Molecular Cell
Article

Regulation of Chromatin Organization and Inducible Gene Expression by a *Drosophila* Insulator

Ashley M. Wood,[1,2] Kevin Van Bortle,[1] Edward Ramos,[1] Naomi Takenaka,[1] Margaret Rohrbaugh,[1] Brian C. Jones,[1] Keith C. Jones,[1] and Victor G. Corces[1,*]
[1]Department of Biology, Emory University, Atlanta, GA 30322, USA
[2]Department of Biology, Johns Hopkins University, Baltimore, MD 21218, USA
*Correspondence: vcorces@emory.edu
DOI 10.1016/j.molcel.2011.07.035

SUMMARY

Insulators are multiprotein-DNA complexes thought to affect gene expression by mediating inter- and intrachromosomal interactions. *Drosophila* insulators contain specific DNA-binding proteins plus common components, such as CP190, that facilitate these interactions. Here, we examine changes in the distribution of *Drosophila* insulator proteins during the heat-shock and ecdysone responses. We find that CP190 recruitment to insulator sites is the main regulatable step in controlling insulator function during heat shock. In contrast, both CP190 and DNA-binding protein recruitment are regulated during the ecdysone response. CP190 is necessary to stabilize specific chromatin loops and for proper activation of transcription of genes regulated by this hormone. These findings suggest that cells may regulate recruitment of insulator proteins to DNA to activate insulator activity at specific sites and create distinct patterns of nuclear organization that are necessary to achieve proper gene expression in response to different stimuli.

INTRODUCTION

Insulators mediate inter- and intrachromosomal interactions that bring together distant regulatory elements in the genome. The specific outcome of these interactions depends on the nature of the connecting insulator sites (Phillips and Corces, 2009). For example, two insulator sites may interact to form a loop that separates an enhancer from the promoter of a gene, in which case enhancer-promoter interactions will be abolished and transcription of the gene will not take place. Alternatively, the interaction between two insulator sites may bring an enhancer in close proximity to a promoter, facilitating activation of transcription (Majumder et al., 2006; Xu et al., 2011). The CTCF insulator appears to be the main type of this class of regulatory sequences discovered thus far in vertebrates. CTCF binds to specific sequences in the genome and mediates interactions among CTCF insulator sites in a process that is stabilized by cohesin (Gaszner and Felsenfeld, 2006; Wendt and Peters, 2009). In *Drosophila*, however, there are various DNA-binding proteins that interact with distinct sites throughout the genome and show insulator activity in transgene assays. These include the *Drosophila* homolog of CTCF (dCTCF), suppressor of Hairy-wing (Su[Hw]), and boundary element-associated factor (BEAF) (Bushey et al., 2008; Maeda and Karch, 2007). Interestingly, all three of these DNA-binding proteins are thought to recruit a common protein, centrosomal protein 190 (CP190), which is necessary for insulator activity (Bushey et al., 2009; Gerasimova et al., 2007; Mohan et al., 2007; Pai et al., 2004). CP190, as well as Mod(mdg4), contain BTB domains and are recruited to insulator sites by the various DNA-binding proteins. CP190 and Mod(mdg4) mediate interinsulator interactions and serve as a bridge to bring together distant insulator sites. These observations suggest that insulator activity can be controlled, in principle, by regulating the binding of Su(Hw), dCTCF, or BEAF to DNA or by modulating the interactions between these proteins and CP190 and Mod(mdg4) (Bushey et al., 2009). Additional regulatory steps may take place by recruitment of the E3 ubiquitin ligase dTopors or the RNA helicase Rm62 (Capelson and Corces, 2005; Lei and Corces, 2006).

Interactions among insulator sites throughout the genome may give rise to a specific three-dimensional organization of the chromatin that can be considered a blueprint of the transcriptional status of the cell. Because patterns of gene expression are cell lineage specific, comparison of the genome-wide distribution of insulator proteins may give insights into the actual strategies used by cells to regulate insulator function. To begin to examine this question, we mapped *Drosophila* insulator protein DNA localization genome-wide in two different cell lines and found that 5%–37% of insulator protein-binding sites for dCTCF, Su(Hw), BEAF, and CP190 are cell type specific (Bushey et al., 2009). Furthermore, a subset of the cell type-specific CP190 sites show localization differences at sites where the amount of dCTCF, Su(Hw), or BEAF is invariant between the two cell lines. This suggests that cells may regulate insulator function by modulating the level of DNA-binding protein recruitment to DNA as well as by controlling the amount of CP190 tethered to insulator sites. Therefore, cells may have insulators in which the DNA-binding protein is bound to DNA but are inactive because they lack CP190; this class of insulators may be poised for rapid activation in response to specific stimuli. Other insulator sites appear to lack all protein components, and their activation would require the recruitment of one of the DNA-binding proteins as well as CP190.

A more controlled system for studying the mechanisms by which insulator function is regulated is to perturb gene expression in a single cell type and monitor how insulator protein localization and gene expression change over time. Two widely used approaches to induce changes in gene expression in *Drosophila* cells are heat shock and ecdysone treatment. *Drosophila* cells respond to an elevation in temperature by inducing transcription of heat shock genes while repressing expression of the remainder of the genome, thus altering transcription on a genome-wide scale (Ashburner and Bonner, 1979). In *Drosophila*, postembryonic development is controlled by the steroid hormone α-ecdysone in its biologically active form, 20-hydroxyecdysone (20-HE). 20-HE binds to the ecdysone receptor complex (ECR-C), a classic nuclear receptor complex, to stimulate insect development (Riddiford et al., 2000). Treatment of cultured cells with ecdysone results in a less dramatic alteration in gene expression than heat shock and allows analysis of expression of individual genes. These two methods of altering transcription afford the possibility of monitoring changes in insulator protein binding as well as insulator-dependent chromatin looping at specific genomic loci.

Here, we assay insulator protein localization during the heat shock and ecdysone responses. Heat shock results in a dramatic loss of CP190 localization to DNA, whereas 20-HE treatment leads to changes in the pattern of CP190, Su(Hw), dCTCF, and BEAF localization at a subset of sites. Specifically, in response to 20-HE, CP190 is recruited to a distinct site at the ecdysone-induced Eip75B locus, resulting in increased interactions between adjacent insulator sites and the formation of chromatin loops. The data presented here suggest that these changes in the three-dimensional architecture of the locus confine ecdysone-induced transcription to a specific set of genes in the region. Together, these findings indicate that the regulation of insulator protein recruitment to DNA is an important mechanism that is used to establish proper chromatin organization and gene expression patterns.

RESULTS

Heat Shock Causes a Genome-wide Reduction in CP190 Localization at Insulator Sites

Drosophila cells undergo dramatic changes in gene expression in response to heat shock. Transcription of most genes is turned off, whereas expression of genes necessary for the heat shock response is induced at high levels (Ashburner and Bonner, 1979). This system, which involves a global change in the transcriptional state of the genome, may be a powerful model to study the relationship between insulator proteins and transcription. We have previously shown that the distribution of Su(Hw) on polytene chromosomes is not significantly altered by heat shock; nevertheless, the formation of Su(Hw) insulator bodies, which represent sites where several individual insulator sites coalesce in the nucleus, is severely disrupted (Gerasimova et al., 2000). Immunolocalization experiments using antibodies against dCTCF and BEAF indicate that the binding of these proteins to DNA is also not notably affected by the heat shock response (Figure 1B, C). Taken together, these results suggest that changes in transcription patterns during the heat shock response are accompanied by alterations in insulator function, but these alterations are not due to large changes in the recruitment of the DNA-binding component of insulator complexes to chromatin. One possibility, suggested by the observed alterations in insulator body formation, is that regulation of insulator function during heat shock is controlled at the level of CP190 recruitment. To test this possibility, we carried out immunolocalization experiments using CP190 antibodies on polytene chromosomes. The results indicate that, upon heat shock, there is a dramatic release of CP190 from most genomic insulator sites in polytene chromosomes from third-instar larvae (Figure 1D) (Oliver et al., 2010). Dismissal of CP190 from insulator sites is accompanied by a dispersion of insulator bodies: 137/160 (85.6%) of nonheat-shocked cells contain CP190 insulator bodies compared to 10/160 (6.25%) of heat-shocked imaginal disc cells (Figures 1E and Figure S1B available online). However, western blot analysis indicates that CP190 protein levels are unchanged upon heat shock, suggesting that temperature elevation results in relocation of this protein from insulator bodies to a diffuse distribution throughout the nuclear space (Figure S1A). This result suggests that the global change in gene expression induced by heat shock correlates with a decrease in insulator interactions, most likely caused by regulation of CP190 recruitment to DNA.

