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# Visualization of chromatin domains created by the *gypsy* insulator of *Drosophila*

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Insulators might regulate gene expression by establishing and maintaining the organization of the chromatin fiber within the nucleus. Biochemical fractionation and in situ high salt extraction of lysed cells show that two known protein components of the *gypsy* insulator are present in the nuclear matrix. Using FISH with DNA probes located between two endogenous Su(Hw) binding sites, we show that the intervening DNA is arranged in a loop, with the two insulators located at the base. Mutations in insulator proteins, subjecting the cells to a brief heat shock, or destruction of the nuclear matrix lead to disruption of the loop. Insertion of an additional *gypsy* insulator in the center of the loop results in the formation of paired loops through the attachment of the inserted sequences to the nuclear matrix. These results suggest that the *gypsy* insulator might establish higher-order domains of chromatin structure and regulate nuclear organization by tethering the DNA to the nuclear matrix and creating chromatin loops.

## Introduction

Multicellular organisms use complex mechanisms to ensure proper spatial and temporal expression of genes. Packaging of the DNA with histones to form chromatin prevents improper gene activation by restricting accessibility of transcription factors to the regulatory sequences of the gene. Current research has focused on the effects of histone modification in promoting or preventing transcription (Jenuwein and Allis, 2001; Berger, 2002). However, much less is known about the three-dimensional arrangement of DNA in the nucleus and the role this organization plays in gene regulation. There is some evidence suggesting that chromatin is packaged into 50–200-kb loops attached to a nuclear matrix by sequences called matrix attachment regions (MARs) or scaffold attachment regions (SARs) (Kaufmann et al., 1986; Gasser et al., 1989; Laemmli et al., 1992; Pederson, 1998). Other results suggest that insulators might similarly be involved in nuclear organization by bringing the chromatin fiber to a perinuclear compartment (Gerasimova and Corces, 1998; Gerasimova et al., 2000; Ishii et al., 2002). Insulators are characterized by two properties consistent with their potential ability to create these loop domains: (1) they prevent an enhancer from activating a promoter when located between the two, and (2) when flanking a transgene, they prevent the local chromatin environment around the integration site from affecting its expression (Bell et al., 2001; Gerasimova and Corces, 2001). The *gypsy* insulator is a 350-bp sequence that requires at least two proteins for function: Su(Hw), a zinc finger DNA binding protein, and Mod(mdg4), a BTB domain protein that binds Su(Hw) and can associate with itself (Gerasimova et al., 1995; Gause et al., 2001; Ghosh et al., 2001). Although the *gypsy* insulator was originally identified in the *gypsy* retrotransposon, the *Drosophila* genome contains ~500 binding sites for the Su(Hw) and Mod(mdg4) proteins that are presumed to be endogenous insulators; out of the multiple isoforms encoded by the *mod(mdg4)* gene, only the Mod(mdg4)2.2 protein appears to be present at the *gypsy* insulator (Mongelard et al., 2002). We will refer to the insulator present in the *gypsy* retrotransposon as the *gypsy* insulator, whereas we will use "endogenous Su(Hw) binding sites" to designate genomic binding sites for the Su(Hw) protein that do not contain copies of the *gypsy* retrotransposon but might act as insulators, although this property has not yet been demonstrated experimentally. We have previously shown that Su(Hw) and Mod(mdg4)2.2 colocalize in large foci, named insulator bodies, located mostly at the nuclear periphery of diploid cells. These insulator bodies are presumed to be formed by multiple individual insulator sites coming together at restricted nuclear locations. This hypothesis is supported by observations indicating that the presence of the *gypsy* insulator at two distant chromosomal sites causes the DNA containing

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Abbreviations used in this paper: *ct*, *cut*; MAR, matrix attachment region; NPC, nuclear pore complex; SAR, scaffold attachment region; SCS, specialized chromatin structures; Ubx, Ultrabithorax.

these *gypsy* sequences to come together at the nuclear periphery (Gerasimova et al., 2000). If insulator bodies bring together individual insulator sites, the intervening DNA should form a loop. These loops might then represent functionally separate chromatin domains that allow independent regulation of transcription within each domain.

Indirect molecular evidence for the possibility that the formation of chromatin loops might serve as a basis for insulator function comes from results obtained during a screen for proteins with boundary function in yeast (Ishii et al., 2002). Proteins involved in nucleo-cytoplasmic transport or components of the nuclear pore complex (NPC) were found to be able to buffer a flanked reporter gene from heterochromatic silencing effects. These results suggest that boundary activity can be accomplished by attachment of both sides of the insulated DNA to a solid substrate, in this case the NPC, in a perinuclear compartment. Although not directly shown in this work, attachment is likely to result in the formation of a small loop with its base at the NPC, and this loop might be functionally similar to the ones hypothesized to mediate *gypsy* insulator activity.

In spite of the widespread discussion of the loop domain model in the context of insulator function, neither the existence of these loops nor the basis for their location at the nuclear periphery has been demonstrated directly. Using FISH analysis of high salt–extracted nuclei, we show here that DNA sequences located between two insulators form a loop in the interphase nucleus. The formation of this loop is dependent on functional insulator proteins and an intact nuclear matrix. The results suggest that insulators might regulate nuclear organization by controlling the formation of higher-order domains of chromatin structure.

## Results

### *Gypsy* insulator proteins are present in the nuclear matrix fraction

The perinuclear localization of the *gypsy* insulator bodies suggests an association of the insulator proteins with a fixed substrate such as the nuclear matrix. The nuclear matrix is defined biochemically as the fraction remaining after extraction of nuclei with 2 M NaCl (Pederson, 1998; Nickerson, 2001). To biochemically confirm the association of Su(Hw) and Mod(mdg4)2.2 proteins with the nuclear matrix, we isolated a karyoskeletal or nuclear matrix fraction (~7% of nuclear proteins) from *Drosophila* embryos ranging in age from 6 to 18 h post–egg laying. Fig. 1 shows the results of Western analyses of different protein fractions. Lamin, as expected, is predominantly located in the nuclear matrix fraction. In contrast, histones are extracted in previous steps and are not present in this fraction (Oegema et al., 1997; Ma et al., 1999). Both *gypsy* insulator components, Su(Hw) and Mod(mdg4)2.2, copurify almost entirely with the nuclear matrix fraction (Fig. 1). As a control, we tested whether the Ultrabithorax (Ubx) transcription factor is also present in the nuclear matrix fraction; the Ubx protein is extracted from nuclei under the same conditions as histones and, therefore, is not associated with the nuclear matrix (Fig. 1). The same association of Su(Hw) and Mod(mdg4)2.2 was found using a different nuclear matrix isolation protocol that involves precipitation with ammonium sulfate (unpublished data). These results suggest that the *gypsy* insulator associates with the nuclear matrix and provides a biochemical foundation for the observed formation of insulator bodies in the nuclear periphery.

