A Chromatin Insulator Determines the Nuclear Localization of DNA

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Summary

Chromatin insulators might regulate gene expression by controlling the subnuclear organization of DNA. We found that a DNA sequence normally located inside of the nucleus moved to the periphery when the gypsy insulator was placed within the sequence. The presence of the gypsy insulator also caused two sequences, normally found in different regions of the nucleus, to come together at a single location. Alterations in this subnuclear organization imposed by the gypsy insulator correlated with changes in gene expression that took place during the heat-shock response. These global changes in transcription were accompanied by dramatic alterations in the distribution of insulator proteins and DNA. The results suggest that the nuclear organization imposed by the gypsy insulator on the chromatin fiber is important for gene expression.

Introduction

Chromatin insulators are DNA sequences with two distinctive properties: They can buffer the expression of a transgene from chromosomal position effects, and they interfere with the ability of an enhancer to activate transcription from a promoter when placed in between (reviewed by Gerasimova and Corces, 1996; Geyer, 1997; Bell and Felsenfeld, 1999). These properties are suggestive of an important role for insulators in the control of eukaryotic gene expression. Studies in the last few years have led to the identification and characterization of insulator sequences in a variety of organisms such as the specialized chromatin structure (scs) and scs sequences of *Drosophila* (Kellum and Schedl, 1991, 1992; Zhao et al., 1995), the Fab-7 element of the *bithorax* complex (Hagstrom et al., 1996; Zhou et al., 1996; Mihaly et al., 1997), and the insulator element present in the 5′ region of the chicken β-globin locus (Chung et al., 1993). In addition to its standard properties, this insulator prevents transgenes from becoming transcriptionally inactive due to DNA hypomethylation and histone deacetylation (Pikaart et al., 1998). The CCTC binding factor (CTCF) protein has been shown to bind to the chicken β-globin and other vertebrate insulators (Bell et al., 1999). Insulator elements have been also found flanking the transcriptionally repressed HML and HMR loci and in the upstream activation sequence of ribosome protein

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genes in yeast (Bi et al., 1999; Bi and Broach, 1999; Donze et al., 1999).

The mechanism of insulator action is not clear, but the peculiar properties of these sequences have led to several models to explain their effects on gene expression. Insulators do not simply inactivate enhancers, because they interfere with the transcriptional activation of a gene only when present between the enhancer and the promoter (Cai and Levine, 1995; Scott and Geyer, 1995). The directionality in the effect of insulators on enhancer-promoter interactions could be explained by assuming that insulators are simply barriers to a process in which a signal from the enhancer is transmitted to the promoter. In an extreme view of this model, the insulator could act as a "promoter decoy," forcing the enhancer-bound transcription factors to interact with the insulator instead of the transcription complex (Geyer, 1997). An alternative view suggests that insulators exert their effects on transcription through changes in chromatin structure. This model is supported by the observation that insulators are usually associated with strong DNase I hypersensitive sites and tend to separate chromatin domains with different degrees of condensation (Udvardy et al., 1985; Prioleau et al., 1999; Bi et al., 1999; Donze et al., 1999).

