

To detect the location of *Fab-8* sequences on polytene chromosomes, we used an 1180 bp DNA fragment obtained by PCR of the bithorax locus using primers 5′-AGTTTTGCCTCCCGACT

TCGACGGC-3′ and 5′-AGGTGTAGCGGGCTTTGGGCTACCA-3′. Digoxigenin-labeled DNA was prepared using the Prime-A-Gene random priming kit (Promega). The labeled probe was ethanol precipitated and stored in hybridization buffer (4× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 50% formamide, and 1× Denhardt's containing 0.4 mg/ml of salmon sperm DNA) until ready to use. Polytene chromosomes from third instar larvae were prepared as described above, except that the glands were fixed in acetic acid-lactic acid mixture (1 acetic acid: 2 water: 3 lactic acid). Slides were heated at 65°C in 2× SSC for 30 min, dehydrated using an ethanol series, and denatured in 0.07 M NaOH. For hybridization of DNA, boiled probes were added to the slide and covered immediately with a coverslip. The slide and coverslip were sealed with rubber cement and incubated at 37°C overnight in a humidified chamber. Following hybridization, coverslips were removed and the slides were washed in 2× SSC at 37°C, then at room temperature in 1× PBS, and finally in antibody dilution buffer. Antibody detection and visualization were carried out as outlined above.

Chromatin Immunoprecipitation

Approximately 1 × 10^7 *Drosophila* Kc cells were crosslinked with 1% formaldehyde for 10 min at room temperature. Nuclear lysates were sonicated to generate 200–1000 bp DNA fragments. Chromatin immunoprecipitation was then performed with rabbit α-CTCF, α-CP190, or α-Su(Hw) antibodies. PCR amplification of the resulting DNA was performed using the following primers: *Fab-8*, 5′-GGCACAATCAAGTTAATGTTGG-3′ and 5′-GCAAGCGAAGAGTTCCATTC-3′ (Moon et al., 2005); 1500 bp upstream of *Fab-8*, 5′-GAGAGTCCAGGCCCAAGGAC-3′ and 5′-CCCACCAGGGGCGATATTTG-3′; 1500 bp downstream of *Fab-8*, 5′-GCCTGTTTCGCGGAACTGAT-3′ and 5′-CGTGTGGAATTCTGTTCTGTCAC-3′; *Abd-B*, 5′-CACTGGTGCATCTTGGCGGC-3′ and 5′-GCAGCTGACCGAGCGACAGG-3′; *gypsy*, 5′-CGCAAAAACATTGCATATTTTCGGC-3′ and 5′-CGGCTAAATGGTATGGCAAGAAAAGG-3′.

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REFERENCES

Barges, S., Mihaly, J., Galloni, M., Hagstrom, K., Muller, M., Shanower, G., Schedl, P., Gyurkovics, H., and Karch, F. (2000). The Fab-8 boundary defines the distal limit of the bithorax complex iab-7 domain and insulates iab-7 from initiation elements and a PRE in the adjacent iab-8 domain. Development *127*, 779–790.

Bell, A.C., and Felsenfeld, G. (2000). Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene. Nature *405*, 482–485.

Bell, A.C., West, A.G., and Felsenfeld, G. (1999). The protein CTCF is required for the enhancer blocking activity of vertebrate insulators. Cell *98*, 387–396.

Belozerov, V.E., Majumder, P., Shen, P., and Cai, H.N. (2003). A novel boundary element may facilitate independent gene regulation in the Antennapedia complex of Drosophila. EMBO J. *22*, 3113–3121.

Blanton, J., Gaszner, M., and Schedl, P. (2003). Protein:protein interactions and the pairing of boundary elements in vivo. Genes Dev. *17*, 664–675.

Buchner, K., Roth, P., Schotta, G., Krauss, V., Saumweber, H., Reuter, G., and Dorn, R. (2000). Genetic and molecular complexity of the position effect variegation modifier mod(mdg4) in Drosophila. Genetics *155*, 141–157.

Byrd, K., and Corces, V.G. (2003). Visualization of chromatin domains created by the gypsy insulator of Drosophila. J. Cell Biol. *162*, 565–574.

Capelson, M., and Corces, V.G. (2004). Boundary elements and nuclear organization. Biol. Cell *96*, 617–629.

Capelson, M., and Corces, V.G. (2005). The ubiquitin ligase dTopors directs the nuclear organization of a chromatin insulator. Mol. Cell *20*, 105–116.

Capelson, M., and Corces, V.G. (2006). SUMO conjugation attenuates the activity of the gypsy chromatin insulator. EMBO J. *25*, 1906–1914.