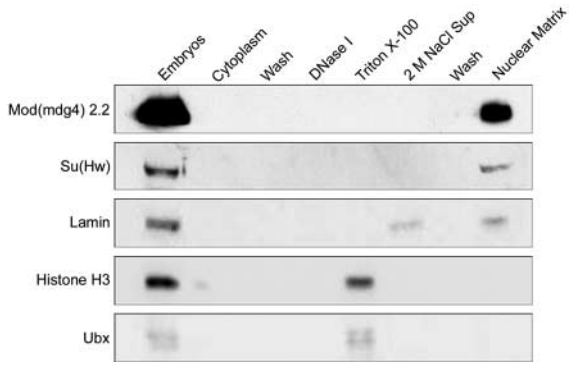


Figure 1. **Protein components of the *gypsy* insulator are present in the nuclear matrix.** A collection of *Drosophila* 6–18-h-old embryos was subjected to a karyoskeletal or nuclear matrix fractionation procedure, and equal amounts of the fractions were run on 7.5 and 10% polyacrylamide gels and subjected to Western blot analysis using antibodies to Mod(mdg4), Su(Hw), lamin, histone H3, and Ubx. Antibodies against Mod(mdg4) were specific to the Mod(mdg4) 2.2 isoform, which is the isoform present in the *gypsy* insulator (Ghosh et al., 2001; Mongelard et al., 2002).

### An intact nuclear matrix is required for the formation of *gypsy* insulator bodies

To further explore the association of *gypsy* insulator proteins with the nuclear matrix under more physiological conditions, we used antibodies against Su(Hw) and Mod(mdg4) 2.2 for immunofluorescence studies on specially prepared nuclear matrices. Using a modified form of a procedure described by Gerdes et al. (1994) to isolate nuclear matrices for cytological analysis, we spun detergent-treated imaginal disk cells from third instar *Drosophila* larvae onto coverslips and then extracted the nuclei with 2 M NaCl. An extracted nucleus appears as a darkly stained nuclear matrix–containing core surrounded by a lightly stained DNA halo (for schematic see Fig. 2 A). The black and white images of nuclei stained with DAPI more clearly show the halo formation around the intensely stained residual nuclear matrix (Fig. 2, F and J). Based on images of extracted nuclei obtained with the electron microscope (McCready et al., 1979), the light blue halo is composed of histone-free DNA loops associated at the base to the more intensely stained nuclear matrix that contains DNA, RNA, ribonucleoproteins, and other proteins resistant to high salt extraction. Along with histones, ~95% of nuclear proteins are extracted with this technique; however, lamin and other known matrix-associated proteins remain (Fey et al., 1986; Ma et al., 1999). We then performed immunofluorescence light microscopy on nuclei that were either untreated or extracted with 2 M NaCl using antibodies to Mod(mdg4)2.2 and lamin. As expected from previous results with paraformaldehyde-fixed nuclei (Gerasimova et al., 2000), Mod(mdg4)2.2 is present

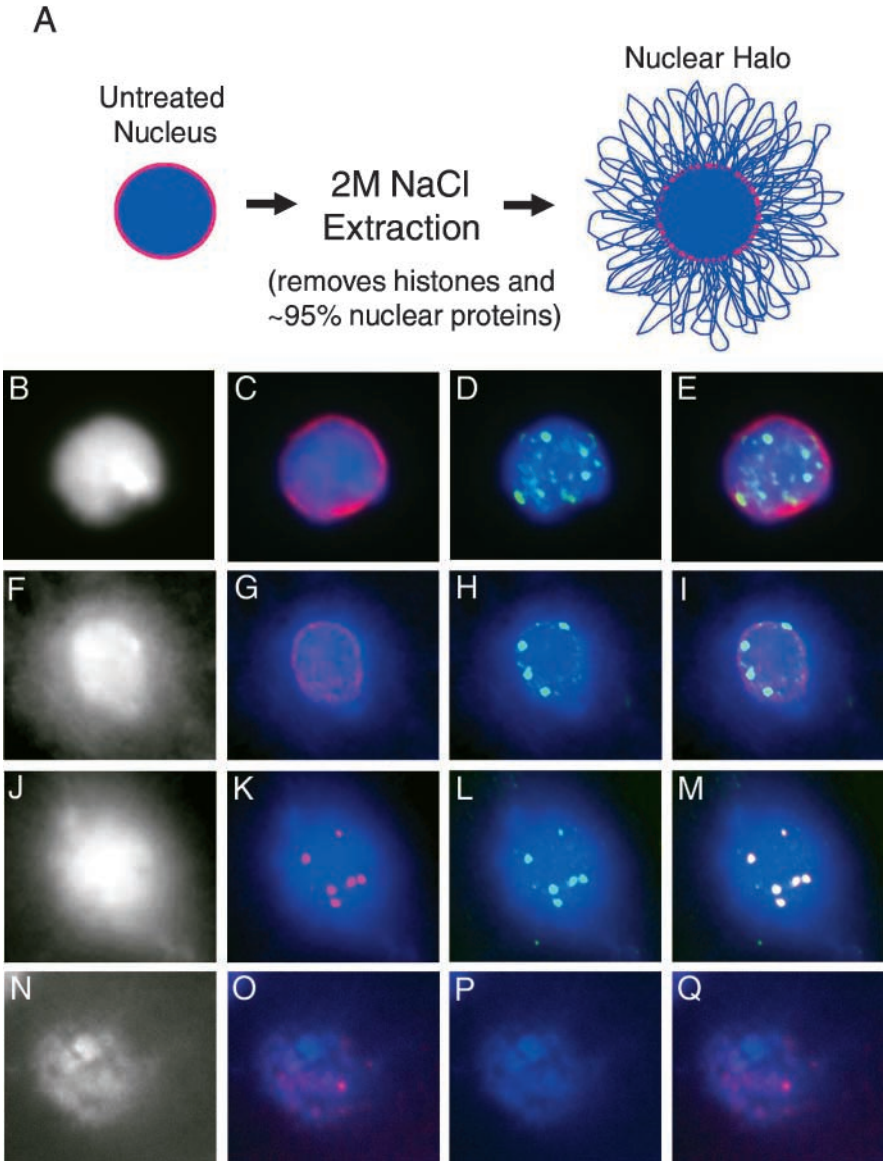


Figure 2. **Distribution of Su(Hw) and Mod(mdg4) insulator proteins after a 2 M NaCl extraction of nuclei.** Imaginal disk cells from wild-type larvae were spun onto coverslips and either extracted with 2 M NaCl or left untreated, and then they were stained with antibodies to Su(Hw), Mod(mdg4), or lamin; in addition, the DNA was stained with DAPI (blue). (A) Schematic representation of a nucleus before and after extraction with 2 M NaCl. The red depicts the nuclear lamin and the blue indicates DNA. Treatment of these cells with 2 M NaCl removes histones and extracts ~95% of nuclear proteins, leaving the DNA in large 50–200-kb loops attached to the residual nuclear matrix (McCready et al., 1979; Ma et al., 1999). B, F, J, and N are black and white images of DNA, corresponding to the panels on their right, visualized by DAPI staining. This staining is shown in blue in the rest of the panels. (C–E) Distribution of lamin (red) and Mod(mdg4)2.2 (green) in untreated nuclei. (G–I) Distribution of lamin (red) and Mod(mdg4)2.2 (green) in 2 M NaCl-extracted nuclei. (K–M) Distribution of Su(Hw) (red) and Mod(mdg4)2.2 (green) in 2 M NaCl-extracted nuclei. (O–Q) Distribution of Su(Hw) (red) and Mod(mdg4)2.2 (green) in nuclei extracted with 2 M NaCl followed by treatment with RNase A.