Ecdysone Treatment Leads to Changes in the Genome-wide Distribution of Insulator Proteins

To determine whether a less dramatic change in gene expression than that induced by heat shock also correlates with alteration of CP190 distribution, we treated Kc cells with the hormone 20-hydroxyecdysone (20-HE). This treatment results in G_2-arrest within 12–24 hr and morphological changes, such as the loss of the spherical cell shape as well as appearance of cellular processes (Stevens et al., 1980). 20-HE has previously been shown to affect the expression of a few hundred *Drosophila* genes in Kc cells over a time course of 1–48 hr (Gauhar et al., 2009). Therefore, we used 3 and 48 hr time points to monitor early and late responses to the hormone. Because the changes in gene expression after 20-HE treatment are less dramatic than those observed after heat shock, we would not expect to see a dramatic rearrangement in CP190 localization after incubation with this hormone. Consequently, insulator bodies remain unchanged in Kc cells after 3 or 48 hr of treatment with 20-HE (Figure S2). In order to more precisely determine possible changes in CP190 localization that accompany changes in gene expression after 20-HE treatment, we performed ChIP-Seq with CP190, Su(Hw), dCTCF, and BEAF at 0, 3, and 48 hr of incubation with this hormone (Figure 2). ChIP-seq data has been deposited in GEO under accession number GSE30740. To verify these data, we compared sites identified at 0 hr of 20-HE treatment to our previously published ChIP-chip data from Kc cells (Bushey et al., 2009). Although we identified more sites by ChIP-Seq than ChIP-chip, as would be expected based on the sensitivity of the techniques, 89% of CP190 sites, 97% of Su(Hw) sites, 90% of CTCF sites, and 93% of BEAF sites identified by ChIP-chip analysis were also identified in the ChIP-Seq data sets presented here.

Analysis of sites that change upon 20-HE treatment indicates that, unlike the dramatic decrease in chromosome-bound

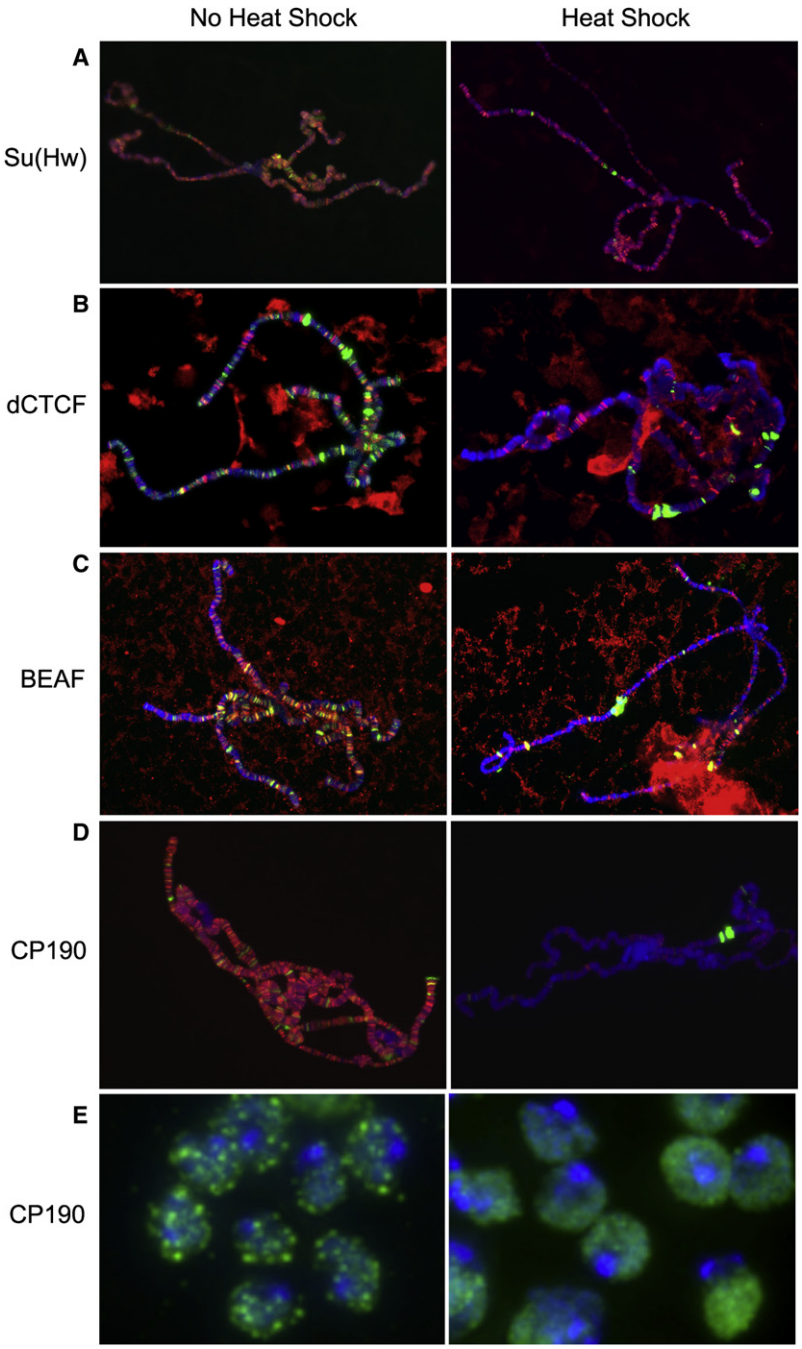


Figure 1. Response of Insulator Proteins to Heat Shock

(A–D) Third-instar larvae were subjected to heat shock, and polytene chromosomes were dissected and stained with antibodies recognizing (A) Su(Hw), (B) dCTCF, (C) BEAF, or (D) CP190, shown in red. All polytene chromosomes (A–D) were costained with antibody against Pol II Ser5, shown in green, to mark the initiating form of RNA polymerase II. Upon heat shock, Pol II Ser5 staining is reduced throughout most of the genome and is found in puffs, indicating relocalization to the highly transcribed heat shock genes. DNA is visualized with DAPI in all panels (blue).

(E) Nuclei from imaginal tissue isolated from third-instar larvae under heat-shock and nonheat-shock conditions were stained with antibodies against CP190 (green), revealing the absence of insulator bodies after heat shock. See also Figure S1B. The loss of CP190 insulator bodies is not due to protein degradation, as shown by western blot analysis in Figure S1A.

