The Gypsy Insulator of Drosophila Affects Chromatin Structure in a Directional Manner

Siquan Chen and Victor G. Corces

Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218

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ABSTRACT

Chromatin insulators are thought to regulate gene expression by establishing higher-order domains of chromatin organization, although the specific mechanisms by which these sequences affect enhancer-promoter interactions are not well understood. Here we show that the gypsy insulator of Drosophila can affect chromatin structure. The insulator itself contains several DNase I hypersensitive sites whose occurrence is dependent on the binding of the Suppressor of Hairy-wing [Su(Hw)] protein. The presence of the insulator in the 5′ region of the *yellow* gene increases the accessibility of the DNA to nucleases in the promoter-proximal, but not the promoter-distal, region. This increase in accessibility is not due to alterations in the primary chromatin fiber, because the number and position of the nucleosomes appears to be the same in the presence or absence of the insulator. Binding of the Su(Hw) protein to insulator DNA is not sufficient to induce changes in chromatin accessibility, and two domains of this protein, presumed to be involved in interactions with other insulator components, are essential for this effect. The presence of Modifier of mdg4 [Mod(mdg4)] protein, a second component of the gypsy insulator, is required to induce these alterations in chromatin accessibility. The results suggest that the gypsy insulator affects chromatin structure and offer insights into the mechanisms by which insulators affect enhancer-promoter interactions.

THE nuclear organization of DNA plays an important role in the regulation of eukaryotic gene expression (Lamond and Earnshaw 1998). This organization is first accomplished by packaging the DNA into a nucleoprotein structure, chromatin, at multiple levels of organization. The chromatin fiber is in turn thought to be organized into loops, perhaps attached to a substrate and arranged within the nucleus according to a specific pattern (Manuelidis and Chen 1990; De Boni 1994; Yokota *et al.* 1995). DNA sequences called MARs (matrix attachment regions) or SARs (scaffold attachment regions) have been proposed to attach the chromosome loops to the nuclear matrix or scaffold and consequently they might delimit higher-order chromatin domains (Mirkovitch *et al.* 1984; Luderus and van Driel 1997). Although MARs or SARs have been mapped near some genes (Gasser and Laemmli 1986), it is still not clear whether these sequences are just structural components of the chromatin or whether they play a functional role. More recently, a group of DNA sequences named chromatin insulators or boundary elements have been shown to share properties suggestive of a functional role in nuclear organization. Insulators have the ability to protect transgenes from chromosomal position effects (Kellum and Schedl 1991; Roseman *et al.* 1995) and to block communication between enhancers and promoters only when present between them (Holdridge and Dorsett 1991; Geyer and Corces 1992; Kellum and Schedl 1992; Chung *et al.* 1993). Furthermore, an enhancer that is blocked from activating a promoter by an insulator placed between the two is still functional and able to activate transcription from a promoter that is not blocked by the insulator (Cai and Levine 1995; Scott and Geyer 1995). Chromatin insulators have been identified in a variety of organisms including yeast, Drosophila, and vertebrates (Corces and Felsenfeld 2000).

The mechanisms of insulator function are not clear, but some evidence suggests an involvement of chromatin structure. For example, insulators are found at the boundaries between active and inactive loci (Zhao *et al.* 1995; Prioleau *et al.* 1999). In the case of the chicken β-globin gene, the active locus is marked by sensitivity to DNase I digestion and has a high level of histone acetylation, whereas the neighboring inactive DNA is resistant to DNase I digestion and is hypoacetylated (Hebbes and Allen 2000; Saitoh *et al.* 2000). This implies that insulators could interrupt the propagation of chromatin changes (positive or negative) along the DNA by forming topologically distinct chromatin domains. The only direct evidence of an altered chromatin structure caused by an insulator comes from studies of the chicken β-globin gene. The locus control region has been shown to be able to activate a reporter gene in transformed cell lines, and this activation is blocked

Corresponding author: Victor G. Corces, Department of Biology, The Johns Hopkins University, 3400 N. Charles St., Baltimore, MD 21218. E-mail: corces@jhu.edu

by the presence of the chicken β-globin insulator. The accessibility to a specific restriction enzyme at the promoter region decreased when the β-globin insulator was present, suggesting a shift in the position of a nucleosome present at the promoter region (Chung *et al.* 1993).

Much of the evidence in favor of an effect of the gypsy insulator on chromatin structure comes from the studies of one of its protein components, Modifier of mdg4 [Mod(mdg4)] (Gerasimova *et al.* 1995, 2000; Gerasimova and Corces 1998). The gypsy insulator has 12 binding sites for the Suppressor of Hairy-wing [Su(Hw)] protein, which is essential for insulator function (Harrison *et al.* 1993). Mod(mdg4) does not bind to DNA directly and, instead, exerts its function through interactions with Su(Hw); the Mod(mdg4) protein contains a BTB domain at its amino terminus that is also present in various transcription factors, including GAGA (Dorn *et al.* 1993; Gerasimova *et al.* 1995). Hypomorphic mutations in *mod(mdg4)* cause a partial inactivation of the gypsy insulator, resulting in groups of cells in which the insulator is active and the enhancers are blocked, whereas in other cells the insulator is inactive and transcription is normal (Gerasimova *et al.* 1995). This phenotype is reminiscent of the typical position effect variegation displayed by mutations caused by rearrangements that bring a gene in close proximity to heterochromatin, a phenomenon that has been shown to involve changes in chromatin structure (Wallrath and Elgin 1995). Furthermore, mutations in *mod(mdg4)* act as enhancers of the variegated phenotype of *white-mottled 4* (w^{m4}), which is caused by a rearrangement of the *white* gene with a breakpoint close to the centromeric heterochromatin, resulting in partial inactivation of *white* gene expression. Therefore, a role of Mod(mdg4) might be to repress the formation of heterochromatin (Dorn *et al.* 1993; Gerasimova *et al.* 1995). A second property of *mod(mdg4)* indicating its involvement in chromatin organization is its genetic behavior as a member of the *trithorax-Group* (*trxG*) gene family (Gerasimova and Corces 1998). TrxG proteins are thought to antagonize the effect of Polycomb-Group (PcG) proteins, which repress the expression of homeotic genes in a manner that is maintained through cell division (Cavalli and Paro 1998). The mechanism for this stable repression is thought to involve alterations of chromatin structure (Cavalli and Paro 1998; Sudarsanam and Winston 2000). This idea is supported by recent findings indicating that a complex of PcG proteins is able to block the ability of nucleosomal arrays to be remodeled by SWI/SNF (Shao *et al.* 1999).

To directly test whether the gypsy insulator has the ability to affect chromatin organization, we studied its effect on chromatin structure by nuclease sensitivity analysis at the *yellow* locus. The data presented here suggest that insulators may play an active role in regulating gene expression by mediating alterations in chromatin structure that in turn affect transcriptional activation.