in insulator bodies formed by the aggregation of multiple individual insulator sites in intact nuclei (Fig. 2, B–E). To confirm that the insulator bodies are associated with the nuclear matrix, we stained nuclei extracted with 2 M NaCl with antibodies to lamin and Mod(mdg4)2.2 (Fig. 2, F–I). The Mod(mdg4)2.2 protein, which marks the localization of *gypsy* insulator bodies, remains associated with the lamin-containing nuclear matrix core. We then extracted nuclei with 2 M NaCl and stained them with antibodies to both insulator proteins, Su(Hw) and Mod(mdg4)2.2, as well as DAPI to visualize the DNA (Fig. 2, J–M). Extraction with such high salt concentrations that remove 95% of nuclear proteins did not disrupt the interaction between the two insulator proteins or their interaction with the nuclear matrix. It has been previously shown that the nuclear matrix, although not composed of one repeating protein unit, is composed of ribonucleoprotein complexes and, thus, is susceptible to destruction by RNase (He et al., 1990; Ma et al., 1999). To test whether disruption of nuclear matrix integrity would affect the formation of *gypsy* insulator bodies, we treated 2 M NaCl-extracted nuclei from wild-type larvae with RNase A. Under these conditions, the nuclear matrix appears fragmented and disorganized. In addition, the insulator bodies are destroyed, as determined by the localization of Su(Hw) and Mod(mdg4)2.2 proteins (Fig. 2, N–Q). This result strongly suggests that the formation of insulator bodies requires the existence of an intact nuclear matrix.

## Chromatin loops form between two insulator sites

If insulator bodies form by attaching multiple individual insulator sites to the nuclear matrix at specific nuclear locations, they might help organize the chromatin fiber into loops representing domains of higher-order organization. The formation of these loops should be testable using the nuclear halo technique and FISH with DNA probes spanning the region contained within the loop. As a loop should form by the DNA located between two individual insulator sites, we used immunostaining with Su(Hw) antibodies to map the location of individual endogenous *gypsy* insulators on polytene chromosomes of third instar larvae. We then

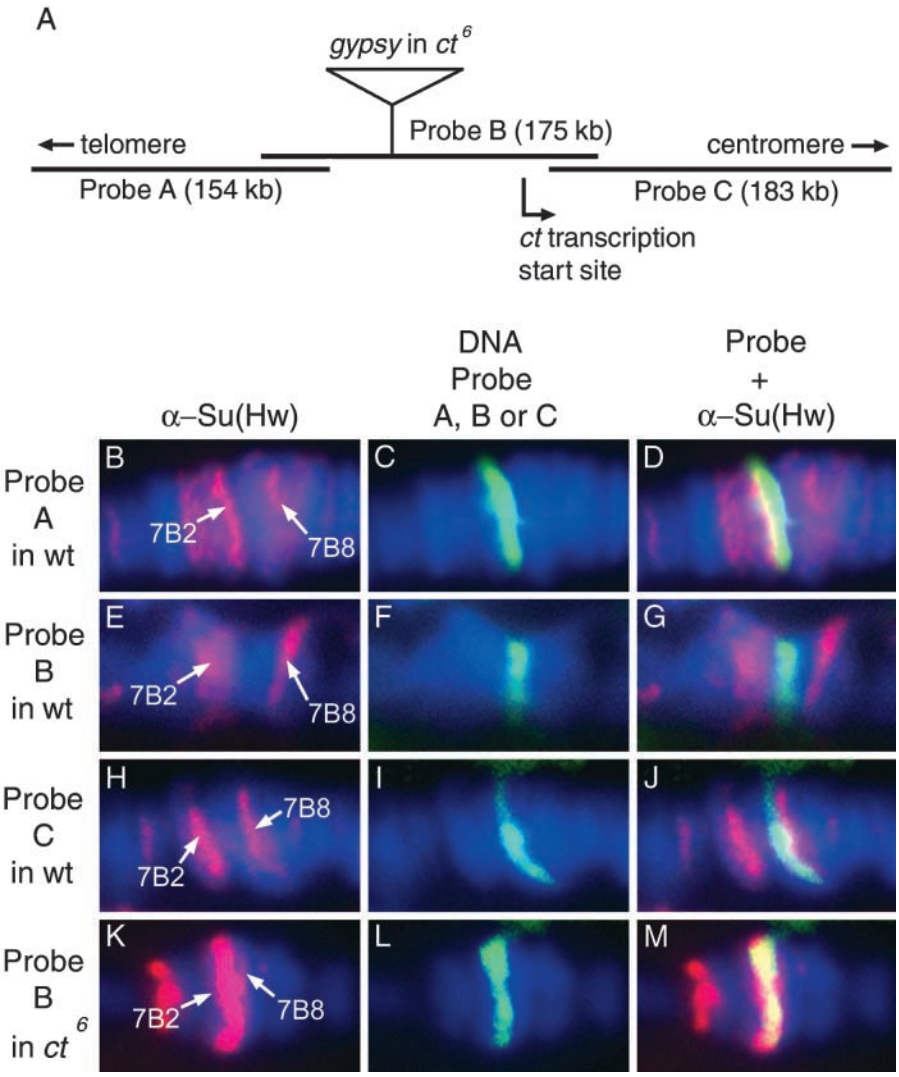


Figure 3. **Localization of DNA probes A, B, and C and Su(Hw) protein on polytene chromosomes.** BAC clones A, B, or C were used as DNA probes for FISH of polytene chromosomes from salivary glands of *Drosophila* third instar larvae from wild-type or $ct^6$ mutant strains. The chromosomes were simultaneously stained with antibodies against the Su(Hw) protein. For all images, the DNA is visualized by DAPI staining (blue), and the locations of endogenous Su(Hw) binding sites at 7B2 and 7B8, indicated by Su(Hw) staining (red), are labeled with arrows. (A) Schematic representation of probes A, B, and C at the *ct* locus. The $ct^6$ mutant contains a copy of the *gypsy* retrotransposon located in the region recognized by probe B. The location of the *gypsy* retrotransposon and overlap of probes A, B, and C are drawn to scale. (B–D) Immunolocalization of Su(Hw) (red) and FISH signal of probe A in polytene chromosomes from wild-type larvae. (E–G) Immunolocalization of Su(Hw) (red) and FISH signal of probe B (green) in polytene chromosomes from wild-type larvae. (H–J) Immunolocalization of Su(Hw) (red) and FISH signal of probe C (green) in polytene chromosomes from wild-type larvae. (K–M) Immunolocalization of Su(Hw) (red) and FISH signal of probe B in polytene chromosomes from $ct^6$ larvae.