The gypsy insulator of *Drosophila* was first identified within the genome of the gypsy retrotransposon (Gdula et al., 1996) and it is composed of a 350-bp sequence and at least two proteins. The suppressor of Hairy-wing [su(Hw)] protein contains 12 zinc fingers and interacts with the insulator DNA (Spana and Corces, 1990). The mod(mdg4) protein contains a BTB domain and interacts with su(Hw) (Dorn et al., 1993; Gerasimova et al., 1995; Gerasimova and Corces, 1998). Results from immunofluorescence experiments using antibodies against su(Hw) and mod(mdg4) indicate that these proteins are present at hundreds of sites in polytene chromosomes from salivary glands. These sites do not contain copies of the gypsy retrotransposon and are presumed to be endogenous insulators, similar to the one found in gypsy, that play a role in the normal regulation of gene expression in *Drosophila*. The mod(mdg4) protein is located in approximately 500 sites and overlaps with su(Hw) at all sites where this protein is present. Surprisingly, similar immunofluorescence experiments using interphase nuclei of diploid cells do not show the diffuse and general nuclear staining one would expect from such a large number of individual binding sites. Instead, the su(Hw) and mod(mdg4) proteins are present in 20 to 25 specific locations within the nucleus (Gerasimova and Corces, 1998). A possible explanation for this arrangement is that several individual binding sites for insulator proteins come together in a single nuclear location to form large protein complexes. We will refer to the large aggregates of several individual gypsy insulator sites as insulator bodies. The implication of this interpretation is that insulator sites and their adjacent DNA, located in different parts of a chromosome, would be brought together to a single nuclear location through interactions mediated by insulator proteins. This model has several predictions
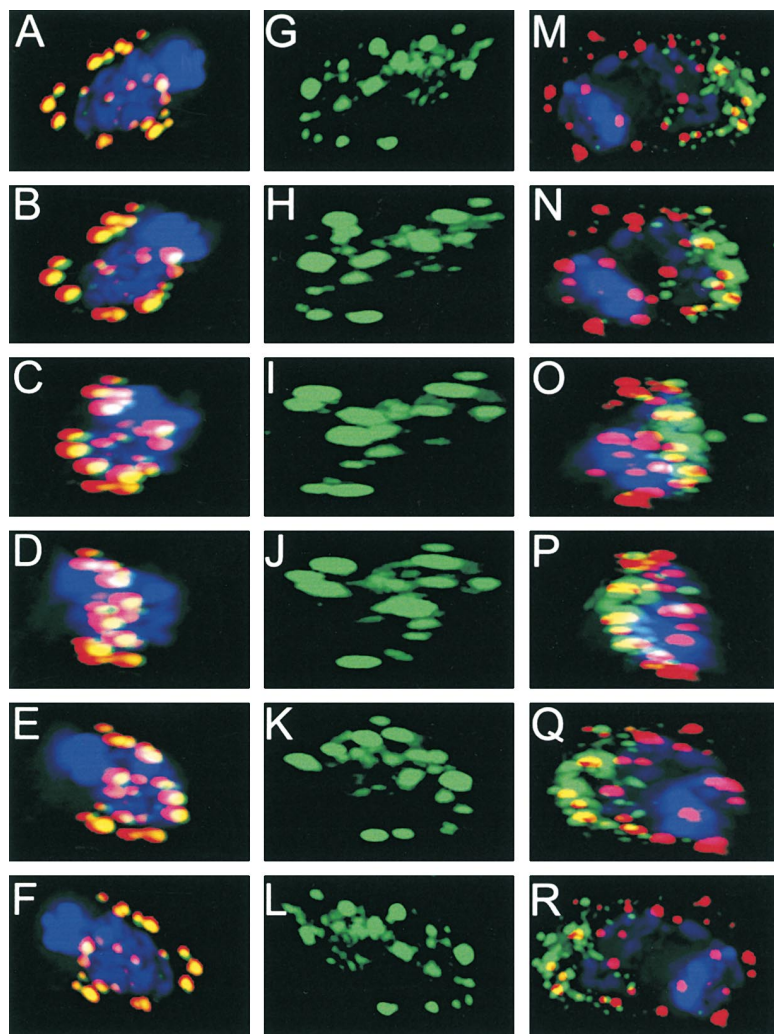
Figure 1. Subnuclear Localization of the Gypsy Insulator in Fixed and Live Interphase Diploid Cells from *Drosophila* Imaginal Discs

Stacks of images obtained as described in the Experimental Procedures section were deconvolved using an empirical point-spread function provided with the Deltavision software package. A 3D reconstruction of the nucleus was rotated around the Z axis. Each series of panels represents a view of the nucleus taken at 30-degree intervals.

(A–F) Distribution of su(Hw) (green) and mod(mdg4) (red) in nuclei. The DNA was stained with DAPI and is represented in blue.