MATERIALS AND METHODS

Chromatin structure analyses: Flies were maintained in standard medium and grown at 22.5° and 75% relative humidity. Third instar larvae were handpicked, weighed, frozen in liquid nitrogen, and stored at −80° until use. Nuclei were purified from 1–1.2 g of frozen larvae according to Lu *et al.* (1993). For DNase I digestion, the isolated nuclei were resuspended in 1 ml of 1× DNase I digestion buffer [60 mm KCl, 15 mm NaCl, 15 mm Tris-HCl pH 7.4, 0.25 m sucrose, 3 mm $MgCl_2$, 0.05 mm dithiothreitol (DTT)] and 250 μl of the nuclear suspension were incubated with 2, 4, 6, or 8 μl of DNase I (10 units/μl; Boehringer Mannheim BioChemicals, Indianapolis) on ice for 3 min with agitation. The digestion was terminated by the addition of 5 μl 0.5 m EDTA. For micrococcal nuclease digestion, the isolated nuclei were resuspended in 1 ml of MNase digestion buffer (60 mm KCl, 15 mm NaCl, 15 mm Tris-HCl pH 7.4, 0.5 mm DTT, 0.25 m sucrose, 1 mm $CaCl_2$) and 250 μl of the nuclear suspension was incubated with 1.0, 1.5, 2.0, or 2.5 units of MNase (Worthington Biochemical, Freehold, NJ) at 23° for 3 min with agitation. The digestion was terminated by the addition of 5 μl 0.5 m EDTA.

After termination of the nuclease digestion reaction, 10 μl 10% SDS and 1.0 μl proteinase K (10 mg/ml) were added and the nuclei were incubated at 37° overnight. The DNA was extracted with an equal volume of phenol-chloroform isoamylalcohol (24:24:1) twice and once with chloroform. One volume of dH_2O and 2 μl 10 mg/ml RNase A were then added and the mixture was incubated at 40° for 2 hr. The DNA was precipitated with 1/10 volumes NaOAc, 2 volumes ethanol, and 1 μl 10 mg/ml yeast tRNA at −20° overnight. The precipitated DNA was collected by spinning at 4° for 30 min and resuspended in 16 μl of dH_2O.

Southern analysis: Purified DNA (10 μg) was fractionated by electrophoresis on a 1.5% agarose gel and transferred to a Nytran Plus membrane (Schleicher & Schuell, Keene, NH). DNA was crosslinked to the membrane by UV irradiation. ^{32}P-labeled DNA probes were prepared by hexamer oligonucleotide random priming. Blot hybridization was carried out as described by Sambrook *et al.* (1989). Quantitation was performed using a PhosphoImager (Molecular Dynamics, Sunnyvale, CA). Blots were reused after stripping the probe by boiling for 5 min in an excess of buffer (10 mm Tris pH 7.5, 1 mm EDTA, 1% SDS). Each experiment was performed at least twice. The probes used in the nuclease sensitivity experiments were constructed as follows: Probe A was generated by cutting a plasmid (p4S-S) containing the entire *yellow* gene (Geyer and Corces 1992) with *Sal*I and *Nco*I and isolating the resulting 667-bp fragment. Probe B was generated by PCR using p4S-S as a template and the primers GGCCGACATAT TATGGCCACCAGTCG and CACCCTTTGTCCTGGAACAT TGC. The resulting 212-bp fragment was cloned into the TA cloning vector pCR II (Invitrogen, San Diego) and this plasmid was called pCRIIy2852-3063. Probe C was generated similarly, but the primers used were GTGTCGCTGGTTGTTTAC and GGTAATTCCACTAGCTGT. The resulting 215-bp fragment was cloned into the pCR II vector and this clone was called pCRIIy1761-1975. Probe D was generated by cutting p4S-S with *Nco*I and *Hin*dIII and isolating the resulting 354-bp fragment. Probe E was generated by isolating the 303-bp fragment after digestion of plasmid p4S-S with *Bam*HI and *Hin*cII.

RESULTS

The gypsy insulator is constitutively sensitive to nucleases: To explore the effect of the gypsy insulator on chromatin organization, we first examined the chromatin structure of the insulator itself. In particular, we ana-