concentrated our analysis on a region of the X chromosome containing the *cut* (*ct*) locus flanked by two endogenous Su(Hw) binding sites located at 7B2 and 7B8. This region is spanned by three different BAC clones, designated as probes A, B, and C. Clone A is 154 kb in length, whereas clones B and C are 175 kb and 183 kb, respectively. The DNA sequence for these three clones has been determined by the *Drosophila* Genome Project, and their location with respect to each other and the *ct* gene is diagrammed in Fig. 3 A. Clones A and B overlap by 28 kb, whereas clones B and C overlap by 16 kb. Using FISH analysis with these three probes and simultaneous immunolocalization with Su(Hw) antibodies, we were able to determine the relative location of each BAC clone with respect to the location of the endogenous Su(Hw) binding sites surrounding the *ct* locus. Fig. 3 D shows that probe A is located adjacent and partially overlapping the Su(Hw) binding site present at chromosomal subdivision 7B2. Probe B is located between the 7B2 and 7B8 Su(Hw) binding sites (Fig. 3 G), whereas probe C partially overlaps the 7B8 site (Fig. 3 J). The $ct^6$ allele is caused by the insertion of a copy of the *gypsy* retrotransposon in the 5′ region of the *ct* gene (Jack, 1985). This insertion site is located 65 kb to the right of the telomere-proximal end of clone B (Fig. 3 A). As the *gypsy* retrotransposon contains the *gypsy* insulator, one would expect to detect a new site of Su(Hw) staining in polytene chromosomes prepared from third instar larvae carrying the $ct^6$ mutation. This is indeed the case, as seen in Fig. 3 (K–M). The *gypsy* insulator present in the *gypsy* retrotransposon contains 12 binding sites for the Su(Hw) protein, giving rise to a strong immunofluorescence signal on polytene chromosomes at chromosomal subdivision 7B4. This signal partially obscures the endogenous Su(Hw) binding sites located at 7B2 and 7B8. In addition, analysis of DAPI-stained chromosomes in this region suggests that the presence of *gypsy* induces a condensation of the local chromatin (unpublished data), further decreasing the resolution between the Su(Hw) signals at 7B2, 7B4, and 7B8; as a consequence, all three signals overlap in one band. Probe B overlaps with the *gypsy* retrotransposon insulator in polytene chromosomes from $ct^6$ mutant larvae (Fig. 3 M), but does not overlap with the two endogenous Su(Hw) binding sites in wild-type animals (Fig. 3 G). Based on the location of the two endogenous Su(Hw) binding sites and the three BAC clones on polytene chromosomes, we expect that, if these sequences form a loop in interphase nuclei of diploid cells as a consequence of the two Su(Hw) binding

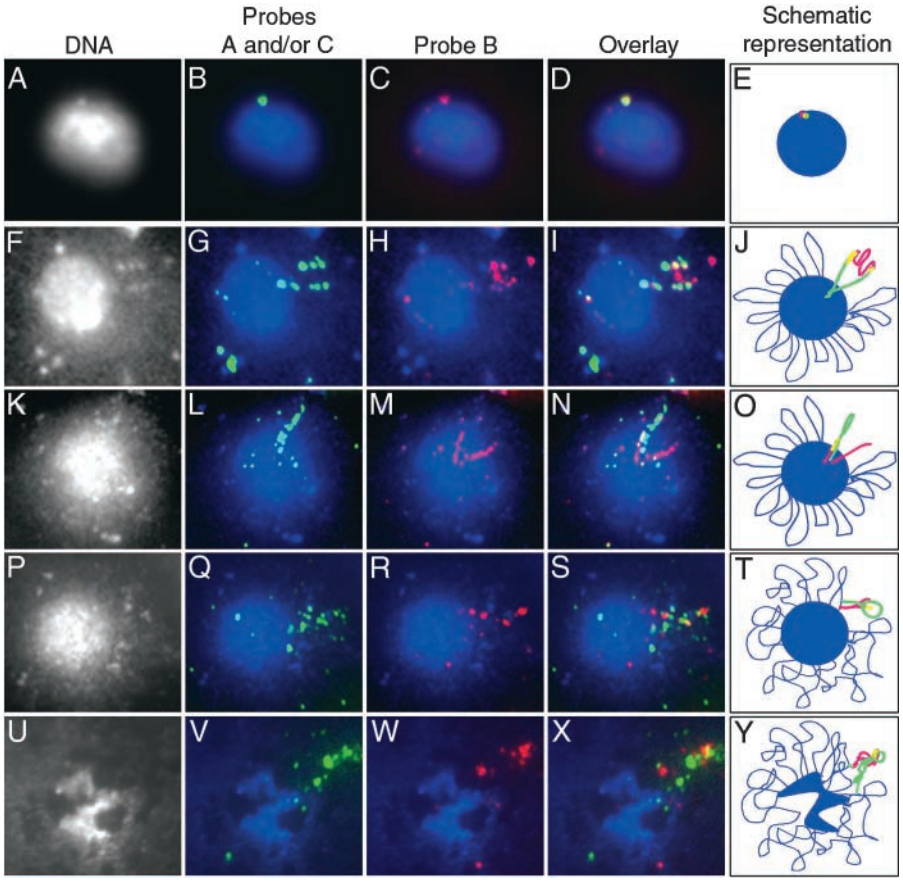


Figure 4. **Effect of the *gypsy* insulator on the distribution of DNA in 2 M NaCl-extracted nuclei from male larvae.** Various combinations of DNA probes A, B, and C from chromosomal subdivision 7B (see Fig. 3) were hybridized to imaginal disk cells that were spun onto coverslips and either extracted with 2 M NaCl or left untreated. Probes A and C are in green and probe B is in red. A, F, K, P, and U are black and white images of DNA visualized by DAPI staining. This staining is shown in blue in the rest of the panels. The red depicts probe B, and the green depicts probes A and C in panels G–I and probe A in the rest. E, J, O, T and Y show schematic representations of the results found to their left. (A–D) Probes A (green) and B (red) in an untreated wild-type nucleus. (F–I) Probes A (green), B (red), and C (green) in a 2 M NaCl-extracted wild-type nucleus. (K–N) Probes A (green) and B (red) in a 2 M NaCl-extracted nucleus from larvae carrying the $ct^6$ mutation. (P–S) Probes A (green) and B (red) in a 2 M NaCl-extracted nucleus from larvae of the genotype $ct^6$; $su(Hw)^V$. (U–X) Probes A (green) and B (red) in a 2 M NaCl-extracted nucleus also treated with RNase A.

sites coming together and attaching to the nuclear matrix, the DNA present in clones A and C would form the two stems of the loop. The loop would be attached at the base of these probes, where the endogenous Su(Hw) binding sites reside, to the nuclear matrix at one location. In contrast, the DNA spanning clone B would be present in the central distal region of the loop, not attached to the nuclear matrix, because the DNA in this region does not contain an insulator in wild-type larvae. This hypothesis rests on the assumption that, as polytene salivary gland cells are in interphase, the location of Su(Hw) and Mod(mdg4)2.2 proteins at endogenous Su(Hw) binding sites would be maintained in diploid cells of larval imaginal disks.