(G–L) Imaginal disc cells from a strain expressing a GFP-su(Hw) fusion protein were viewed by fluorescence microscopy.

(M–R) Localization of gypsy insulator sites within the male X chromosome of *Drosophila*. Distribution of msl-2 is marked in green and mod(mdg4) in red. The DNA was stained with DAPI and is represented in blue. The distribution of the msl-2 protein marks the location of the X chromosome.

that can be tested experimentally to determine its validity. Results presented here suggest that the gypsy chromatin insulator might regulate gene expression by organizing the DNA within the nucleus, presumably through the establishment of higher-order chromatin domains that might play a role in the normal regulation of transcription.

Results

Gypsy Insulator Proteins Are Preferentially Located in the Nuclear Periphery

To further test the functional significance of the aggregation of large clusters of individual gypsy insulator sites at specific nuclear locations, we first determined their precise arrangement using antibodies against the su(Hw) and mod(mdg4) proteins. We used immunofluorescence light microscopy to collect images at regularly spaced focal planes; stacks of images were then deconvolved using the point-spread function of Agard et al., 1989. Three-dimensional (3D) reconstructions of nuclei were examined for the location of su(Hw) and mod(mdg4) proteins. Figures 1A through 1F show a nucleus from a third instar larva imaginal disc cell in interphase stained

with DAPI (blue) to visualize the location of the DNA. The location of the su(Hw) protein is indicated in green; and that of the mod(mdg4), in red. Both proteins colocalized in approximately 21 sites to form an equivalent number of insulator bodies (the precise number varied slightly from cell to cell, oscillating between 20 and 25). Snapshots of the nucleus as it rotated 180 degrees around a vertical axis (Figures 1A through 1F) show that all gypsy insulator bodies, with the exception of five located in the center of the nucleus, were located in the nuclear periphery. These interior bodies tended to be smaller and less intense than those located in the periphery. Because the total number of individual sites for the mod(mdg4) protein is approximately 500 (Gerasimova and Corces, 1998), these results suggest that, on average, approximately 25 individual gypsy insulator sites come together to form one insulator body; and these structures are present mostly in the nuclear periphery.

To test that the aggregation of individual insulator sites was not caused during the fixation step required by the immunofluorescence procedure, we decided to examine the distribution of su(Hw) protein in live cells. We constructed transgenic flies expressing a green fluorescent protein (GFP)-su(Hw) protein under the control
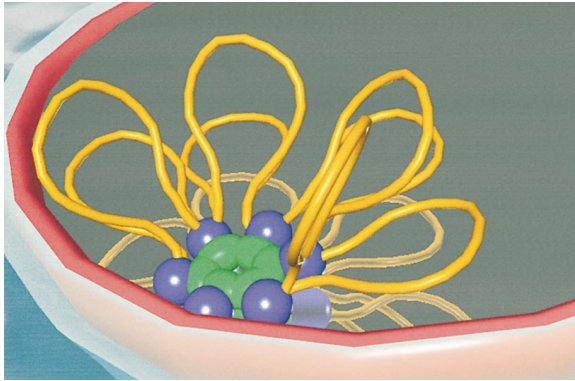
Figure 2. Artistic Representation of the Hypothesized Arrangement of Insulator Sites in the Nucleus of Interphase Cells

The interior of the nucleus is represented in gray, the nuclear lamina is depicted in red, and the nuclear membrane and cytoplasm are indicated in light blue. Dark blue and green spheres represent the su(Hw) and mod(mdg4) proteins, respectively. The chromatin fiber is represented in gold.