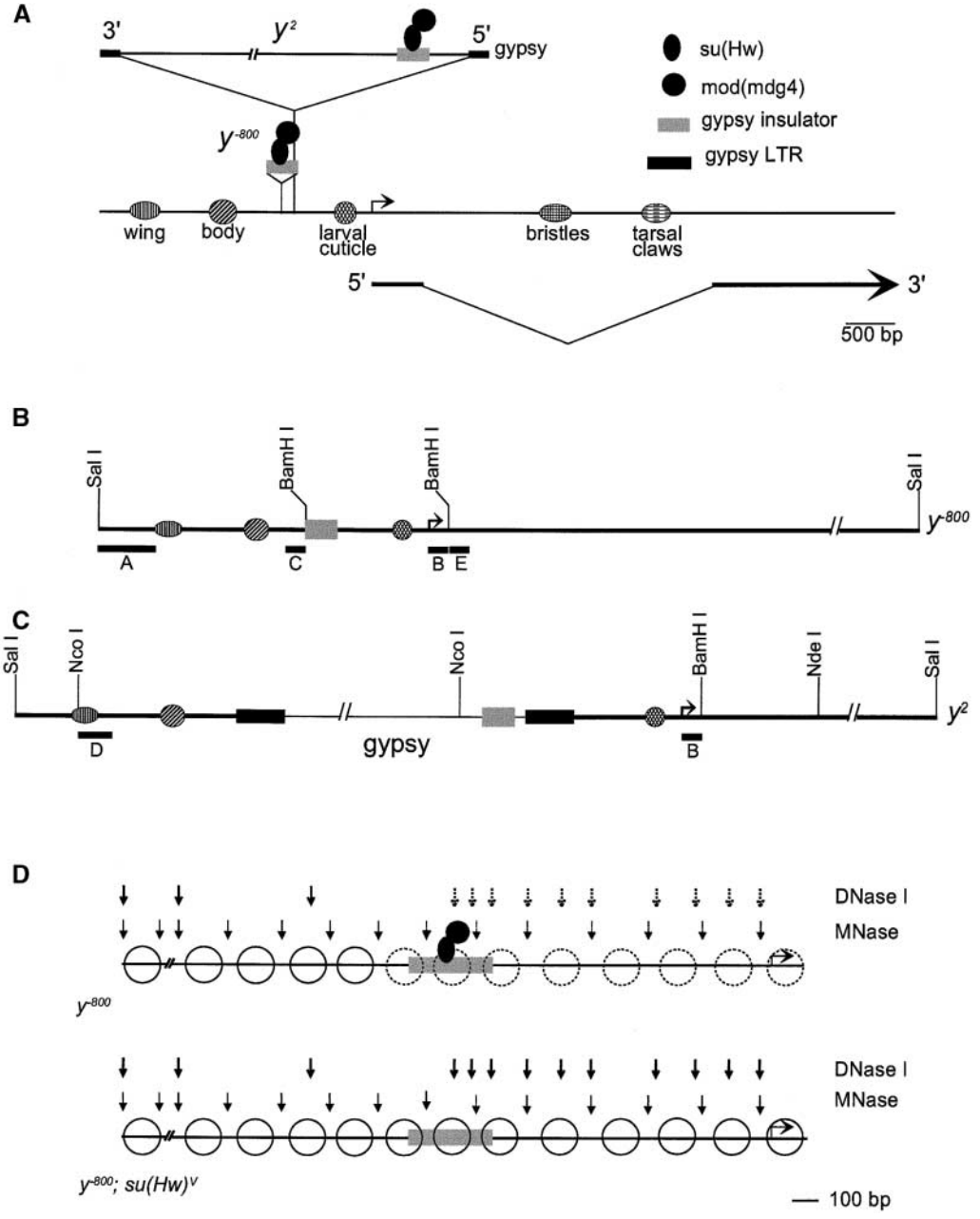

FIGURE 1.—Structure and chromatin organization of the *yellow* locus. (A) Organization of the *yellow* gene and the y^2 and y^{-800} alleles. The *yellow* gene has five tissue-specific enhancers represented by ovals with different shadings; these enhancers are located in the 5′ region and the intron of the gene, and they control its expression in wings, adult body cuticle, larval cuticle, adult bristles, and tarsal claws. The y^2 allele contains a complete *gypsy* element inserted 700 bp upstream from the transcription start site. The gypsy insulator in the *gypsy* element is represented by a shaded box with the two known protein components, Su(Hw) and Mod(mdg4), represented as solid ovals. Solid boxes flanking the *gypsy* element represent the long terminal repeats (LTRs). The y^{-800} allele was constructed by inserting the gypsy insulator 800 bp upstream from the transcription start site of the *yellow* gene; the construct was then inserted into $Df(1)y^-\ ac^-$ flies by germline transformation (GEYER and CORCES 1992). (B) Restriction map of the *yellow* gene in y^{-800} flies with the enzymes used in the studies described in the text. The gypsy insulator is shown as a shaded box. Probes used in the Southern analysis described in the text are shown as solid bars. (C) Restriction map of the *yellow* gene in y^2 flies. The *gypsy* element is depicted as a thin line, with the LTRs represented as solid boxes and the insulator as a shaded box. (D) Schematic drawings summarizing the results of the nuclease accessibility analyses in the promoter-proximal and distal regions of the *yellow* gene; the location of the promoter is indicated by a bent arrow. The top part of the diagram shows the location of MNase and DNase I sites in y^{-800} flies, which contain a functional gypsy insulator (represented by a shaded box); the location of nucleosomes, inferred from the position of MNase sites, is indicated by circles; dotted circles and arrowheads denote a higher accessibility to nucleases. The bottom part of the diagram shows the same information for $y^{-800}; su(Hw)^V$ flies, which lack a functional insulator.

lyzed the *gypsy* element inserted in the *yellow* (*y*) gene in two different *y* alleles. The *yellow* gene has at least five enhancers that control its expression in five different tissues (Figure 1A). The y^2 mutation is caused by the insertion of the *gypsy* element 700 bp upstream from the transcription start site between the two enhancers that control *yellow* expression in the wing and body cuticle and the promoter. The insulator present in the *gypsy* element disrupts the interaction between the wing and body enhancers and the promoter, giving rise to a mutant phenotype in the wing and body cuticle (GEYER *et al.* 1986). The y^{-800} strain shows a similar phenotype. This strain was constructed by inserting a 350-bp region, containing all 12 Su(Hw) binding sites present in the *gypsy* insulator, 800 bp upstream from the transcription start site (Figure 1A); the resulting plasmid was then introduced into a strain carrying a deletion of the endogenous *yellow* gene by *P*-element-mediated transformation (GEYER and CORCES 1992).

We first studied the chromatin structure of the gypsy

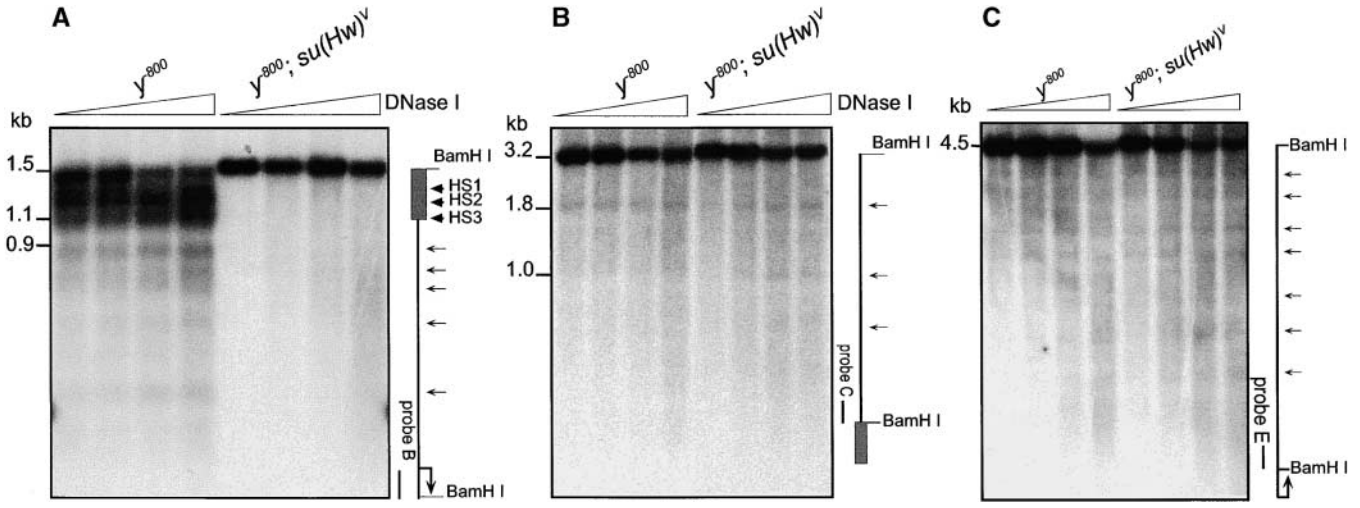

FIGURE 2.—DNase I accessibility analysis of the gypsy insulator and the surrounding regions of the *yellow* gene. Sizes in kilobases are indicated on the left of A, B, and C. A schematic drawing of the positions of the DNase I hypersensitive sites is shown on the right of A, B, and C; the *yellow* gene is shown as a thin line and the gypsy insulator is shown as a solid box. The transcription start site is indicated by an arrow. (A) DNase I accessibility of the gypsy insulator in y^{-800} and y^{-800}; *su(Hw)*^{V} flies. Three DNase I hypersensitive sites present within the gypsy insulator are indicated by arrowheads; hypersensitive sites present between the insulator and the promoter are indicated by arrows. (B) DNase I accessibility analysis of the region upstream from the gypsy insulator in y^{-800} and y^{-800}; *su(Hw)*^{V} flies. (C) DNase I hypersensitivity analysis of the region downstream from the *yellow* promoter. The *Bam*HI site located at the 3′ end is not present in the genome; it is located in the plasmid used to construct the y^{-800} strain.

insulator in y^{-800} flies by examining the sensitivity to digestion by DNase I. Isolated nuclei from third instar larvae were incubated with increasing amounts of DNase I. Genomic DNA was then purified and digested with *Bam*HI. The samples were subjected to Southern analysis using probe B (Figure 1B). The results of this experiment are shown in Figure 2A; the 1.5-kb band corresponds to the undigested *Bam*HI-*Bam*HI fragment present in the y^{-800} strain (Figure 1B). In y^{-800} flies, the gypsy insulator contains three strong DNase I hypersensitive sites, named HS1, HS2, and HS3 (indicated by arrowheads to the right of Figure 2A). Interestingly, these three DNase I hypersensitive sites located in the Su(Hw) binding region are not present in y^{-800}; *su(Hw)*^{V} flies (Figure 2A). These hypersensitive sites are also absent in chromatin preparations from wild-type Canton-S larvae (data not shown). These results suggest that binding of the Su(Hw) protein to the gypsy insulator causes changes in chromatin structure that increase the accessibility of the region to DNAse I.