To test this hypothesis, we performed FISH on untreated and 2 M NaCl-extracted imaginal disk nuclei spun onto coverslips. As the *ct* locus is located in the X chromosome, we initially restricted our analysis to nuclei of cells from imaginal disks of male larvae. We first probed untreated wild-type nuclei with probes A and B and found, as expected, that they colocalize specifically in one region of the nucleus (Fig. 4, A–D). We then performed FISH with probes A, B, and C on nuclei extracted with 2 M NaCl from imaginal disk cells of wild-type male larvae. Probes A and C frequently appear attached to the nuclear matrix at the same location at one of their ends, presumably through the putative endogenous Su(Hw) binding sites located at the outer boundaries of probes A and C (Fig. 4 G). In contrast, we found that DNA sequences complementary to probe B are usually located in the halo region and do not associate with the nuclear matrix (Fig. 4 H). The DNA complementary to the other ends of probes A and C, overlapping probe B and lacking endogenous Su(Hw) binding sites, is present in the nuclear halo region partially colocalizing with the small overlapping regions at the ends of probe B (Fig. 4 I). To determine the statistical significance of these results, we measured the number of nuclei in which probes A and C form a V structure with the vertex located in the darkly stained core region. We scored the V structure based on the appearance of two linear FISH signals meeting at one location in the nuclear matrix. 86% of wild-type nuclei show the characteristic V structure formed by probes A and C. The other 14% had either nonlinear signal(s), signal(s) that did not have a clear matrix association, and/or signals that did not meet to form a vertex. Differences are significant ($P < 0.001$, chi-squared test) when the presence of the V structure in wild type is compared with $ct^6$; $su(Hw)^V$, a null for Su(Hw) protein (Table I). In imaginal disk cells from *su(Hw)* mutant male larvae, only 16% of nuclei show the presence of a V structure formed by probes A and C. This lack of matrix association of probes A and C in nuclei that lack Su(Hw) indicates that a functional Su(Hw) protein is required for DNA attachment to the nuclear matrix at that location.

### Insertion of a new *gypsy* insulator in the center of a loop results in the formation of two smaller loops

To further test the idea that the observed chromatin loops are formed between *gypsy* insulator sites, we then performed similar FISH experiments on nuclei that were ex-

Table I. **Statistical analysis of FISH data presented in Fig. 4**

| Genotype | No. of nuclei | Probes A and C in V structure | No. of nuclei | Probe B in V structure |
|---|---|---|---|---|
| | | % | | % |
| Wild-type males | 96 | 86 | 127 | 16 |
| $ct^6$ males | 94 | 81 | 189 | 78 |
| $ct^6$; $su(Hw)^V$ males | 83 | 16 | 112 | 19 |
| Wild-type males, RNase treated | 28 | 0 | 28 | 3 |
| $ct^6$ heat-shocked males | ND | ND | 80 | 25 |
| Wild-type females | ND | ND | 79 | 18 |
| $ct^6$ females | ND | ND | 95 | 80 |

Nuclei were scored blindly by two researchers independently, based on the presence or absence of a V structure in the DNA complementary to probe B, indicating an attachment to the nuclear matrix. Nuclei were also scored based on the presence of a V structure complementary to probes A and C with the vertex located in the nuclear matrix core.

tracted with 2 M NaCl from male larvae carrying the $ct^6$ mutation, known to contain an additional *gypsy* insulator in the 7B4 region. In these nuclei, the DNA hybridizing to probe B acquired a V shape, with the vertex in the darkly stained central core, suggesting that this region of the chromosome is now attached to the nuclear matrix through the new insulator present at the site of insertion of the *gypsy* retrotransposon in the $ct^6$ allele (Fig. 4 M). Furthermore, the shape of this V structure is asymmetric, with one side of the V longer than the other, as would be expected given the asymmetric location of the *gypsy* element in the *ct* locus within the region covered by probe B (Fig. 3 A). We scored the formation of a V structure by the DNA complementary to probe B based on the appearance of a continuous linear FISH signal attached to the nuclear matrix at one location. Probe B acquires a V shape in 78% of nuclei from $ct^6$ male larvae. Differences are significant ($P < 0.001$, chi-squared test) when we compare $ct^6$ nuclei with wild-type nuclei in which such a structure is only observed in 16% of nuclei (Table I). In many cases, it was possible to observe the position of the proximal region of probe A overlapping with the distal end of probe B (Fig. 4 N). This allowed us to conclude that the location of the vertex of the V approximately maps to the region where the *gypsy* retrotransposon is inserted in the $ct^6$ allele. The 22% of nuclei from $ct^6$ larvae not scored as having a V structure had either a nonlinear signal and/or a signal that did not have a clear matrix association. The state of the cell cycle might explain the lack of V formation in those cells, as late G2 and mitotic cells do not have insulator bodies. In contrast, a possible explanation for the V formation of probe B observed in 16% of nuclei from wild-type larvae could be the presence of replicating or transcribing DNA in the region complementary to probe B associated with the nuclear matrix at the time of the 2 M NaCl extraction. This interpretation is supported by findings suggesting that actively transcribing and replicating sequences associate with the nuclear matrix (Gerdes et al., 1994). These results suggest that the presence of a new *gypsy* insulator in the 7B4 region between the two endogenous Su(Hw) binding sites located at 7B2 and 7B8 causes the original loop formed between 7B2 and 7B8 to become divided into two smaller loops, due to the attachment of the new insulator at 7B4 to the nuclear matrix (Fig. 4 N).

To ensure that the formation of an additional loop was due to the presence of a new functional *gypsy* insulator, we examined 2 M NaCl-extracted nuclei from male larvae of the genotype $ct^6$; $su(Hw)^V$, which carry the *gypsy* retrotransposon in the *ct* locus but lack Su(Hw) protein. Nuclei from these larvae failed to show DNA structures with the characteristic V shape in the region complementary to probe B (Fig. 4 R). The number of nuclei showing a V structure, scored as described above, is 19%, which is significantly different ($P < 0.001$, chi-squared test) when compared with nuclei carrying the $ct^6$ mutation but wild type for *su(Hw)* (Table I). The formation of a V structure by probes A and C is also disrupted in nuclei from $ct^6$; $su(Hw)^V$ mutant male larvae. In these nuclei, the DNA complementary to probes A and C only occasionally appears as a straight line going from the central region toward the surrounding halo; instead, the DNA appears disorganized in the nuclear halo region surrounding the residual nuclear matrix core (Fig. 4, Q–S). The number of times the V structure was observed in nuclei from $ct^6$; $su(Hw)^V$ larvae is significantly lower ($P < 0.001$, chi-squared test) than wild type (Table I). In the absence of Su(Hw) protein, both the insulator present in the *gypsy* retrotransposon in the *ct* locus as well as the putative insulators present at endogenous Su(Hw) binding sites are not functional. The disorganized appearance of the DNA in nuclei from $ct^6$; $su(Hw)^V$ male larvae implies that the lack of Su(Hw) protein results in the absence of loop structures, probably because of a failure to form functional insulators, suggesting that the insulators serve as attachment points of the DNA to the nuclear matrix. Similar experiments were also performed with nuclei from male larvae of the genotype $ct^6$; $mod(mdg4)^{u1}$. The results were comparable to those obtained with the $ct^6$; $su(Hw)^V$ (unpublished data), suggesting that both proteins, Su(Hw) and Mod(mdg4)2.2, are required for the formation of a functional insulator capable of attaching the DNA to the nuclear matrix.