of the normal su(Hw) promoter; in addition, these flies carried a null mutation in the su(Hw) gene. Analysis of nuclei from imaginal disc cells shows the same pattern of su(Hw) distribution previously observed in fixed cells. Figures 1G through 1L illustrate views of a live nucleus as it rotated around a vertical axis; the image of the nucleus was obtained by 3D reconstruction of stacks of images after deconvolution analysis, as described above. These results support the idea that the large insulator bodies are present in live cells and might be the result of the aggregation of multiple individual insulator sites at specific single nuclear locations. This aggregation might be mediated through interactions between the su(Hw) and mod(mdg4) proteins or other putative, and as yet unknown, components of the gypsy insulator. The final outcome of these interactions might be to physically attach the chromatin fiber to a nuclear peripheral substrate–possibly the nuclear lamina–and in the process, organize the chromatin fiber into distinct domains. A model to visualize these interactions and the ensuing subnuclear organization of the DNA is depicted in Figure 2.

The Organization of Gypsy Insulator Bodies in the Male X Chromosome

To obtain further insight into the significance of the organization imposed by the presence of gypsy insulator sites on the structure of a specific chromosome during interphase, we determined the number of gypsy insulator bodies formed in a single X chromosome. To this end, we stained diploid interphase cells from imaginal discs of a wild-type Drosophila male with antibodies against the male-specific lethal 2 (msl-2) and mod(mdg4) proteins; msl-2 is present specifically in the X chromosome of males (Kelley et al., 1995). Serial optical sections through a nucleus were deconvolved and the information was used to build 3D reconstructions of individual nuclei. The results of this analysis are shown in Figures

1M through 1R in the form of different views of a nucleus as it rotated 180 degrees around a vertical axis. The location of the X chromosome is demarcated by the green staining due to the msl-2 antibody. The location of gypsy insulator bodies is indicated by the red staining of anti-mod(mdg4) antibodies. It is apparent from Figure 1 that the territory occupied by the X chromosome contained five large aggregates of individual gypsy insulator sites. This number varied between four and six in different nuclei, and was also the average of ten different nuclei examined in the same fashion (data not shown). Each large aggregate represented several individual gypsy insulator sites, suggesting that the X chromosome of Drosophila is organized in approximately five different rosettes similar to that represented in Figure 2. Because the X chromosome contains approximately 21,000 kb of DNA, each of these rosettes would contain approximately 4,000 kb of DNA, and the sequences from 4B and 7D (described below) would be present within the same rosette. The X chromosome contains approximately 80 individual gypsy insulator sites (Gerasimova and Corces, 1998), and, therefore, each rosette should be composed, on average, of 16 individual sites. Two individual sites coalescing into a gypsy insulator body form a loop or domain of higher-order chromatin organization. The length of the DNA contained within each loop would be approximately 250 kb.

Gypsy Insulator DNA Preferentially Localizes to the Nuclear Periphery

The model for the organization of the chromatin fiber presented in Figure 2 is based solely on the observed localization of the protein components of the gypsy insulator (Figure 1). The model has several testable predictions on the nuclear location of insulator-containing DNA sequences. For example, if the model is correct, a DNA sequence containing a copy of the gypsy insulator should be located, in most cases, in the nuclear periphery, because the protein components of the gypsy insulator bodies appear to reside preferentially in this nuclear compartment. But the location of the sequence should move toward the center of the nucleus in cells mutant for one of the insulator components, such as su(Hw), because this protein binds to the gypsy insulator DNA and is essential for its function.