The presence of the gypsy insulator changes the chromatin structure in the promoter-proximal, but not the promoter-distal, region: The effect of the insulator is not to repress transcription by silencing gene expression, but rather to interfere with enhancer-promoter communication. The nature of this communication is at the heart of how enhancers activate transcription, which involves the transmission of a signal from the enhancer to the promoter. This signal could be a direct interaction between enhancer-bound transcription factors and components of the transcription complex at the promoter or alterations in chromatin structure propagated along the primary chromatin fiber or at a higher level of chromatin structure. To test whether the gypsy insulator could interfere with such a process by affecting chromatin, we examined possible effects of this insulator on the chromatin structure of the surrounding regions. Five DNase I hypersensitive sites are present between the site of the gypsy insulator and the promoter in y^{-800} flies (labeled by thin arrows at the right of Figure 2A). Interestingly, these hypersensitive sites disappear when the Su(Hw) protein is not present and the insulator is not functional in a y^{-800}; *su(Hw)*^{V} mutant. These sites are also absent in wild-type Canton-S larvae (data not shown), suggesting that their absence in y^{-800}; *su(Hw)*^{V} larvae is not due to a global effect on chromatin structure caused by the lack of Su(Hw) protein but rather to a local effect due to the lack of a functional insulator.

The same Southern blot shown in Figure 2A was stripped and reprobed with probe C (Figure 1B) to examine possible changes in DNase I accessibility in the region upstream from the gypsy insulator. The results show the presence of three minor DNase I hypersensitive sites in the region upstream from the insulator in y^{-800} flies (labeled by arrows on the side of Figure 2B), and these sites remain unchanged in y^{-800}; *su(Hw)*^{V} (Figure 2B) and the wild-type Canton-S strain (data not shown). The results of this experiment also serve as a control for equal loading and digestion of the different DNA samples present on the gel. We also analyzed the effect of the gypsy insulator on the chromatin structure downstream from the promoter of the *yellow* gene. The blot shown in Figure 2A was rehybridized with probe E (Figure 1B), and the result is shown in Figure 2C. The

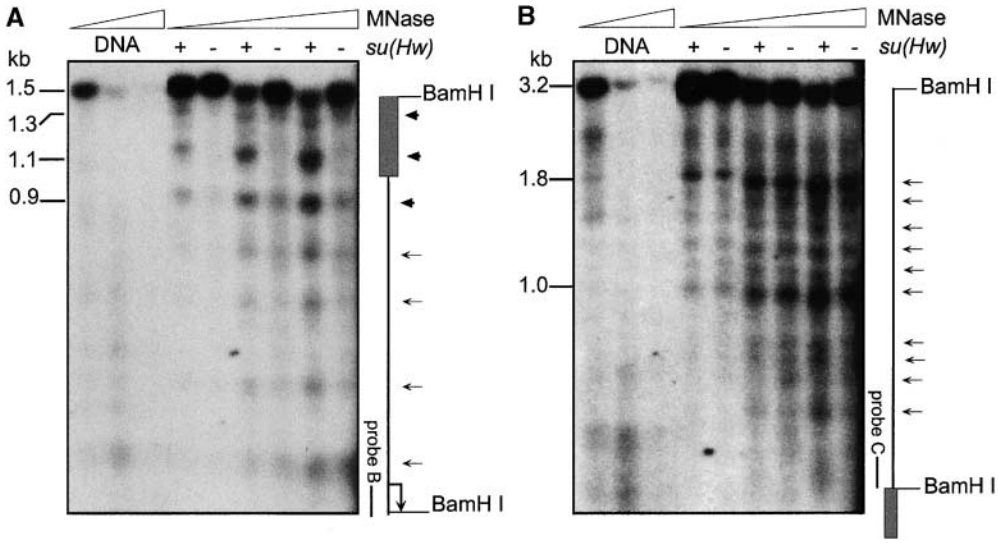

FIGURE 3.—MNase accessibility of the gypsy insulator and the surrounding regions in y^{-800} and y^{-800}; $su(Hw)^V$ flies. Sizes in kilobases are indicated on the left of A and B. A schematic drawing of the positions of the MNase hypersensitive sites is shown on the right of A and B; the *yellow* gene is shown as a thin line and the gypsy insulator is shown as a solid box. The transcription start site of the *yellow* gene is indicated by an arrow. A control of naked DNA from y^{-800} flies digested in the same conditions is also shown. (A) MNase analysis of the gypsy insulator and the downstream promoter-proximal region; a + in the top of the figure indicates y^{-800} flies and a − indicates y^{-800}; $su(Hw)^V$ flies. (B) MNase analysis of the region upstream of the gypsy insulator in y^{-800} (lanes labeled +) and y^{-800}; $su(Hw)^V$ flies (lanes labeled −).

region downstream from the *yellow* promoter is relatively insensitive to DNase I. Several hypersensitive sites distributed throughout the region are the same in the presence or absence of mutations in the *su(Hw)* gene. The results of these experiments suggest that the presence of the gypsy insulator induces several DNase I hypersensitive sites in the promoter-proximal region with respect to its insertion site, whereas the insulator does not alter the chromatin structure in the promoter-distal region upstream from its location or downstream from the promoter.