## Loop formation requires an intact nuclear matrix

As these results suggest the requirement of the nuclear matrix for the formation of DNA loops in the nucleus, we then examined the effect of destroying the nuclear matrix, with RNase, on the organization of the DNA flanked by endogenous Su(Hw) binding sites. Nuclei from wild-type male larvae were extracted with 2 M NaCl, treated with RNase A,

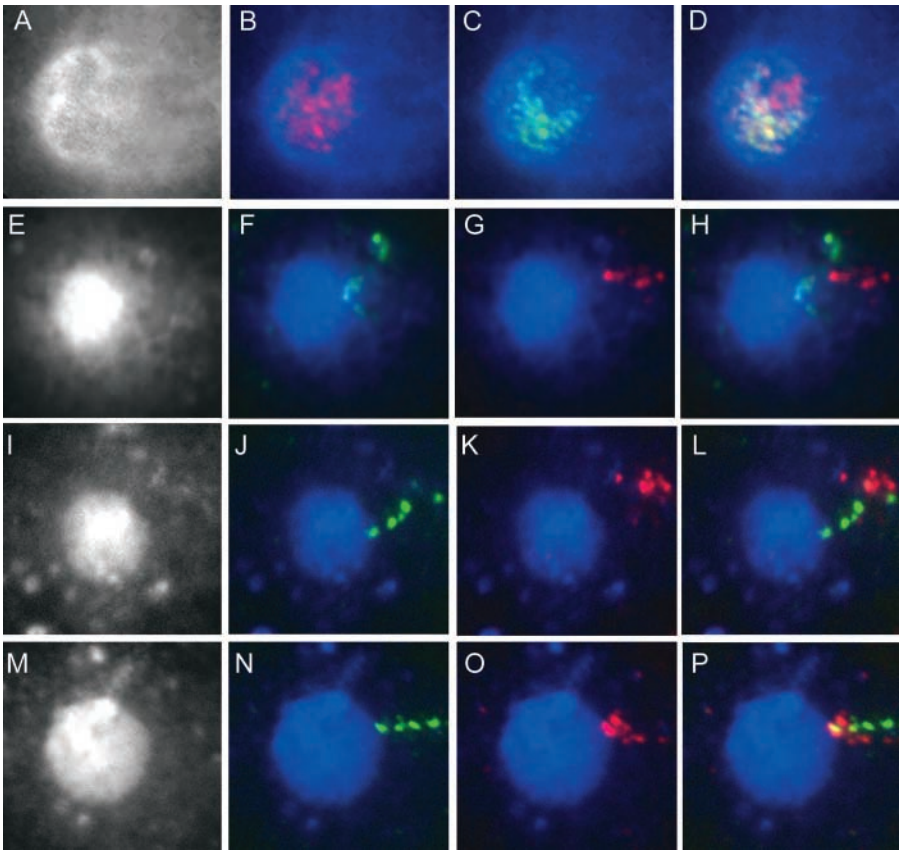


Figure 5. **Insulator-mediated loop organization after heat shock and in nuclei from female larvae.** (A–D) Immunofluorescence analysis of 2 M NaCl-extracted nuclei from imaginal disk cells of $ct^6$ male larvae subjected to a 30-min heat shock at 37°C using antibodies against Su(Hw) and Mod(mdg4)2.2. The Su(Hw) protein is shown in red and the Mod(mdg4)2.2 protein in green. DNA was stained with DAPI and is shown in A; DAPI staining is shown in blue in B–D. The rest of the panels in the figure show FISH analysis of 2 M NaCl-extracted nuclei from imaginal disk cells using probes A (green) and B (red); in all panels, DAPI staining is shown in gray (E, I, and M) or in blue. (E–H) Nuclei from $ct^6$ male cells subjected to a 30-min heat shock at 37°C. (I–L) Nuclei from imaginal disk cells of wild-type female larvae. (M–P) Nuclei from imaginal disk cells of $ct^6$ female larvae.

and then labeled with probes A, B, and C. The DNA complementary to probes A and C fails to form the characteristic V structure with the vertex attached to the nuclear matrix (Fig. 4, compare V with G). The frequency of the V structure in the RNase-treated nuclei is significantly lower ($P < 0.001$, chi-squared test) when compared with wild-type nuclei that were not RNase treated using probes A and C or to $ct^6$ nuclei using probe B (Table I). This result suggests that the nuclear matrix is important for the attachment of the DNA and the establishment of the loop structures.

The formation of DNA loops should require not only attachment to the nuclear matrix, but also interactions between individual Su(Hw) binding sites to form insulator bodies. We have previously shown that the heat shock response causes *gypsy* insulator bodies to fall apart, as a brief increase in temperature to 37°C results in the disappearance of the typical punctate pattern of Su(Hw) and Mod(mdg4) observed in imaginal disk cells (Gerasimova et al., 2000). Under these conditions, the insulator bodies disappear, and, instead, Su(Hw) and Mod(mdg4) proteins are diffusely spread throughout the nucleus; this is accompanied by changes in the subnuclear localization of insulator-containing sequences, suggesting a dissociation of the chromatin loops (Gerasimova et al., 2000). To confirm the effect of heat shock on insulator body structure and distribution under the assay conditions used in the experiments described above, we performed immunolocalization experiments using Su(Hw) and Mod(mdg4) 2.2 antibodies on 2 M NaCl-extracted nuclei from imaginal disk cells of $ct^6$ male larvae. The typical distribution of insulator bodies observed in cells grown at normal temperature (Fig. 3) is dramatically affected by heat shock. Nuclei from heat-shocked cells do not show large insulator bodies; instead, the Su(Hw) and Mod(mdg4)2.2 proteins are distributed throughout the nucleus and do not appear to be located in the nuclear periphery (Fig. 5, A–D). This result suggests that heat shock interferes with the ability of individual Su(Hw) binding sites to come together at specific nuclear locations to form insulator bodies. If this is the case, heat shock should also interfere with the formation of DNA loops, which are presumed to be caused by interactions between Su(Hw) binding sites. To test this prediction, we performed in situ hybridizations with probes A and B to 2 M NaCl-extracted nuclei from $ct^6$ male larvae. As we have described above, nuclei from these larvae should show the formation of an asymmetric V structure by DNA homologous to probe B (Fig. 4, L–N). Instead, in most nuclei from cells subjected to heat shock, the DNA homologous to probe B appears disorganized and located in the halo region of the extracted nuclei, away from the darkly stained core (Fig. 5, E–H). The number of times the V structure was observed in nuclei from heat-shocked $ct^6$ male larvae is significantly lower ($P < 0.005$, chi-squared test) than in non–heat-shocked animals (Table I). These results suggest that interactions between Su(Hw) binding sites, which are disrupted by heat shock, are normally required for the formation of the V structure and, therefore, for the formation of a loop mediated by the *gypsy* insulator present in the *ct* locus of $ct^6$ flies.