CP190 observed after heat shock, CP190 sites remain unchanged in response to 20-HE after 3 hr of treatment, and there is no significant loss of CP190 binding at either time point (Figures 2 and 3A). However, 20-HE treatment results in new or increased CP190 association with DNA at 306 sites (5%) after 48 hr. Comparison of the binding profiles of the three DNA-binding proteins—BEAF, dCTCF, and Su(Hw) —reveals similar alterations. BEAF exhibits decreased binding at 135 sites (2%) at 3 hr and 65 sites (1%) at 48 hr (Figure 3A), of which 58 sites are common between the two time points. dCTCF shows lost or decreased binding at 34 sites (0.6%) at 3 hr, followed by a decrease or loss of binding at 11 sites (0.2%) at 48 hr (Figure 3A). Su(Hw) behaves in the opposite fashion, showing reduced binding at 70 sites (1%) at 3 hr and then new or increased binding at 200 sites (2%) at 48 hr (Figure 3A). The discrepancy between the responses of BEAF, dCTCF, and Su(Hw) at 48 hr is not surprising because we and others have previously reported that insulators defined by these various DNA-binding proteins show distinct properties (Bushey et al., 2009; Nègre et al., 2010). Therefore, these observations further support the idea that BEAF, dCTCF, and Su(Hw) insulators play different roles in regulating gene expression.

Analysis of Insulator Sites Regulated in Response to 20-HE Treatment

Because recruitment of CP190 is necessary for insulator function (Gerasimova et al., 2007; Mohan et al., 2007; Pai et al., 2004), we concentrated our analysis on the CP190 sites that are upregulated after 48 hr of treatment in order to examine the role of regulated insulator sites in the 20-HE response. A comparison between the 306 CP190 sites upregulated at 48 hr of 20-HE treatment and the DNA-binding proteins present at 48 hr of treatment reveals that upregulated CP190 sites colocalize with at least one DNA-binding protein at 86% of sites (Figure 3B). This is very similar to the 89% of CP190 sites that colocalize with at least one DNA-binding protein at 0 hr (Figure 3B), suggesting that these upregulated CP190 sites are authentic insulator sites. Notably, the distribution of DNA-binding proteins that colocalize with these upregulated CP190 sites is very different than that for the total population of CP190 sites (Figures 3B and 3C). There is a strong enrichment for Su(Hw) sites alone or in combination with dCTCF at

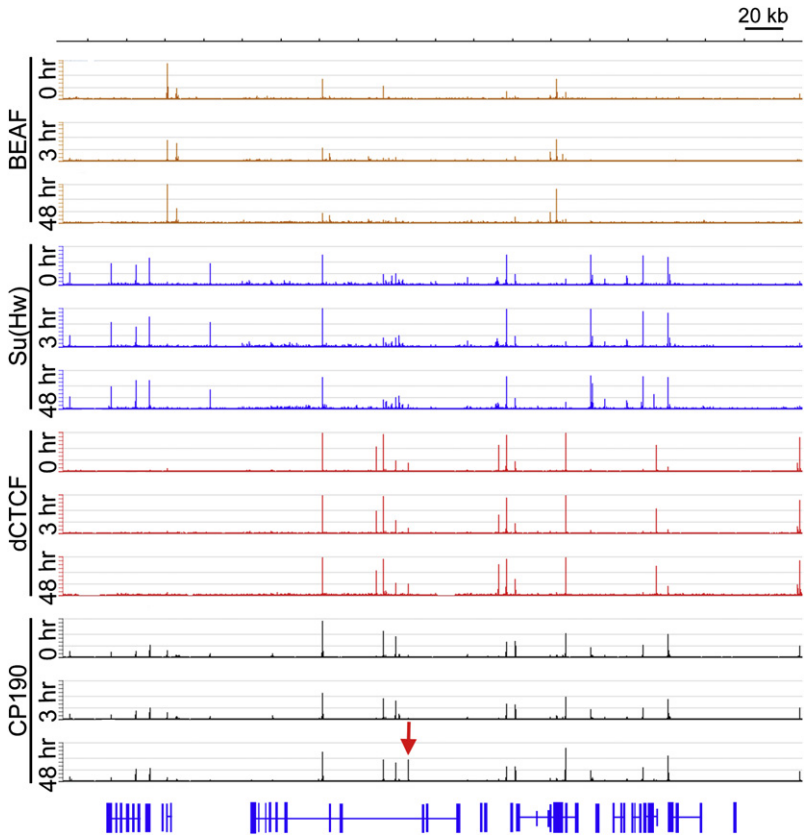


Figure 2. Response of Insulator Proteins to 20-HE Treatment

The 360 kb region of chromosome 3L including the Eip75B gene. ChIP-Seq analysis of BEAF, Su(Hw), dCTCF, and CP190 at 0, 3, and 48 hr of 20-HE treatment in Kc cells. Genes from the UCSC Genome Browser are shown in blue. The upregulated CP190 site used for 3C analysis is indicated (red arrow). Changes in insulator protein localization upon 20-HE treatment do not lead to alterations in insulator body formation as shown in Figure S2.

upregulated CP190 sites and a depletion in BEAF sites, as well as total dCTCF sites. Overall, 220 (72%) upregulated CP190 sites colocalize with a Su(Hw) site. Of these 220 CP190/Su(Hw) sites, 61 (27%) also show an increase in Su(Hw) binding. This is much greater than the percentage of Su(Hw) sites upregulated at 48 hr genome-wide (2%), indicating that upregulated CP190 sites are significantly enriched for upregulated Su(Hw) sites ($p < 1 \times 10^{-100}$). These observations suggest that the recruitment of CP190 after hormone induction occurs at a specific subset of insulator sites, characterized by the presence of Su(Hw), that may represent a distinct functional class of these elements.

The data suggest that, in some cases, the formation of a new active insulator after 20-HE induction first requires recruitment of Su(Hw), followed by binding of CP190. Alternatively, at other sites, induced CP190 recruitment occurs at sites that already have one of the DNA-binding proteins. This result suggests that the upregulated CP190 sites that display constant levels of DNA-binding proteins may be poised insulator sites and that 20-HE induction is necessary for the recruitment of CP190 and activation of insulator function.

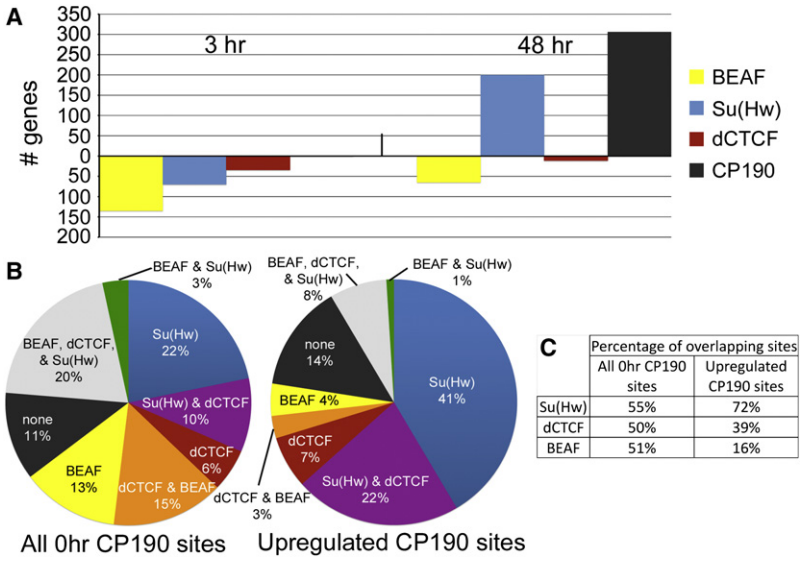
20-HE Stimulates a CP190-Dependent Increase in Chromatin Looping at the Eip75B Locus

We have previously suggested that CP190 is involved in the formation of chromatin loops between insulator sites (Pai et al., 2004). The ecdysone-induced protein 75B (Eip75B) locus contains a dCTCF site at which the levels of CP190 increase dramatically after 48 hr of 20-HE treatment (Figure 2). Analysis of ChIP-seq data at this locus reveals an increase in dCTCF localization as well as induced Su(Hw) localization upon 20-HE treatment, but no change in BEAF localization. These changes were validated by ChIP-qPCR analysis (Figure S2B). To determine the role of the increased recruitment of CP190 to this dCTCF site in the regulation of chromatin structure after hormone induction, we performed chromatin conformation capture (3C) analysis at the locus (Dekker et al., 2002).