The gypsy insulator does not affect chromatin structure at the nucleosome level: The DNase I hypersensitivity results suggest that the presence of the gypsy insulator selectively creates a more open chromatin structure in the promoter-proximal *vs.* promoter-distal regions. To further study the basis for these changes in chromatin organization, we used micrococcal nuclease (MNase) digestion to determine the arrangement of nucleosomes in this region. Nuclei from third instar larvae were incubated with increasing amounts of MNase for a period of time. The purified DNA was digested with *Bam*HI and subjected to Southern analysis using Probe B (Figure 1B). Figure 3A shows the results of these experiments. The 1.5-kb band corresponds to the parental *Bam*HI-*Bam*HI fragment undigested by MNase. The ladder of bands obtained after MNase digestion shows the location of internucleosome cutting sites in the gypsy insulator and in the downstream region of y^{-800} flies [labeled *su(Hw)*+ at the top of Figure 3A]. The position of these nucleosomes within the *yellow* locus is shown schematically in Figure 1D. Three very intense bands of ~1.3, 1.1, and 0.9 kb correspond to MNase sites within the gypsy insulator and the immediately adjacent downstream region (indicated in the map on the right of Figure 3A by arrowheads). On the basis of the intensity of these bands, it is apparent that the insulator region is more sensitive to the nuclease than the region downstream in flies with a functional gypsy insulator. In addition, both the insulator and the downstream region are insensitive to MNase in y^{-800}; $su(Hw)^V$ flies that lack a functional insulator [labeled *su(Hw)*− at the top of Figure 3A], supporting previous results obtained with DNase I. Even at high MNase concentration, these sequences are still more accessible in y^{-800} than in y^{-800}; $su(Hw)^V$ flies. Although the accessibility to MNase in y^{-800}; $su(Hw)^V$ larvae of both the insulator and the downstream region is low at low MNase concentrations, there is sufficient cutting with higher amounts of MNase to allow the mapping of the position of nucleosomes in both strains. These results suggest that, in the absence of a functional gypsy insulator [in y^{-800}; $su(Hw)^V$ flies], the DNA in the 5′ region of the *yellow* gene is assembled into an array of regularly spaced nucleosomes and this chromatin is relatively insensitive to digestion by nucleases. The presence of a functional insulator in y^{-800} flies does not alter the relative position of the nucleosomes in the adjacent region, but increases the accessibility of the chromatin to nucleases, suggesting that the insulator opens the chromatin. This increased accessibility might not take place at the level of the primary fiber but rather at a higher-order level of organization. This opening of the chromatin takes place in the region between the insulator and the promoter (summarized in Figure 1D).

To test whether the effect of the gypsy insulator also extends to the upstream promoter-distal chromatin, the blot in Figure 3A was stripped and reprobed with probe C (Figure 1B). The results are shown in Figure 3B. The accessibility to MNase in the upstream region is the same in both y^{-800} and y^{-800}; $su(Hw)^V$ flies. Eight regularly spaced nucleosomes were detected in this region. The two predominant sites sensitive to MNase (at 1.0 and

1.8 kb) were also detected in the DNase I hypersensitive assay (Figure 2B). The results suggest that, as is the case for the promoter-proximal region, the promoter-distal sequences are also organized in a nucleosomal array that is relatively insensitive to nucleases, but contrary to the downstream sequences, the accessibility of the upstream region to MNase is not altered by the presence of a functional gypsy insulator.

Specific domains of the Su(Hw) protein mediate the effects of the gypsy insulator on chromatin structure: The gypsy insulator contains at least two protein components characterized to date, Su(Hw) and Mod(mdg4), but additional proteins not yet identified could also be important for the function of this insulator. The effects of the gypsy insulator on chromatin structure could be due simply to the binding of Su(Hw) to DNA or to the assembly of a functional insulator. To distinguish between these two possibilities, we examined the effect of mutations in Su(Hw) known to affect its interactions with other proteins and insulator function, while not affecting its ability to bind DNA. The leucine zipper domain of the Su(Hw) protein and the adjacent sequences known as region B have been shown to be essential for the ability of the gypsy insulator to block enhancer function but not required for binding of Su(Hw) to DNA (Harrison *et al.* 1993; Gdula and Corces 1997); these two regions of Su(Hw) have been shown to be necessary and sufficient for its interaction with Mod(mdg4) (Ghosh *et al.* 2001). The $su(Hw)^{L775K}$ allele encodes a nonfunctional protein that can bind DNA but is unable to block the function of upstream enhancers; it is caused by a point mutation in the leucine zipper region that results in a change of the last leucine in the zipper to a lysine. The $su(Hw)^{D765N}$ allele has a point mutation in an aspartic acid residue not conserved among Su(Hw) proteins of different Drosophila species; this alteration has no effect on the function of the Su(Hw) protein or the gypsy insulator (Harrison *et al.* 1993). The $su(Hw)^{\Delta B}$ allele carries a deletion of the B region and has a similar phenotype to $su(Hw)^{L775K}$. To investigate whether the leucine zipper and B domains of Su(Hw) are important for the effects of the gypsy insulator on chromatin structure, we examined nuclease accessibility to the insulator region in flies carrying these *su(Hw)* mutations; in this case, we analyzed the *yellow* gene present in the y^2 allele, which contained a complete copy of the gypsy element instead of just insulator sequences (Figure 1A). Purified nuclei from third instar larvae of different strains were incubated with increasing amounts of DNase I for a period of time. Purified genomic DNA was digested with *Bam*HI and *Nco*I; the later restriction enzyme has a recognition site adjacent to the Su(Hw) binding region in the *gypsy* element (Figure 1C). The Southern blot was probed with probe B (Figure 1C) and the results are shown in Figure 4A. The 2.3-kb band corresponds to the *Bam*HI-*Nco*I parental fragment undigested by DNase I. Three intense bands between 2.3 and 1.6 kb correspond to hypersensitive sites located within the insulator, in agreement with the results obtained with the y^{-800} strain. A series of additional DNase I hypersensitive sites are located in the promoter-proximal region, both within the *gypsy* element and in the downstream adjacent sequences (see the right side of Figure 4A). The DNase I hypersensitive sites located in the 5′ region of the *yellow* gene are present at the same locations as in the y^{-800} strain (Figure 2A), but four new DNase I hypersensitive sites map to *gypsy* sequences located between the insulator and the *yellow* gene in the y^2 allele. The accessibility of both the insulator region and promoter-proximal sequences decreases in flies carrying mutations in the leucine zipper [$su(Hw)^{L775K}$] or B region [$su(Hw)^{\Delta B}$] of Su(Hw) with respect to wild-type flies. The Asp to Asn change in the $su(Hw)^{D765N}$ allele, which has no effect on the function of the Su(Hw) protein, also has no effect on the accessibility of the insulator or adjacent sequences to DNase I (Figure 4A).

To test whether mutations in the leucine zipper and B regions have an effect on chromatin structure of the upstream promoter-distal region, the same blot was stripped and reprobed with probe D (Figure 1C). Figure 4B shows three major DNase I hypersensitive sites present in this region that remain unaltered in the Su(Hw) mutants. These results support the previous conclusion that changes in chromatin structure caused by the presence of the gypsy insulator affect the downstream but not the upstream sequences. The findings also suggest that these changes are not due simply to binding of the Su(Hw) protein to the *gypsy* DNA. Interactions of Su(Hw) with other proteins through the leucine zipper and B regions are required for normal insulator function and for the observed changes in chromatin organization.