To test this prediction, we carried out fluorescence in situ hybridization (FISH) with DNA sequences that span a region of the Drosophila genome that contains a copy of the gypsy retrotransposon. Figure 3 shows the result of such an experiment using DNA sequences corresponding to the cut (ct) locus located at subdivision 7B in the X chromosome. This probe was hybridized to imaginal disc cells from animals carrying the ct^6 allele caused by gypsy insertion into the ct gene (Jack, 1985). Note the location of the hybridization signal in the nuclear periphery, at the very edge of the blue staining corresponding to DNA (Figure 3A). In contrast, in imaginal disc cells from flies of the genotype ct^6; $su(Hw)^V$, which carry a null mutation in the su(Hw) gene, the location of the hybridization signal was preferentially distributed in the central part of the nucleus (Figure 3B). The same experiment was carried out using sequences from
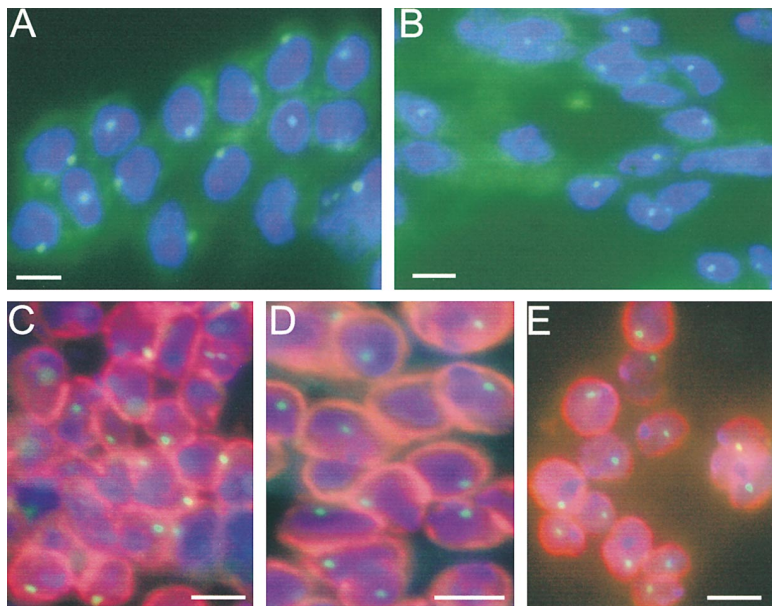
Figure 3. Effect of the Gypsy Insulator on the Nuclear Localization of DNA

DNA probes from chromosomal subdivision 7B (A and B) or 4D (C, D, and E) were used as hybridization probes on cells from imaginal discs of *Drosophila* third instar larvae from a *ct⁶* strain containing gypsy elements at 4D and 7B. The hybridization signal corresponding to the probe is seen in green, the nuclear DNA is depicted in blue, and the nuclear lamina is visualized in red (C, D, and E).
(A) ct^6 strain, 7B probe.
(B) ct^6; $su(Hw)^V$ strain, 7B probe.
(C) ct^6 strain, 4D probe.
(D) ct^6; $su(Hw)^V$ strain, 4D probe.
(E) ct^6 strain, 4D probe after a 20-min heat shock at 37°C.

the 4D chromosomal subdivision and imaginal disc cells from the same ct^6 strain, which also contains a gypsy element at 4D. In this case, antibodies to lamin were used simultaneously to mark the peripheral location of the nuclear lamina. As in the case of the 7B probe, sequences from 4D were preferentially located in the nuclear periphery, immediately adjacent to or overlapping the nuclear lamina, in cells from the ct^6 strain in which the gypsy element was present at 4D (Figure 3C). In contrast, the hybridization signal corresponding to the 4D subdivision was located in a more central region of the nucleus in cells from flies of the genotype ct^6; $su(Hw)^V$ (Figure 3D). A similar result was obtained when sequences from the *bithorax* locus were used as hybridization probes to compare their nuclear localization in cells from a strain carrying the gypsy-induced bx^{34e} mutation (Modolell et al., 1983) and from bx^{34e} $su(Hw)^V$ flies (data not shown).

Data obtained from the second set of experiments were analyzed by measuring the ratio of the distance between the dot of FISH signal and the lamina to the radius of the nucleus. When nuclei had an ovoid shape, we used the longest radius to avoid biasing the results. The data from this analysis are presented in Figure 4A. In flies wild type for *su(Hw)*, the hybridization signals in 25% of the nuclei were at a distance of zero from the lamina, that is, the center of the hybridization signal was inside of the nuclear lamina; 50% of the nuclei showed hybridization signals within 0.1 units from the lamina. In flies carrying a mutation in the *su(Hw)* gene, the hybridization signal was at a distance of 0.3 from the nuclear lamina in 25% of the nuclei; 50% of the nuclei showed hybridization signals at a distance of almost 0.4 units from the lamina. This analysis shows a clear and statistically significant difference ($p \leq 0.00001$) in the nuclear distribution of insulator-containing DNA sequences between nuclei from cells expressing the su(Hw) protein or lacking this essential component of the gypsy insulator, suggesting that this insulator and associated proteins

contribute to the peripheral localization of a DNA sequence.