### Su(Hw)-mediated loop organization in nuclei from female larvae

The results described so far were obtained in experiments performed with X-linked DNA probes and cells from male larvae, which have only one X chromosome. In *Drosophila*, both the homologous autosomes and the two X chromosomes in females are paired during interphase (Hiraoka et al., 1993; Fung et al., 1998). The ability of the two homologous chromosomes to pair is responsible for the phenomenon of transvection, in which two different mutant alleles of the same gene can complement each other, resulting in a pairing-dependent wild-type phenotype (Lewis, 1954; Henikoff, 1997). The nature of the factors responsible for chromosome pairing during interphase is not known. It is possible that, if insulator proteins contribute to the organization of the interphase chromatin fiber into loop domains, the same proteins might also contribute to homologous chromosome pairing. To test this possibility, we determined whether DNA sequences surrounding the *ct* locus are present in one or two loops in nuclei obtained from female larvae. Probes A and B were used for in situ hybridization on 2 M NaCl-extracted nuclei from wild-type imaginal disk cells. As in the case of male nuclei, DNA homologous to probe A appears arranged in a straight line, with one end located in the darkly stained core and the other end pointing toward the surrounding halo region (Fig. 5, I–L). Interestingly, only one such hybridization signal is observed per nucleus, suggesting that the two homologous chromosomes remain paired after 2 M NaCl treatment and that the proteins involved in chromosome pairing are resistant to high salt extraction. As in male cells, sequences homologous to probe B are located in the halo region (Fig. 5, I–L). We then undertook the same type of analysis using nuclei from female larvae carrying the $ct^6$ mutation. Again, only one signal homologous to probe A was observed; in these nuclei, probe B gives rise to a single hybridization signal displaying an asymmetric V structure (Fig. 5, M–P). Probe B acquires a V shape in 80% of nuclei from $ct^6$ female larvae. Differences are significant ($P < 0.005$, chi-squared test) when we compare $ct^6$ nuclei with wild-type nuclei in which such a structure is only observed in 18% of nuclei (Table I). The observation of only one V structure suggests that the two new loops formed as a consequence of the presence of the *gypsy* retrotransposon in the *ct* locus from each chromosome homologue are still paired after high salt extraction. These results again support a conclusion suggesting that insulator proteins and/or other proteins present in the nuclear matrix are involved in maintaining homologous chromosome pairing during interphase.

## Discussion

We have presented evidence suggesting that the *gypsy* insulator creates chromatin loop domains via association to the nuclear matrix. The existence and exact composition of the nuclear matrix has been a subject of intense debate (Nickerson, 2001). Lamin, which is the main component of the nuclear lamina, is present in the nuclear matrix fraction. It is possible that protein components of the *gypsy* insulator interact with the nuclear lamina or with other components of the nuclear matrix. In our experiments, we have used two different biochemical nuclear matrix purification procedures. In both cases, it is clear that the Su(Hw) and Mod(mdg4)2.2 proteins associate with the nuclear matrix fraction, whereas other proteins, such as histones and Ubx, are extracted by high salt. We have also used an in situ cell extraction procedure followed by visualization of the extracted nuclei using light microscopy as a means of confirming the association of insulator proteins with the nuclear matrix. Using this approach, *gypsy* insulator proteins also appear to be associated with the nuclear residue that is resistant to salt extraction. Whether this resistant fraction is a filamentous network of defined composition or a matrix formed by interactions among different proteins and nucleic acids is not known, but it is clear that insulator proteins are not extractable by 2 M NaCl and are not present in the chromosomal regions that extrude from the nucleus after high salt extraction. The interaction of Su(Hw) and Mod(mdg4)2.2 with nuclear matrix components supports previous observations indicating that *gypsy* insulator proteins are preferentially present in the nuclear periphery of the interphase nucleus (Gerasimova et al., 2000).

Although the insulator DNA and associated proteins remain in the nuclear matrix fraction, the intervening DNA is extruded from the nucleus by high salt extraction and is found in the form of a large loop. The DNA contained within this loop appears only as a small dot after FISH analysis of unextracted nuclei. These two observations suggest that the chromatin fiber present in the loop formed by two insulators is not completely decondensed during interphase and only becomes extended after extraction of histones and other associated proteins. This suggests that the loop formed by two insulators might represent a domain of higher-order chromatin structure. This higher-order structure might be established and/or maintained by specific covalent modifications of histone tails. For example, it has been found that the chicken β-globin locus, which is flanked by two CTC-binding factor insulators, contains histones H3 and H4, acetylated in various lysine residues (Litt et al., 2001; Mutskov et al., 2002). Covalent modification of histone tails might modulate internucleosome interactions, which, in turn, determine the degree of higher-order chromatin structure (Tse and Hansen, 1997).

The existence of chromatin insulator-induced domains would explain their unusual gene regulatory property of preventing an enhancer from activating a promoter in a different domain while not preventing the same enhancer from activating a promoter located in its own domain. The ability of the insulator, when flanking a transgene, to provide position-independent expression of the transgene is also consistent with the formation of a loop domain. This domain appears to be created by the interaction of flanking insulators with each other and the nuclear matrix. In fact, recent experiments by Ishii et al. (2002) have shown that boundary function in yeast can be elicited by tethering boundary-associated proteins to the NPC. This tethering would presumably result in the formation of a loop, similar to the ones we observe here, by the DNA located between the two boundary elements, which would attach the base of the loop to the NPC. In the case of *Drosophila*, the requirement for interac-

tions between individual Su(Hw) binding sites for the formation of the loops is underscored by the observation that a brief heat shock interferes both with the formation of insulator bodies and with the ability of the *gypsy* retrotransposon to form a new loop when inserted in the *ct* locus.