The Mod(mdg4) protein mediates changes in chromatin structure induced by the gypsy insulator: Since domains of the Su(Hw) protein thought to interact with Mod(mdg4), and perhaps with other proteins, are important for changes in chromatin structure induced by the gypsy insulator, we decided to test directly whether *mod(mdg4)* mutations also affect chromatin accessibility to DNase I. We used a combination of two *mod(mdg4)* alleles that allow survival to early pupal stages. The $mod(mdg4)^{T16}$ allele was induced by EMS, causes lethality in late embryonic/early larval stages, and behaves as a null (T. Gerasimova and V. Corces, unpublished data); $mod(mdg4)^{E(var)3\text{-}93D}$ is a *P*-element-induced allele that displays pupal lethality with some escapers developing to the adult stage (Dorn *et al.* 1993). The combination of both alleles in y^2; $mod(mdg4)^{T16}$/ $mod(mdg4)^{E(var)3\text{-}93D}$ flies causes early pupal lethality and allows the selection of third instar larvae for DNase I sensitivity experiments. Figure 5A shows that the three strong hypersensitive sites present in the gypsy insulator region (marked by arrowheads on the right side of Figure 5A) in wild-type flies are also present in y^2; $mod(mdg4)^{T16}$/ $mod(mdg4)^{E(var)3\text{-}93D}$ flies, but the hyper-

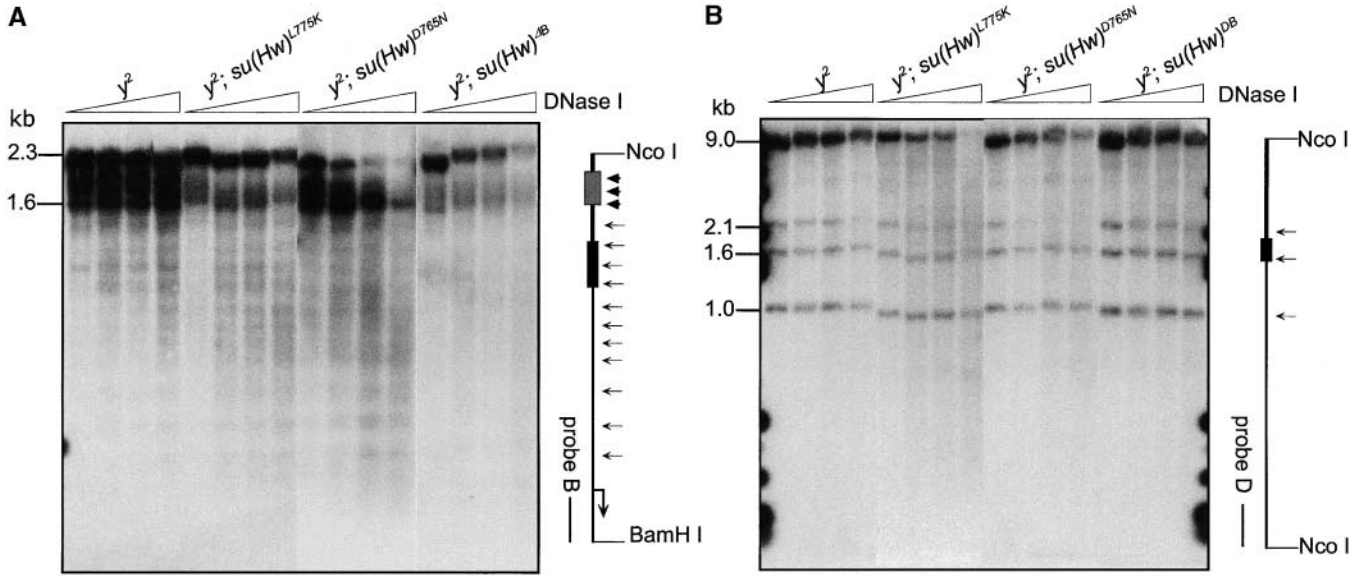

FIGURE 4.—DNase I accessibility analyses of the gypsy insulator and surrounding regions in y^2 flies and different *su(Hw)* mutants. Sizes in kilobases are indicated on the left and a schematic drawing of the positions of the DNase I hypersensitive sites is shown on the right of A and B. The *yellow* gene is shown as a thin line and the gypsy element is depicted as a thick line, with the LTRs represented as solid boxes and the gypsy insulator shown as a shaded box. The transcription start site is indicated by an arrow. Arrowheads represent hypersensitive sites within insulator sequences and arrows represents sites within the gypsy element, excluding the insulator, or the *yellow* gene. (A) DNase I analysis of the gypsy insulator and the promoter-proximal region in y^2 flies and different *su(Hw)* mutants. (B) DNase I analysis of the promoter-distal region of the gypsy insulator in y^2 flies and different *su(Hw)* mutants.

sensitive sites present in the promoter-proximal region disappear in flies carrying mutations in the *mod(mdg4)* gene. When the same blot was stripped and reprobed with Probe D (Figure 1C), no dramatic changes were observed in the pattern of DNase I hypersensitive sites in the promoter-distal region between wild-type and *mod(mdg4)* mutant flies, although three sites present within the gypsy element are more pronounced in the *mod(mdg4)* mutant larvae (Figure 5B). These results suggest that the observed changes in chromatin structure induced by the gypsy insulator in the promoter-proximal region require the binding of Su(Hw) to insulator sequences as well as the presence of Mod(mdg4) protein. The observed effects of *mod(mdg4)* mutations on the chromatin structure of sequences located upstream and downstream of the insulator correlate with the observed effect of these mutations on enhancers located proximal and distal to the insulator with respect to the promoter (GERASIMOVA *et al.* 1995).

DISCUSSION

Insulators are thought to play a role in chromatin organization. This idea originally came from the observation that insulators flank the regions of polytene chromosomes that become puffed in response to heat shock (UDVARDY *et al.* 1985). More recently, insulators at the chicken globin locus have been found to separate regions with different degrees of chromatin condensation and histone acetylation (PRIOLEAU *et al.* 1999; SAITOH *et al.* 2000). Additional support for the idea that insulators are involved in the establishment of higher-order chromatin domains also came from the observation that groups of individual gypsy insulator sites coalesce at specific nuclear locations forming rosette-like structures. These structures could be the basis for the hypothesized role of insulators, as the rosette formations could create independent domains of chromatin organization (GERASIMOVA *et al.* 2000). An important question that still needs to be answered is whether the establishment of higher-order chromatin structures also has an impact on the organization of the primary chromatin fiber. To address this question, we examined the effect of the gypsy insulator on the accessibility of adjacent chromatin to various types of nucleases. This analysis was carried out on the *yellow* gene during third instar larval development. At this time of development, the *yellow* gene is not transcribed and it will not be turned on until several days later in the middle of pupal development. This situation allows the study of possible effects of the insulator on chromatin organization without interference from effects due to binding of transcription factors, which could themselves influence chromatin structure. We have used two different strains containing the gypsy insulator in the 5′ region of the *yellow* gene. One of the strains is the original gypsy-induced y^2 allele, in which the gypsy element is inserted between the enhancers that control expression in the wing and body cuticle of