For the 3C experiments, we used HindIII to digest genomic DNA and also used fragment 0, which contains the 20-HE-induced CP190 site, as an anchor (Figure 4A, shaded region). Crosslinking frequencies between the anchor and downstream fragments were quantified using a Taqman probe and qPCR after 48 hr of either 20-HE or ethanol (mock) treatment. 3C analysis was also performed with cells treated with either control or CP190 RNAi. Under conditions simulating normal growth, mock hormone treatment and control RNAi, we observed weak but significant interactions between the anchor fragment and fragments 6/7 and 9/10 (Figure 4B). Although CP190 is only present within the anchor fragment at very low levels before 20-HE treatment, dCTCF is present at this site and may be sufficient to stabilize these weak interactions (Figure 2B). The strength of these two interactions increases after 20-HE treatment, but not in cells treated with CP190 RNAi. Interestingly, fragments 7 and 9 contain CP190 sites before and after 20-HE induction, suggesting that the upregulated CP190 site in the anchor fragment mediates intrachromosomal interactions with existing sites to enhance chromatin loop formation upon 20-HE treatment. Together, these data suggest that de novo recruitment of CP190 to an existing dCTCF site results in alterations in the chromatin organization of the Eip75B locus upon 20-HE treatment.

CP190-Mediated Chromatin Organization Modulates 20-HE-Induced Transcription

Recruitment of CP190 to a poised insulator site increases intrachromosomal interactions and alters the three-dimensional structure of chromatin at the Eip75B locus. In order to explore the functional significance of the activation of this dCTCF insulator site by CP190 recruitment, we monitored gene expression in response to 20-HE treatment in the presence of either control



C

	Percentage of overlapping sites	
	All 0hr CP190 sites	Upregulated CP190 sites
Su(Hw)	55%	72%
dCTCF	50%	39%
BEAF	51%	16%

Figure 3. Insulator Sites Regulated in Response to 20-HE

(A) Graph indicates the number of insulator sites that are regulated for each protein at each time point of 20-HE treatment. Bars above the x axis indicate upregulated genes, and genes below the axis indicate downregulated genes. For a list of up- and downregulated sites, see Table S2.

(B) Colocalization of CP190 sites and each of the DNA-binding proteins. All CP190 sites identified at 0 hr of 20-HE treatment are represented in the pie chart on the left, and those upregulated at 48 hr of 20-HE treatment are represented in the pie chart on the right.

(C) The percentage of CP190 sites that overlap with each DNA-binding protein is indicated. These percentages add up to more than 100% because some CP190 sites overlap with more than one DNA binding protein, as indicated in (B).

or CP190 RNAi using Nimblegen microarrays that cover more than 16,000 predicted genes. Expression data has been deposited in GEO under accession number GSE30686. 20-HE-responsive genes were identified by comparing RNA isolated from cells at 0 hr to that at either 3 or 48 hr of 20-HE induction ($p < 0.1$ and 1.5-fold cutoff).

In cells treated with control RNAi, we found 38 genes that were upregulated at the early (3 hr) time point and no genes that were significantly downregulated. At the 48 hr time point, we found 2,127 20-HE responsive genes, 1,184 upregulated and 943 downregulated (Tables 1 and S2). In cells treated with CP190 RNAi, we found 290, 447, and 415 genes misregulated at 0 hr, 3 hr, and 48 hr of 20-HE treatment, respectively (Tables 2 and S2). We observed that genes are up- and down-regulated upon CP190 knockdown at all time points, but more genes are misregulated at 3 and 48 hr of 20-HE treatment than at 0 hr. This may be a result of DNA-bound CP190 being more stable than the free pool of CP190 during RNAi treatment. As a consequence, the pre-existing CP190 sites may not be affected to the same extent as the upregulated CP190 sites after 20-HE treatment. Alternatively, CP190 may be more important for gene regulation in response to stimuli than for steady-state growth. However, both scenarios suggest that upregulated CP190 sites are important for proper regulation of gene expression in response to 20-HE. Further support of this conclusion comes from the finding that 24% (9 out of 38) of 3 hr 20-HE-responsive genes and 11% (226 out of 2,127) of 48 hr 20-HE-responsive genes are misregulated upon CP190 knockdown at the respective time point, suggesting that CP190 is necessary for a proper transcriptional response to 20-HE.

Surprisingly, the microarray data indicate that transcription of the Eip75B gene, which contains the dCTCF insulator site to which CP190 is recruited after 20-HE induction, is properly induced in response to 20-HE, even in a CP190 RNAi knockdown (Figure 5B). Nevertheless, a closer look at the genes upstream of Eip75B reveals an increase in expression of four genes after 48 hr of 20-HE treatment in CP190 knockdown compared to control RNAi treatment. This observation suggests that CP190 is necessary to prevent genes surrounding Eip75B from being upregulated upon 20-HE induction (Figure 5B).

Eip75B encodes four different transcripts expressed from four different alternative promoters (Figure 5A). However, the microarrays used for gene expression analysis only include probes for the common exons at the 3′ end of the gene. Therefore, we used qPCR with primers that distinguish the four Eip75B transcripts to analyze the levels of each of the mRNAs. Results indicate that the four Eip75B transcripts respond differently to 20-HE treatment (Figure 5C). Transcripts Eip75B-RA and Eip75B-RC are dramatically induced in response to 20-HE, whereas expression of Eip75B-RD does not change upon hormone treatment. In cells treated with control RNAi, the Eip75B-RB transcript is induced at 3 hr of 20-HE treatment, but levels begin to decrease by 48 hr. Interestingly, in cells treated with CP190 RNAi, upregulation of Eip75B-RB continues at 48 hr of 20-HE treatment. Therefore, the Eip75B-RB transcript behaves in a similar manner as the four misregulated genes upstream of Eip75B in cells lacking the CP190 protein. These data can be interpreted in the context of the location of binding sites for the ecdysone receptor complex (ECR-C) (Gauhar et al., 2009), which act as regulatory elements for the transcriptional activation of ecdysone-induced genes. Binding sites for ECR-C have been mapped throughout the coding region of Eip75B, such that the ecdysone-induced CP190 insulator separates ECR-C sites from the promoters of ecdysone-induced genes misregulated in cells treated with CP190 RNAi (Figure 5A).

These data suggest that activation of a poised dCTCF insulator by recruitment of CP190 is necessary to strengthen intra-chromosomal interactions with pre-existing insulator sites. These interactions create chromatin loops that may block the spread of 20-HE-induced transcription to genes surrounding Eip75B-RA. The loops formed between insulator sites could segregate the Eip75B-RB promoter as well as upstream genes from regulatory elements acting on Eip75B-RA, which continues to be upregulated at 48 hr of 20-HE treatment.