The organization of the chromatin fiber into loops has also been shown for the *Drosophila* specialized chromatin structures (scs) and scs' boundary sequences. The proteins that interact with these elements have been shown to interact with each other both in vitro and in vivo. Consistent with the idea that interaction between the two proteins might facilitate pairing of boundary elements and formation of chromatin loops, sequences corresponding to the scs and scs' elements can be found in close proximity to each other in *Drosophila* nuclei (Blanton et al., 2003). The formation of similar loops by the *gypsy* insulator could also explain results by Cai and Shen (2001) and Muravyova et al. (2001), who demonstrated that two *gypsy* insulators inserted between an enhancer and promoter have no enhancer-blocking effect. These results could be explained in the context of the loop organization observed here by assuming that two closely linked insulators, due to their proximity, may preferentially interact with each other. This interaction would take place at the expense of interactions with other insulators, and it would result in the formation of a minidomain within a larger domain. Enhancers located within the larger domain would then be free to activate transcription from promoters in the same domain (Mongelard and Corces, 2001). The ability of the *gypsy* insulator to establish these chromatin loops raises the question of whether insulators/boundary elements are functionally equivalent to MARs/SARs. These sequences have been defined biochemically, based on their ability to attach to the nuclear matrix protein fraction in vitro. In some cases, MARs/SARs have also been shown to possess boundary activity using in vivo assays (McKnight et al., 1992; Namciu et al., 1998), although most MARs lack this activity (Poljak et al., 1994; van der Geest and Hall, 1997). It is possible that insulators and MARs have similar properties but play very different roles in the cell. MARs might have a fixed structural function in establishing chromatin organization within the nucleus. MARs might only be functional during mitosis, when the interphase to metaphase and back to interphase transition requires orderly changes in chromosome condensation and organization. Alternatively, MARs might create a scaffold of proteins and DNA that is more or less permanent during cell differentiation and among various cell types. Insulators, on the other hand, might act at a different level by creating an organization superimposed to that of MARs. Contrary to MARs, insulator activity might be regulatable, allowing this organization to change during development as cells differentiate and different patterns of gene expression are established.

Functional analyses of genome-wide expression patterns in yeast, *Drosophila*, and mammals also support the idea of the compartmentalization of the chromatin fiber into domains of gene expression (Cohen et al., 2000; Caron et al., 2001; Lercher et al., 2002; Spellman and Rubin, 2002). Studies in this diverse group of organisms have shown that genes located together in the same chromosomal region are transcriptionally coregulated. Although coregulation has not been shown to depend on insulator function, the existence of these transcriptional domains could have a structural basis in the formation of chromatin loops in which gene expression is globally regulated.

## Materials and methods

### *Drosophila* strains

Fly stocks were maintained in standard medium and grown at 22.5°C and 75% relative humidity. The $su(Hw)^{V}$ allele is caused by a small deletion that also affects the *RpII15* gene, resulting in embryonic lethality. The strain $su(Hw)^{V}$ used in these studies carries a transgene containing the *RpII15* gene that rescues this lethality (Harrison et al., 1992). The $mod(mdg4)^{u1}$ allele has been described previously (Gerasimova et al., 1995); this mutation affects only the Mod(mdg4)2.2 protein isoform (Mongelard et al., 2002). The $ct^{6}$ strain was obtained from the *Drosophila* Stock Center.

### Nuclear matrix preparations

Nuclear matrices were prepared from wild-type, Oregon R, *D. melanogaster* embryos 6–18 h old, following the protocol described by Fisher et al. (1982), except that 2 M NaCl was used for the final fractionation step. Western analysis was performed according to standard protocols. Nuclear halos were prepared according to Gerdes et al. (1994), except that the cells were obtained by dissecting and manually disrupting imaginal disk cells from third instar larvae with dissecting needles and spun onto coverslips at 350 *g* for 15 min. Samples that were treated with RNase were extracted with 2 M NaCl, as before, and then were incubated with 200 μg/ml of RNase A for 1 h at 4°C.

### Immunocytochemistry and FISH analysis

Antibodies against Su(Hw) and Mod(mdg4)2.2 were prepared as previously described (Gerasimova and Corces, 1998; Mongelard et al., 2002). Antibodies against Mod(mdg4)2.2 recognize only this isoform, which is the only one present in the *gypsy* insulator (Mongelard et al., 2002). Monoclonal antibodies against lamin were obtained from P. Fisher (The State University of New York at Stony Brook, Stony Brook, NY), H. Saumweber (Humboldt Universitaet Berlin, Berlin, Germany), and Y. Gruenbaum (The Hebrew University of Jerusalem, Jerusalem, Isreal); Ubx antibodies were obtained from J. Botas (Bayer College of Medicine, Houston, TX). Immunolocalization of proteins on nuclear halos was performed as follows: coverslips with samples were incubated 3 × 20 min in 1% BSA, 0.1% TX-100, 1× PBS (BBST) and then incubated in 1:100 dilution of primary antibody overnight at 4°C in a humidified chamber. Samples were then washed 3 × 1 min and 3 × 15 min in 1× BBST and then incubated in 1:500 FITC- or Texas red–conjugated secondary antibody in 1× BBST for 1 h at 37°C in a humidified chamber. The samples were then washed 3 × 1 min and then 3 × 15 min in 1× BBST without BSA, coated with DAPI-containing Vectashield (Vector Laboratories), and then visualized with a Carl Zeiss MicroImaging, Inc. fluorescence light microscope using MetaMorph (Universal Imaging Corp.) imaging software. For FISH analysis, probes A, B, and C were made from DNA from BAC clones 20K1, 35A, and 26L11 respectively representing the X chromosome at subdivisions 7B2–7B8. BAC clones were obtained from BACPAC Resources, and DNA was prepared following their protocol. Probes were synthesized using DIG-UTP or Biotin-UTP following a protocol from Boehringer. FISH on the untreated and 2 M NaCl-extracted halos was performed following protocols established by Gerdes et al. (1994) with the following exceptions. The probes were denatured for 5 min at 95°C. Washes after 16 h incubation with probe were 3 × 5 min in 2× SSC at 42°C, 3 × 5 min in 0.1× SSC at 60°C, and 2 × 30 min in 4× SSC with 1.5% BSA at 37°C. The samples were then incubated in 1:500 secondary antibody in 4× SSC with 1.5% BSA at 37°C in a humidified chamber for 1 h. The samples were washed 3 × 1 min in 4× SSC, 1 × 10 min in 4× SSC, 1 × 10 min in 4× SSC with 0.1% TX-100, 1 × 10 min in 4× SSC, 3 × 5 min in 1× PBS with 0.1% TX-100, and then 3 × 1 min in 1× PBS. The samples were then coated with Vectashield and visualized as described above. Chi-squared analyses were done using Statistica 4.0 (Statsoft Inc.). Polytene chromosomes for immunostaining and FISH were prepared as previously described (Gerasimova and Corces, 1998) with the following exceptions. Before immunostaining, FISH was performed by placing the liquid nitrogen–frozen slides in −70°C 95% EtOH at RT for 3 h. Then the samples were air dried, incubated in 2× SSC at 65°C for 1 h, incubated in 65°C 70% EtOH at RT 3 × 10 min, incu-

bated in 95% EtOH at RT 2 × 10 min, denatured in 0.14 M NaOH for 3 min, rinsed in 2× SSC for 5 min, rinsed in 70% EtOH three times, rinsed in 95% EtOH two times, and then air dried. Probes were prepared and denatured as above and added to each slide, covered with coverslips, sealed with rubber cement, and incubated in humidified chambers overnight at 37°C. After hybridization, the rubber cement was removed, and the slides were placed in 2× SSC at 37°C for 2 min, washed 2 × 10 min in 2× SSC at 37°C, 1 × 5 min in 1× SSC at 30°C, 1 × 10 min in 0.1× SSC, 2 × 5 min in 1× PBS, 0.1% TX-100, and then rinsed 1 × 5 min in 1× PBS at room temperature. Immunostaining was performed as previously described (Gerasimova and Corces, 1998).

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