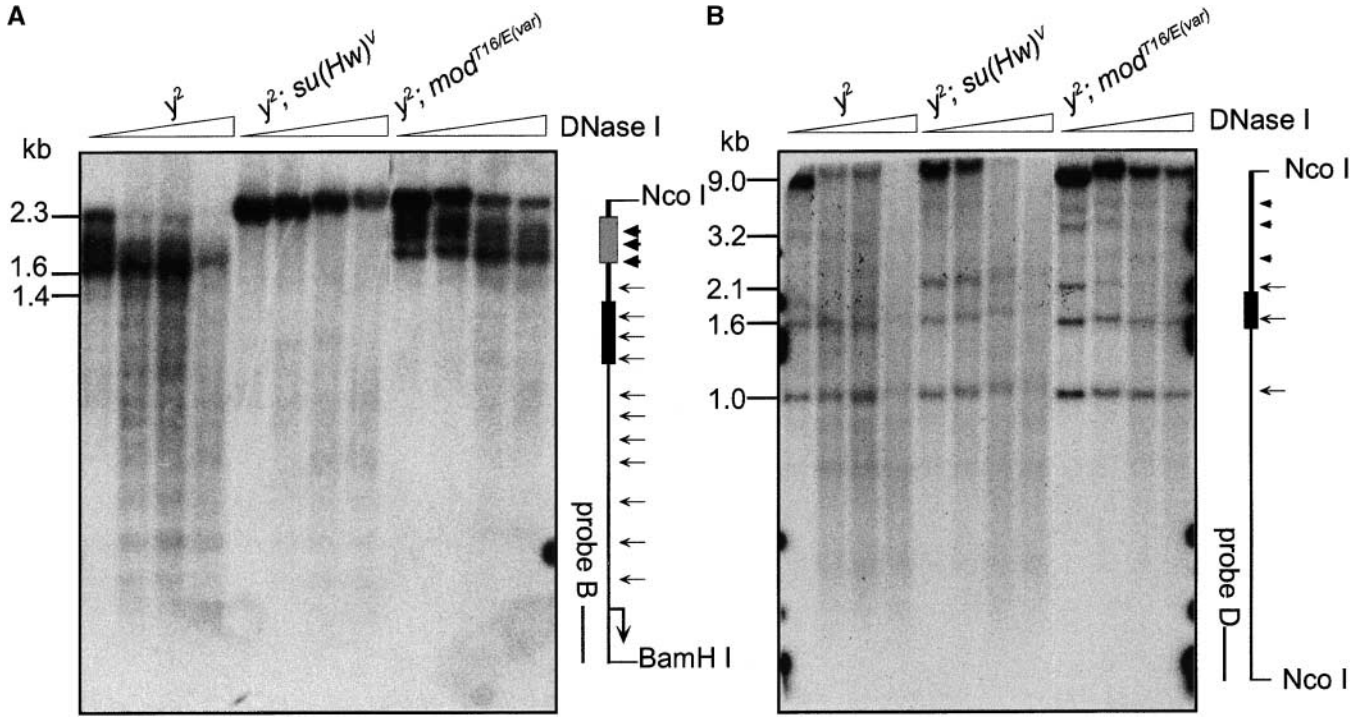

FIGURE 5.—DNase I accessibility analyses of the *yellow* locus in *mod(mdg4)* mutants. Sizes in kilobases are indicated on the left and a schematic drawing of the positions of the DNase I hypersensitive sites is shown on the right of A and B; the *yellow* gene is shown as a thin line, the gypsy element is depicted as a thick line with the LTR shown as a solid box, and the gypsy insulator is shown as a shaded box. The transcription start site is indicated by an arrow. Arrowheads represent hypersensitive sites in the insulator and arrows represent hypersensitive sites within *yellow* sequences or the gypsy element, excluding the insulator. (A) Effect of mutations in the *su(Hw)* and *mod(mdg4)* genes on the DNase I accessibility of the region located between the gypsy insulator and the promoter. (B) Effect of mutations in the *su(Hw)* and *mod(mdg4)* genes on the DNase I accessibility of the region upstream of the gypsy insulator.

the adult and the promoter; a second strain, y^{-800}, carries a transgene containing only sequences of the gypsy insulator also inserted between the body cuticle enhancer and promoter. Both strains show the same phenotypes as a consequence of the effect of the gypsy insulator on *yellow* gene expression, and both gave the same results when tested for nuclease accessibility.

We used two different nucleases that have low sequence specificity. In particular, the use of micrococcal nuclease, which digests chromatin in the internucleosomal regions, allows one to determine whether nucleosomes are present in the region under analysis and their precise position. Both DNase I and MNase have very low accessibility to the DNA in the 5′ region of the *yellow* gene in the absence of a functional gypsy insulator. This is manifested in both the absence of bands corresponding to digested products and in the resistance of the parental restriction fragment to digestion by these nucleases. The lack of MNase digestion could be interpreted as due to the absence of nucleosomes in the region or the absence of phasing between nucleosomes in different DNA molecules, which would result in a smear instead of a tight band. This is not the case because, at high MNase concentration, a nucleosomal ladder is clearly visible, suggesting the presence of regularly arranged nucleosomes in the 5′ region of the wild-type *yellow* gene. The lack of accessibility to this enzyme must then be due to a highly condensed chromatin, not at the level of the primary fiber but rather at a higher-order level of organization. In the presence of the gypsy insulator, both in the y^2 and y^{-800} strains, the accessibility to nucleases increases dramatically within the insulator itself and in the sequences located between the insulator and the promoter. The position of the nucleosomes appears to be the same in the presence or absence of the insulator, but the accessibility to nucleases is higher when an active insulator is present, suggesting that the gypsy insulator causes a more open chromatin structure in the promoter-proximal sequences. The opening of the chromatin is not due to the absence of nucleosomes and must therefore be the result of alterations in the higher-order structure. Interestingly, this opening of the chromatin does not take place in the promoter-distal or the coding regions of the *yellow* gene, as these sequences show low accessibility to nucleases both in the presence or absence of the gypsy insulator.

The observed effect of the gypsy insulator in inducing a more open chromatin structure agrees with its predicted role in establishing higher-order domains of chromatin organization. These results also agree with alterna-

tive models to explain insulator function. For example, it has been proposed that interactions between enhancers and promoters require facilitator factors involved in the establishment of chromatin structures that bring transcription factors bound to the enhancer close to the transcription complex (Dorsett 1999). Insulators might then affect transcription by interfering with facilitator factors, and the observed changes in chromatin structure resulting from the presence of the gypsy insulator might hinder the function of facilitators. Insulator-induced changes in chromatin structure could also obstruct the direct interaction of enhancer-bound transcription factors with insulator proteins, as proposed by the promoter decoy model (Geyer 1997).

An important conclusion of the results presented here is that changes in chromatin structure are not simply the result of the binding of Su(Hw) protein to insulator DNA. Further interactions between Mod(mdg4) and Su(Hw) are needed to induce changes in chromatin structure, a conclusion supported both by the requirement of the Mod(mdg4) protein and of domains of Su(Hw) involved in mediating interactions between both proteins (Gause *et al.* 2001; Ghosh *et al.* 2001). Since interactions between Su(Hw) and Mod(mdg4) lead to the establishment of loops or domains of higher-order chromatin organization (Ghosh *et al.* 2001), this observation further supports the role of the gypsy insulator in the regulation of chromatin structure at this level of organization. The establishment of these domains by insulators might be a first step and a prerequisite to allow further steps necessary for the activation of transcription. The differences in the effect of the gypsy insulator on promoter-proximal *vs.* promoter-distal sequences could be due to the ability of the insulator to discern between either side with respect to its own location. This property would require the insulator to orient itself with respect to the promoter and should be based on interactions between insulator proteins and the transcription complex. Alternatively, insulators might just affect chromatin structure at a global level in a manner that allows subsequent interactions of the chromatin fiber with components of the transcriptional machinery. These transcription factors could then further affect chromatin structure in a promoter-specific manner, resulting in the observed promoter-proximal specific effects.