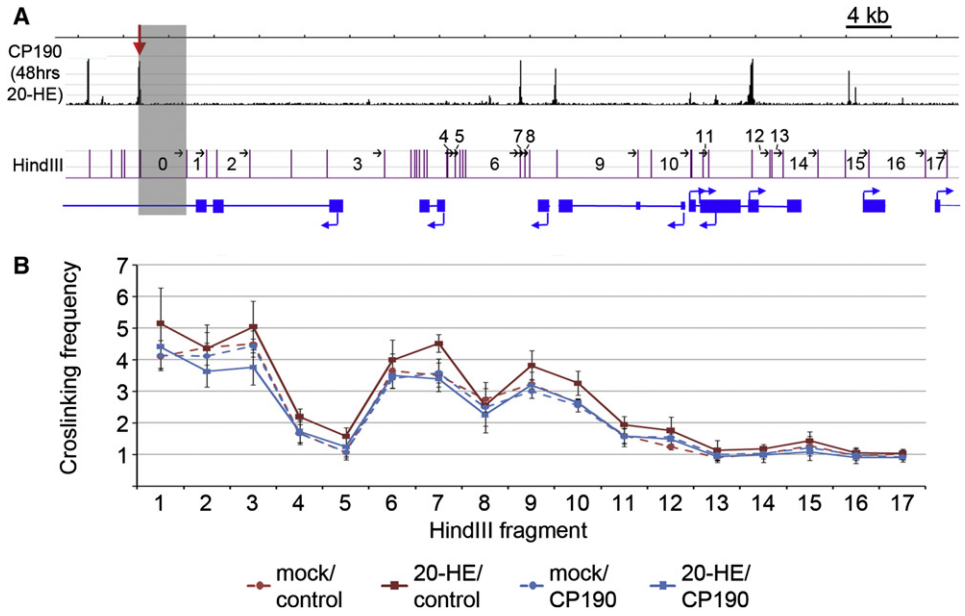


Figure 4. 3C Analysis of the Eip75B Locus in Response to 20-HE Treatment

(A) The 80 kb region of chr3L containing part of the Eip75B gene. CP190 ChIP-Seq data from 48 hr of 20-HE treatment is shown in black with the upregulated CP190 site from Figure 4B indicated with a red arrow. The fragments used for 3C analysis and generated by digestion of genomic DNA with HindIII are shown in purple. Fragment 0, which was used as the anchor in 3C analysis, is shaded in gray.

(B) 3C analysis covering the HindIII fragments labeled as in (A). Analysis was done with mock hormone treatment and control RNAi (dashed red line), 20-HE treatment for 48 hr and control RNAi (solid red line), mock hormone treatment and CP190 RNAi (dashed blue line), and 20-HE treatment and CP190 RNAi (solid blue line). Error bars represent the standard deviation of the mean of four biological replicates.

DISCUSSION

Chromatin insulators mediate inter- and intrachromosomal interactions that establish a three-dimensional nuclear architecture and impact gene expression by controlling the juxtaposition of regulatory sequences within the nuclear space. Insulators are composed of DNA-binding proteins that recruit other factors to establish or maintain these interactions. Genome-wide analyses of the distribution of these proteins in different *Drosophila* cell lines suggest the possibility that cells regulate insulator activity by modulating the recruitment of these two classes of proteins. To test this hypothesis, we examined changes in insulator protein localization in response to two different stimuli, heat shock and ecdysone hormone induction, which result in changes in the transcription output of the cell. We find that insulator protein recruitment to chromatin is regulated during both the heat shock and ecdysone responses.

Results from the analysis of polytene chromosomes during the heat shock response suggest that dismissal of CP190 from insulator sites may be a prerequisite for the broad changes in transcription that accompany temperature elevation. This implicates CP190 as a critical contributor to insulator function and suggests that its recruitment to insulator sites may play a role in regulating gene expression. Upon recovery from heat shock, a cell must return to its original transcription profile. The observed persistence in the localization of DNA-binding proteins on polytene chromosomes suggests that these proteins could act as a memory for CP190 to return to the DNA after heat shock. Nevertheless, these findings suggest that regulatory mechanisms, perhaps in the form of covalent modifications of Su(Hw), dCTCF, and BEAF, must exist to control the targeting of CP190 to a subset of sites where these proteins are present in the genome.

Table 1. Gene Expression upon Ecdysone Treatment

20HE Treatment	Number of 20HE-Responsive Genes	Number of Genes Upregulated	Number of Genes Downregulated
3 hr	38	38	0
48 hr	2,127	1,184	943

The number of genes that are up- and downregulated at 3 and 48 hr of 20-HE treatment compared to 0 hr of 20-HE treatment are given. For expression levels of individual genes, see Table S2.

Table 2. Gene Expression in CP190 Knockdown

20HE Treatment	Number of CP190 RNAi- Responsive Genes	Number of Genes Upregulated	Number of Genes Downregulated
0 hr	290	222	68
3 hr	447	356	91
48 hr	415	251	164

The numbers of genes that are misregulated upon CP190 RNAi treatment compared to control RNAi treatment are given. This analysis was performed at 0, 3, and 48 hr of 20-HE treatment. For expression levels of individual genes, see Table S2.

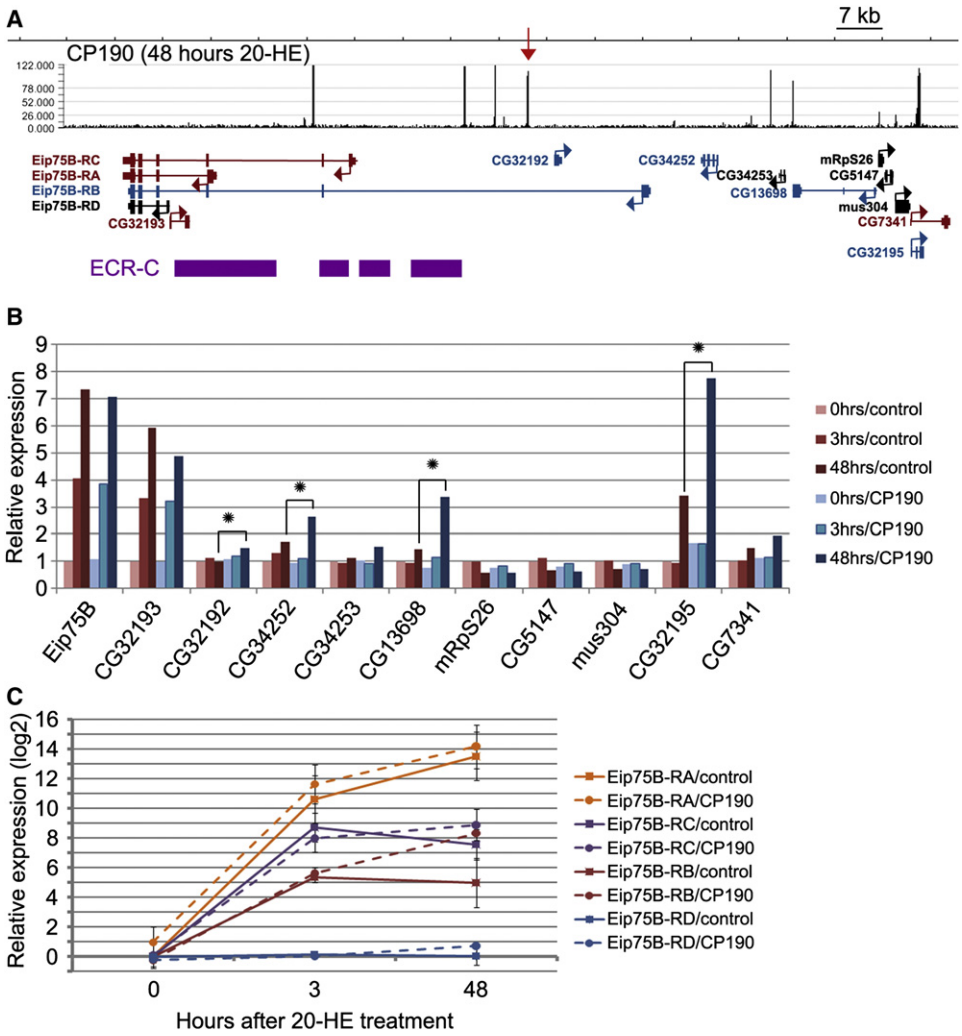


Figure 5. Gene Expression at the Eip75B Locus in Response to 20-HE Treatment

(A) The 130 kb region of chr3L containing the Eip75B gene. CP190 ChIP-Seq data from 48 hr of 20-HE treatment is shown in black, with the upregulated CP190 site indicated by a red arrow and the y axis representing the number of tags. An expanded representation of genes, including the four transcripts of Eip75B, is shown. Transcripts upregulated at 48 hr of 20-HE treatment in a CP190 RNAi-dependent manner are shown in blue, transcripts upregulated at 48 hr of 20-HE treatment but not affected by CP190 RNAi are shown in red, and transcripts that do not change are shown in black. Binding sites for the ECR-C are shown in purple (Gauhar et al., 2009).