Chao, W., Huynh, K.D., Spencer, R.J., Davidow, L.S., and Lee, J.T. (2002). CTCF, a candidate trans-acting factor for X-inactivation choice. Science *295*, 345–347.

Estrada, B., Casares, F., Busturia, A., and Sanchez-Herrero, E. (2002). Genetic and molecular characterization of a novel iab-8 regulatory domain in the Abdominal-B gene of Drosophila melanogaster. Development *129*, 5195–5204.

Felsenfeld, G., Burgess-Beusse, B., Farrell, C., Gaszner, M., Ghirlando, R., Huang, S., Jin, C., Litt, M., Magdinier, F., Mutskov, V., et al. (2004). Chromatin boundaries and chromatin domains. Cold Spring Harb. Symp. Quant. Biol. *69*, 245–250.

Filippova, G.N., Thienes, C.P., Penn, B.H., Cho, D.H., Hu, Y.J., Moore, J.M., Klesert, T.R., Lobanenkov, V.V., and Tapscott, S.J. (2001). CTCF-binding sites flank CTG/CAG repeats and form a methylation-sensitive insulator at the DM1 locus. Nat. Genet. *28*, 335–343.

Gaszner, M., and Felsenfeld, G. (2006). Insulators: exploiting transcriptional and epigenetic mechanisms. Nat. Rev. Genet. *7*, 703–713.

Gaszner, M., Vazquez, J., and Schedl, P. (1999). The Zw5 protein, a component of the scs chromatin domain boundary, is able to block enhancer-promoter interaction. Genes Dev. *13*, 2098–2107.

Gerasimova, T.I., Gdula, D.A., Gerasimov, D.V., Simonova, O., and Corces, V.G. (1995). A Drosophila protein that imparts directionality on a chromatin insulator is an enhancer of position-effect variegation. Cell *82*, 587–597.

Gerasimova, T.I., Byrd, K., and Corces, V.G. (2000). A chromatin insulator determines the nuclear localization of DNA. Mol. Cell *6*, 1025–1035.

Hark, A.T., Schoenherr, C.J., Katz, D.J., Ingram, R.S., Levorse, J.M., and Tilghman, S.M. (2000). CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus. Nature *405*, 486–489.

Harrison, D.A., Gdula, D.A., Coyne, R.S., and Corces, V.G. (1993). A leucine zipper domain of the suppressor of Hairy-wing protein mediates its repressive effect on enhancer function. Genes Dev. *7*, 1966–1978.

Kim, T.H., Abdullaev, Z.K., Smith, A.D., Ching, K.A., Loukinov, D.I., Green, R.D., Zhang, M.Q., Lobanenkov, V.V., and Ren, B. (2007). Analysis of the vertebrate insulator protein CTCF-binding sites in the human genome. Cell *128*, 1231–1245.

Labrador, M., and Corces, V.G. (2002). Setting the boundaries of chromatin domains and nuclear organization. Cell *111*, 151–154.

Labrador, M., and Corces, V.G. (2003). Extensive exon reshuffling over evolutionary time coupled to trans-splicing in Drosophila. Genome Res. *13*, 2220–2228.

Lei, E.P., and Corces, V.G. (2006). RNA interference machinery influences the nuclear organization of a chromatin insulator. Nat. Genet. *38*, 936–941.

Moon, H., Filippova, G., Loukinov, D., Pugacheva, E., Chen, Q., Smith, S.T., Munhall, A., Grewe, B., Bartkuhn, M., Arnold, R., et al. (2005). CTCF is conserved from Drosophila to humans and confers enhancer blocking of the Fab-8 insulator. EMBO Rep. *6*, 165–170.

Pai, C.Y., Lei, E.P., Ghosh, D., and Corces, V.G. (2004). The centrosomal protein CP190 is a component of the gypsy chromatin insulator. Mol. Cell *16*, 737–748.

Yusufzai, T.M., and Felsenfeld, G. (2004). The 5′-HS4 chicken {beta}-globin insulator is a CTCF-dependent nuclear matrix-associated element. Proc. Natl. Acad. Sci. USA *101*, 8620–8624.

Yusufzai, T.M., Tagami, H., Nakatani, Y., and Felsenfeld, G. (2004). CTCF tethers an insulator to subnuclear sites, suggesting shared insulator mechanisms across species. Mol. Cell *13*, 291–298.

Zhao, K., Hart, C.M., and Laemmli, U.K. (1995). Visualization of chromosomal domains with boundary element-associated factor BEAF-32. Cell *81*, 879–889.