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LITERATURE CITED

Cai, H., and M. Levine, 1995 Modulation of enhancer-promoter interactions by insulators in the Drosophila embryo. Nature **376:** 533–536.

Cavalli, G., and R. Paro, 1998 Chromo-domain proteins: linking chromatin structure to epigenetic regulation. Curr. Opin. Cell Biol. **10:** 354–360.

Chung, J. H., M. Whiteley and G. Felsenfeld, 1993 A 5′ element of the chicken beta-globin domain serves as an insulator in human erythroid cells and protects against position effect in Drosophila. Cell **74:** 505–514.

Corces, V. G., and G. Felsenfeld, 2000 Chromatin boundaries, pp. 278–299 in *Chromatin Structure and Gene Expression*, edited by S. C. R. Elgin and J. L. Workman. Oxford University Press, Oxford.

De Boni, U., 1994 The interphase nucleus as a dynamic structure. Int. Rev. Cytol. **150:** 149–171.

Dorn, R., V. Krauss, G. Reuter and H. Saumweber, 1993 The enhancer of position-effect variegation of Drosophila, E(var)3-93D, codes for a chromatin protein containing a conserved domain common to several transcriptional regulators. Proc. Natl. Acad. Sci. USA **90:** 11376–11380.

Dorsett, D., 1999 Distant liaisons: long-range enhancer-promoter interactions in Drosophila. Curr. Opin. Genet. Dev. **9:** 505–514.

Gasser, S. M., and U. K. Laemmli, 1986 Cohabitation of scaffold binding regions with upstream/enhancer elements of three developmentally regulated genes of D. melanogaster. Cell **46:** 521–530.

Gause, M., P. Morcillo and D. Dorsett, 2001 Insulation of enhancer-promoter communication by a gypsy transposon insert in the Drosophila cut gene: cooperation between suppressor of hairy-wing and modifier of mdg4 proteins. Mol. Cell. Biol. **21:** 4807–4817.

Gdula, D. A., and V. G. Corces, 1997 Characterization of functional domains of the su(Hw) protein that mediate the silencing effect of mod(mdg4) mutations. Genetics **145:** 153–161.

Gerasimova, T. I., and V. G. Corces, 1998 Polycomb and trithorax group proteins mediate the function of a chromatin insulator. Cell **92:** 511–521.

Gerasimova, T. I., D. A. Gdula, D. V. Gerasimov, O. Simonova and V. G. Corces, 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., K. Byrd and V. G. Corces, 2000 A chromatin insulator determines the nuclear localization of DNA. Mol. Cell **6:** 1025–1035.

Geyer, P. K., 1997 The role of insulator elements in defining domains of gene expression. Curr. Opin. Genet. Dev. **7:** 242–248.

Geyer, P. K., and V. G. Corces, 1992 DNA position-specific repression of transcription by a Drosophila zinc finger protein. Genes Dev. **6:** 1865–1873.

Geyer, P. K., C. Spana and V. G. Corces, 1986 On the molecular mechanism of gypsy-induced mutations at the yellow locus of Drosophila melanogaster. EMBO J. **5:** 2657–2662.

Ghosh, D., T. I. Gerasimova and V. G. Corces, 2001 Interactions between the Su(Hw) and Mod(mdg4) proteins required for gypsy insulator function. EMBO J. **20:** 2518–2527.

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

Hebbes, T. R., and S. C. Allen, 2000 Multiple histone acetyltransferases are associated with a chicken erythrocyte chromatin fraction enriched in active genes. J. Biol. Chem. **275:** 31347–31352.

Holdridge, C., and D. Dorsett, 1991 Repression of hsp70 heat shock gene transcription by the suppressor of hairy-wing protein of Drosophila melanogaster. Mol. Cell. Biol. **11:** 1894–1900.

Kellum, R., and P. Schedl, 1991 A position-effect assay for boundaries of higher order chromosomal domains. Cell **64:** 941–950.

Kellum, R., and P. Schedl, 1992 A group of scs elements function as domain boundaries in an enhancer-blocking assay. Mol. Cell. Biol. **12:** 2424–2431.

Lamond, A. I., and W. C. Earnshaw, 1998 Structure and function in the nucleus. Science **280:** 547–553.

Lu, Q., L. L. Wallrath, H. Granok and S. C. Elgin, 1993 (CT)n (GA)n repeats and heat shock elements have distinct roles in chromatin structure and transcriptional activation of the Drosophila hsp26 gene. Mol. Cell. Biol. **13:** 2802–2814.

Luderus, M. E. E., and R. van Driel, 1997 Nuclear matrix-associated regions, pp. 99–115 in *Nuclear Organization, Chromatin Structure, and Gene Expression*, edited by R. van Driel and A. P. Otte. Oxford University Press, Oxford.

Manuelidis, L., and T. L. Chen, 1990 A unified model of eukaryotic chromosomes. Cytometry **11:** 8–25.

Mirkovitch, J., M. E. Mirault and U. K. Laemmli, 1984 Organization of the higher-order chromatin loop: specific DNA attachment sites on nuclear scaffold. Cell **39:** 223–232.

Prioleau, M. N., P. Nony, M. Simpson and G. Felsenfeld, 1999 An insulator element and condensed chromatin region separate the chicken beta-globin locus from an independently regulated erythroid-specific folate receptor gene. EMBO J. **18:** 4035–4048.

Roseman, R. R., J. M. Swan and P. K. Geyer, 1995 A Drosophila insulator protein facilitates dosage compensation of the X chromosome mini-white gene located at autosomal insertion sites. Development **121:** 3573–3582.

Saitoh, N., A. C. Bell, F. Recillas-Targa, A. G. West, M. Simpson *et al.*, 2000 Structural and functional conservation at the boundaries of the chicken beta-globin domain. EMBO J. **19:** 2315–2322.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Scott, K. S., and P. K. Geyer, 1995 Effects of the su(Hw) insulator protein on the expression of the divergently transcribed Drosophila yolk protein genes. EMBO J. **14:** 6258–6267.

Shao, Z., F. Raible, R. Mollaaghababa, J. R. Guyon, C. T. Wu *et al.*, 1999 Stabilization of chromatin structure by PRC1, a Polycomb complex. Cell **98:** 37–46.

Sudarsanam, P., and F. Winston, 2000 The Swi/Snf family nucleosome-remodeling complexes and transcriptional control. Trends Genet. **16:** 345–351.

Udvardy, A., E. Maine and P. Schedl, 1985 The 87A7 chromomere. Identification of novel chromatin structures flanking the heat shock locus that may define the boundaries of higher order domains. J. Mol. Biol. **185:** 341–358.

Wallrath, L. L., and S. C. Elgin, 1995 Position effect variegation in Drosophila is associated with an altered chromatin structure. Genes Dev. **9:** 1263–1277.

Yokota, H., G. van den Engh, J. E. Hearst, R. K. Sachs and B. J. Trask, 1995 Evidence for the organization of chromatin in megabase pair-sized loops arranged along a random walk path in the human G0/G1 interphase nucleus. J. Cell Biol. **130:** 1239–1249.

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

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