(B) Expression level of genes within the locus (A) pulled from our microarray analysis. Expression in control RNAi is shown in red, and expression in CP190 RNAi is shown in blue. For each gene, expression levels are shown for 0, 3, and 48 hr of 20-HE treatment in light, medium, and dark bars, respectively. Stars indicate genes that show a change in gene expression (FDR < 10%, 1.5-fold change) between control and CP190 RNAi at 48 hr of 20-HE treatment.

(C) Relative expression of the four different Eip75B transcripts determined by qPCR at 0, 3, and 48 hr of 20-HE treatment. Dashed lines show expression under control RNAi and solid lines under CP190 RNAi. Error bars represent the standard deviation of the mean of three biological replicates. For levels of CP190 knockdown by RNAi, see Figure S3.

The steroid hormone 20-HE induces changes in gene expression that are necessary to initiate and coordinate cell proliferation, differentiation, and death that occur during molting and metamorphosis in *Drosphila* development (Riddiford et al., 2000). The Kc cell response to 20-HE, which includes exiting the cell cycle and altering cell morphology, resembles cell differentiation. Contrary to heat shock, which changes the expression of all transcribed genes, Kc cells respond to treatment with 20-HE by altering transcription of a relatively small subset of genes. Therefore, it is not surprising that alterations in insulator protein distribution after 20-HE treatment are not as dramatic as those taking place after heat shock. Although the majority of insulator protein-binding sites remain constant upon 20-HE treatment, a subset is up- and downregulated at 3 and 48 hr after hormone treatment. Interestingly, some sites upregulated at 3 hr of treatment are not maintained at 48 hr, when several dCTCF and BEAF sites are downregulated and Su(Hw) and CP190 sites become upregulated. This agrees with the changes in gene expression observed upon 20-HE treatment, as there are distinct sets of early and late ecdysone-responsive genes, and products

of the early genes repress their own transcription and induce expression of late genes (Ashburner, 1974; Burtis et al., 1990; Segraves and Hogness, 1990). Therefore, it is not surprising that many insulator sites regulated at 3 hr are distinct from those regulated at the later 48 hr time point.

We have previously shown that Su(Hw), dCTCF and BEAF show distinct distribution patterns with respect to genomic features, with BEAF found preferentially at promoters and Su(Hw) found more often at sites distant from genes (Bushey et al., 2009). Based on this distribution, we have suggested that BEAF may be involved in mediating interactions directly required for activation of transcription, whereas Su(Hw) may affect gene expression more indirectly, perhaps by bringing together regions of the genome to establish different chromatin domains. In this context, it is interesting that CP190 is preferentially recruited to pre-existing Su(Hw) sites after hormone treatment. This result suggests that new CP190-mediated interactions after exposure to 20-HE may play a more general role in chromatin organization as opposed to direct regulation of the expression of specific genes.

Comparison between the genomic location of regulated insulator sites and ecdysone responsive genes does not reveal any significant correlation. This further supports the idea that insulator proteins are not typical transcription factors, and their role in regulating gene expression is more complex than protein binding to the promoter of target genes. Mechanistically, the 3C data presented here support the idea that CP190 functions by modulating chromatin looping. Although intriguing, such a role makes it difficult to link a regulated insulator site with affected genes because insulator sites may act over long distances and a change in one insulator protein-binding site can potentially regulate the expression of multiple genes.

The significance of the preferential upregulation of CP190 and Su(Hw) at 48 hr of 20-HE treatment and the downregulation of dCTCF and BEAF is unclear. Because the downregulated dCTCF and BEAF sites do not localize to ecdysone-responsive genes, it is possible that these are actually insulator sites that, under normal growth conditions, are poised for activation. However, upon ecdysone treatment, this poised state is lost. In the future, it will be interesting to test whether ecdysone-treated cells lose the potential to respond to other environmental stimuli, thus supporting the idea that these downregulated dCTCF and BEAF sites represent a reduction in poised insulator sites.

To gain a better understanding of the functional significance of the new 20-HE-induced CP190 sites, we examined the role of a single insulator site. To this end, we selected a CP190 site within the Eip75B locus that is strongly upregulated after 48 hr of 20-HE treatment. This site contains dCTCF before and after hormone induction and, thus, may be poised for activation by CP190 recruitment. Interestingly, the site contains barely detectable amounts of CP190 before 20-HE treatment that are not considered statistically significant by standard peak analysis software but that may be significant. This site can mediate interactions with adjacent insulator sites in untreated cells, suggesting that dCTCF alone or in the presence of low levels of CP190 is sufficient to mediate interactions. Alternatively, other BTB domain-containing insulator proteins such as Mod(mdg4) may be responsible for low-level interactions in the absence of CP190. Nevertheless, interactions between this site and adjacent insulators are strengthened considerably after 48 hr of 20-HE treatment in a CP190-dependent manner. Additionally, a new Su(Hw) signal appears at 48 hr of 20-HE treatment, which is notable because the interactions identified by 3C are with sites that contain CP190, dCTCF, and Su(Hw). Therefore, this increase in the Su(Hw) ChIP signal may result indirectly from an increase in chromatin looping with a site directly bound by Su(Hw). Alternatively, binding of multiple insulator proteins may be necessary to achieve stable chromatin loops, and the pattern of insulator protein overlap at a given genomic location may direct interaction with other insulator sites with similar protein occupancy. Although at this point we do not understand the significance of having multiple DNA-binding proteins at one genomic location, it is clear from the pie charts in Figure 3B that this is a common occurrence and therefore must have a functional output.

In addition to the 20-HE-induced Eip75B gene, the locus under study contains several additional genes whose expression is not affected by hormone treatment under normal growth conditions. Enhancer elements bound by the ecdysone receptor complex (ECR-C) responsible for 20-HE-dependent transcription of Eip75B are located within this gene (Gauhar et al., 2009). The dCTCF-poised insulator site separates these regulatory sequences and the promoters of the CG32193, Eip75B-RA, Eip75B-RC, and Eip75B-RD genes from the Eip75B-RB and nonecdysone-inducible genes located in the vicinity. In the absence or at 3 hr of 20-HE stimulation, this insulator is presumably not functional because it lacks CP190, a conclusion supported by the increased transcription of Eip75B-RB after 3 hr of ecdysone treatment. Recruitment of CP190 after 48 hr of hormone induction causes activation of this insulator, explaining the drop in transcription of the Eip75B-RB RNA. This newly activated insulator may also be responsible for the absence of hormone-induced expression of genes located distal to the Eip75B-RB promoter. This hypothesis is corroborated by the activation of these genes, including Eip75B-RB, in cells lacking CP190 protein. Therefore, this new CP190 site is in a position that would enable it to block communication between the ECR-C and the promoters of Eip75B-RB and other misregulated genes. Importantly this CP190 site is induced only at 48 hr, when the downreguation of Eip75B-RB begins. Hence, recruitment of CP190 to a poised insulator site happens at the correct time and place for a classic enhancer-blocking insulator function. Significantly, dCTCF is found at this site at 0, 3, and 48 hr of 20-HE treatment, though the insulating effect on Eip75B-RB and the other upstream genes is observed only at 48 hr after CP190 recruitment, suggesting that dCTCF alone is not sufficient for insulator activity. This suggests that regulation of CP190 recruitment is a mechanism of regulating insulator activity and that insulator sites without CP190 may be inactive sites poised for activation.

The effect of the Eip75B 20-HE-induced insulator on the expression of adjacent genes appears to be mediated by the establishment of intrachromosomal interactions with adjacent sites that create a specific chromatin organization. The loops formed by these interactions seem to be already in place prior to ecdysone treatment, but recruitment of CP190 to the poised

insulator increases the strength of the interactions or the frequency of loop formation. This suggests that, at least at this locus, there is a threshold in the plasticity of the chromatin that is critical for an insulator to affect enhancer-promoter interactions. Recruitment of CP190 regulates the level of chromatin looping within the region such that a more dynamic chromatin organization may allow for enhancer-promoter contacts, whereas a more structured and less dynamic chromatin organization may prevent these interactions. Furthermore, the promoters of transcripts affected by CP190 knockdown do not all localize within one CP190-mediated loop, as suggested by classic insulator function models, again suggesting that it may be an overall decrease in chromatin mobility and not loop formation itself that causes the enhancer-blocking activity of insulator elements.

Insulator proteins have been implicated in chromatin organization and gene expression, but there have been few studies that shed light on the mechanisms by which insulator activity can be regulated to orchestrate the establishment of different patterns of gene expression during cell differentiation. Here, we provide evidence that insulator protein recruitment is regulated in response to various stimuli and that this regulation plays a role in stabilizing chromatin loops. How CP190 is recruited to the specific site in Eip75B-RB upon 20-HE stimulation remains unclear, as dCTCF is present at the site before induction. There must be other forms of regulation, such as protein modifications, binding of additional cofactors, or release of inhibitors, that lead to CP190 recruitment to specific sites after 20-HE treatment. However, the ability to regulate CP190 recruitment to chromatin provides an important mechanism by which cells may control the organization and expression of the genome within the three-dimensional nuclear space.

EXPERIMENTAL PROCEDURES

Treatment of Cells with 20-HE and dsRNA

Kc167 cells were grown in CCM3 serum-free insect media (HyClone SH30065.01) at 25°C. For RNAi treatment, cells were plated at 0.5 × 10^6 cells/ml and incubated with 4μg/ml of either control dsRNA targeting the *β-galactosidase* gene or dsRNA targeting the *CP190* gene using Cellfectin II reagent (Invitrogen 10362-100). After addition of dsRNA, cells were grown for 3 days, replated at 0.5 × 10^6 cells/ml, and retreated with 4μg/ml of dsRNA as before. Cells were harvested after 3 additional days of growth (day 6). For ecdysone treatment, cells were plated at 0.5 × 10^6 cells/ml and grown overnight. Cells were treated with 20-HE (Sigma) at a final concentration of 5 × 10^{-7} M for 3 or 48 hr. For experiments in which mock 20-HE treatment is indicated, cells were treated with EtOH. For cells treated with RNAi and 20-HE, the hormone was added on day 4 of knockdown for 48 hr treatment and day 6 of knockdown for 3 hr treatment; all cells were harvested on day 6. The degree of CP190 knockdown is depicted in Figure S3.

Heat-Shock and Immunofluorescence Analysis of Tissues from Third-Instar Larvae

Heat shock treatment and immunofluorescence analysis of polytene chromosomes and imaginal tissue was done as described previously (Ivaldi et al., 2007). Cells were stained with primary antibodies in antibody dilution buffer (1 × PBS, 0.1% Tween20, 1% BSA) overnight at 4°C (1:1000 rabbit α-CP190 [Pai et al., 2004], 1:100 rabbit α-Su[Hw] [Gerasimova et al., 1995], 1:50 rabbit α-BEAF [Bushey et al., 2009], 1:100 guinea pig α-dCTCF [gift from Elissa Lei], and 1:200 mouse α-Pol IIo^{Ser5} [H14, Covance]). For immunofluorescence of imaginal discs, tissue was processed as previously described (Gerasimova et al., 2007) and slides were stained with 1:2000 rabbit α-CP190 and 1:500 mouse α-Pol IIo^{Ser5} (H14, Covance).

ChIP-Seq Analysis

Chromatin immunoprecipitation was performed as described (Bushey et al., 2009). To generate sequencing libraries, ChIP DNA was prepared for adaptor ligation by end repair (End-It DNA End Repair Kit, Epicenter Cat# ER0720) and addition of "A" base to 3′ ends (Klenow 3′-5′ exo–, NEB Cat# M0212S). Illumina adaptors (Illumina Cat# PE-102-1001) were titrated according to prepared DNA ChIP sample concentration and ligated with T4 ligase (NEB Cat# M0202S). Ligated ChIP samples were PCR-amplified using Illumina primers and Phusion DNA polymerase (NEB Cat# F-530L) and size selected for 200–300 bp by gel extraction. ChIP libraries were sequenced at the HudsonAlpha Institute for Biotechnology, using an Illumina GAII sequencer. Sequences were mapped to the dm3 genome with Bowtie 0.12.3 (Langmead et al., 2009) using default settings. Peaks were then called with MACS 1.4.0alpha2 (Zhang et al., 2008) using equal numbers of unique reads for input and ChIP samples and a p value cutoff of 1 × 10^{-10}. To determine up- and downregulated insulator sites at each time point, ChIP-Seq data for each insulator protein were normalized by total read count between the different time points. The data were then filtered by a second normalization for lane effect differences of the mean plus 1/2 standard deviation of lane-specific read counts. The ChIP-time point-A/ChIP-time point-B $Log_2()$ ratio was determined and assigned for every 100 bp. Significant changes in insulator occupancy were then called as up- or downregulated using CMARRT, a modified correlation and moving average algorithm (Kuan et al., 2008), and a stringent p value cutoff of 1 × 10^{-10}. For a list of regulated insulator sites, see Table S2.

3C Analysis

3C-qPCR analysis was done essentially as described (Hagège et al., 2007) with a few minor adaptations. 1 × 10^7 cells were crosslinked with 1% formaldehyde for 10 min at room temperature. Cell lysis was performed in 10 mM Tris (pH 8), 10 mM NaCl, 0.2% NP-40, and complete EDTA-free mini protease inhibitors (Robbins et al., 2007) for 15 min on ice. Following the initial phenol-chloroform/EtOH extraction, the sample was resuspended in 200 μl H_2O. A second phenol-chloroform /EtOH extraction was then performed, and the pellet was washed three times in 70% EtOH before final resuspension in 10 mM Tris (pH 7.5). Total DNA concentration was then measured using qPCR and Sybr green detection, and all samples were diluted to 50 ng/μl. Crosslinking frequency was determined using a control sample generated with BACR05B18 and a TaqMan MGB probe (Applied Biosystems) homologous to the anchor fragment (6FAM-TCCTATAACAGAATTTGATCTGA-MGBNFQ). Primers used for anchor and bait fragments can be found in Table S3. qPCR was performed with TaqMan Universal PCR Master Mix (Applied Biosystems 4324018). The data presented represent an average of four biological replicates.

Gene Expression Analysis

RNA was purified from Kc cells with the RNeasy kit (QIAGEN), and cDNA synthesis was performed with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). For qPCR analysis, primers were designed to span transcript-specific exon junctions. Primer sequences can be found in Table S4. Real-time PCR was performed, and data were normalized to Act5C. For microarray analysis, cDNA was digested with RNase A for 30 min at 37°C and cleaned by running through a PCR purification column (QIAGEN). Sample labeling, hybridization, and data extraction were performed by the FSU-Nimblegen Microarray facility (http://www.bio.fsu.edu/nimblegen_microarray.php) using Nimblegen 12 × 135K arrays. Two biological replicates were performed for each sample. To determine genes that change in expression after 20-HE induction, we used ArrayStar software (DNASTAR, Inc.) and included a statistical cutoff of 10% FDR generated with a moderated t test with Benjamini Hochberg multiple testing correction and a fold change of 1.5.

ACCESSION NUMBERS

ChIP-seq and gene expression data are deposited in NCBI's Gene Expression Omnibus (GEO) under accession numbers GSE30740 and GSE30686, respectively. Data can also be found under SuperSeries accession number GSE30741.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at doi:10.1016/j.molcel.2011.07.035.

ACKNOWLEDGMENTS

We would like to thank Dr. Elissa Lei for sharing antibodies and RNAi protocols. We also thank The Genomic Services Lab at the HudsonAlpha Institute for Biotechnology for their help in performing Illumina sequencing of ChIP-Seq samples. This work was supported by U.S. Public Health Service Award GM35463 from the National Institutes of Health. K.V.B. was supported by NIH training grant 2T32GM008367.

Received: February 8, 2011
Revised: June 2, 2011
Accepted: July 29, 2011
Published: October 6, 2011

REFERENCES

Ashburner, M. (1974). Sequential gene activation by ecdysone in polytene chromosomes of Drosophila melanogaster. II. The effects of inhibitors of protein synthesis. Dev. Biol. *39*, 141–157.

Ashburner, M., and Bonner, J.J. (1979). The induction of gene activity in drosophilia by heat shock. Cell *17*, 241–254.

Burtis, K.C., Thummel, C.S., Jones, C.W., Karim, F.D., and Hogness, D.S. (1990). The *Drosophila* 74EF early puff contains E74, a complex ecdysone-inducible gene that encodes two ets-related proteins. Cell *61*, 85–99.

Bushey, A.M., Dorman, E.R., and Corces, V.G. (2008). Chromatin insulators: regulatory mechanisms and epigenetic inheritance. Mol. Cell *32*, 1–9.

Bushey, A.M., Ramos, E., and Corces, V.G. (2009). Three subclasses of a Drosophila insulator show distinct and cell type-specific genomic distributions. Genes Dev. *23*, 1338–1350.

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

Dekker, J., Rippe, K., Dekker, M., and Kleckner, N. (2002). Capturing chromosome conformation. Science *295*, 1306–1311.

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

Gauhar, Z., Sun, L.V., Hua, S., Mason, C.E., Fuchs, F., Li, T.R., Boutros, M., and White, K.P. (2009). Genomic mapping of binding regions for the Ecdysone receptor protein complex. Genome Res. *19*, 1006–1013.

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.

Gerasimova, T.I., Lei, E.P., Bushey, A.M., and Corces, V.G. (2007). Coordinated control of dCTCF and gypsy chromatin insulators in *Drosophila*. Mol. Cell *28*, 761–772.

Hagège, H., Klous, P., Braem, C., Splinter, E., Dekker, J., Cathala, G., de Laat, W., and Forné, T. (2007). Quantitative analysis of chromosome conformation capture assays (3C-qPCR). Nat. Protoc. *2*, 1722–1733.

Ivaldi, M.S., Karam, C.S., and Corces, V.G. (2007). Phosphorylation of histone H3 at Ser10 facilitates RNA polymerase II release from promoter-proximal pausing in Drosophila. Genes Dev. *21*, 2818–2831.

Kuan, P.F., Chun, H., and Keleş, S. (2008). CMARRT: a tool for the analysis of ChIP-chip data from tiling arrays by incorporating the correlation structure. Pac. Symp. Biocomput. 515–526.

Langmead, B., Trapnell, C., Pop, M., and Salzberg, S.L. (2009). Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. *10*, R25.

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

Maeda, R.K., and Karch, F. (2007). Making connections: boundaries and insulators in Drosophila. Curr. Opin. Genet. Dev. *17*, 394–399.

Majumder, P., Gomez, J.A., and Boss, J.M. (2006). The human major histocompatibility complex class II HLA-DRB1 and HLA-DQA1 genes are separated by a CTCF-binding enhancer-blocking element. J. Biol. Chem. *281*, 18435–18443.

Mohan, M., Bartkuhn, M., Herold, M., Philippen, A., Heinl, N., Bardenhagen, I., Leers, J., White, R.A., Renkawitz-Pohl, R., Saumweber, H., and Renkawitz, R. (2007). The Drosophila insulator proteins CTCF and CP190 link enhancer blocking to body patterning. EMBO J. *26*, 4203–4214.

Nègre, N., Brown, C.D., Shah, P.K., Kheradpour, P., Morrison, C.A., Henikoff, J.G., Feng, X., Ahmad, K., Russell, S., White, R.A., et al. (2010). A comprehensive map of insulator elements for the Drosophila genome. PLoS Genet. *6*, e1000814.

Oliver, D., Sheehan, B., South, H., Akbari, O., and Pai, C.Y. (2010). The chromosomal association/dissociation of the chromatin insulator protein Cp190 of Drosophila melanogaster is mediated by the BTB/POZ domain and two acidic regions. BMC Cell Biol. *11*, 101.

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.

Phillips, J.E., and Corces, V.G. (2009). CTCF: master weaver of the genome. Cell *137*, 1194–1211.

Riddiford, L.M., Cherbas, P., and Truman, J.W. (2000). Ecdysone receptors and their biological actions. Vitam. Horm. *60*, 1–73.

Robbins, M.J., Starr, K.R., Honey, A., Soffin, E.M., Rourke, C., Jones, G.A., Kelly, F.M., Strum, J., Melarange, R.A., Harris, A.J., et al. (2007). Evaluation of the mGlu8 receptor as a putative therapeutic target in schizophrenia. Brain Res. *1152*, 215–227.

Segraves, W.A., and Hogness, D.S. (1990). The E75 ecdysone-inducible gene responsible for the 75B early puff in Drosophila encodes two new members of the steroid receptor superfamily. Genes Dev. *4*, 204–219.

Stevens, B., Alvarez, C.M., Bohman, R., and O'Connor, J.D. (1980). An ecdysteroid-induced alteration in the cell cycle of cultured *Drosophila* cells. Cell *22*, 675–682.

Wendt, K.S., and Peters, J.M. (2009). How cohesin and CTCF cooperate in regulating gene expression. Chromosome Res. *17*, 201–214.

Xu, Z., Wei, G., Chepelev, I., Zhao, K., and Felsenfeld, G. (2011). Mapping of INS promoter interactions reveals its role in long-range regulation of SYT8 transcription. Nat. Struct. Mol. Biol. *18*, 372–378.

Zhang, Y., Liu, T., Meyer, C.A., Eeckhoute, J., Johnson, D.S., Bernstein, B.E., Nusbaum, C., Myers, R.M., Brown, M., Li, W., and Liu, X.S. (2008). Model-based analysis of ChIP-Seq (MACS). Genome Biol. *9*, R137.