Sequences Containing the Gypsy Insulator Overlap with Sites Where Insulator Proteins Aggregate in the Nucleus to Form Insulator Bodies

According to the model proposed to explain the formation of gypsy insulator bodies visible with the light microscope (Figure 2), these aggregates are formed by the combination of individual binding sites for insulator proteins. As a consequence, a specific DNA sequence should be present in the nucleus in or immediately adjacent to one of these insulator bodies when the sequence contains a gypsy insulator, but the two should localize in different nuclear regions when the gypsy insulator is not present in the sequence. To test this prediction, we carried out in situ hybridization to cells from imaginal discs using as a probe sequences from the *bithorax* complex. At the same time, we determined the distribution of gypsy insulator bodies by immunofluorescence using antibodies against the mod(mdg4) protein. The results of these experiments are displayed in Figure 5. When imaginal disc cells from a strain carrying the gypsy-induced bx^{34e} allele were used in the experiment, the site of DNA localization determined by FISH frequently overlapped with one of the large aggregates of gypsy insulator proteins visualized with antibodies against mod(mdg4) (Figures 5A–5D). In contrast, the two signals failed to overlap when cells from wild-type flies, in which the gypsy element is absent from the *bithorax* locus, were used (Figures 5E–5H). In order to determine the statistical significance of this observation, we examined 540 nuclei from bx^{34e} flies and 455 nuclei from the wild-type *Oregon R* strain. The FISH and immunofluorescence signal overlapped in 73.8% of nuclei from imaginal disc cells of the bx^{34e} strain, but only overlapped in 19.6% of nuclei from *Oregon R*. The p value of this analysis was $p \leq 0.001$, indicating a highly significant probability that the correlation was not random.

A

Distance between FISH signals and lamina/radius of the nucleus

- Min-Max
- 25%-75%
- Median

ct^6 22 °C $ct^6; su(Hw)^V$ 22 °C ct^6 37 °C

B

Distance between FISH signals/ radius of the nucleus

- Min-Max
- 25%-75%
- Median

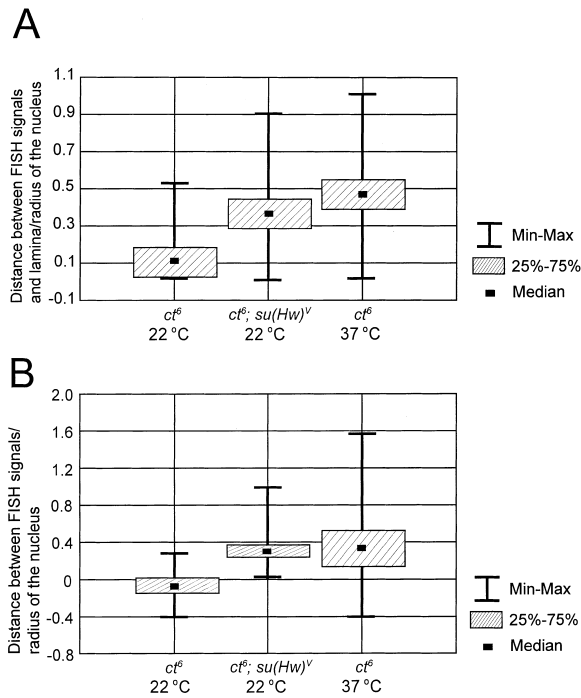ct^6 22 °C $ct^6; su(Hw)^V$ 22 °C ct^6 37 °C

Figure 4. Statistical Analysis of FISH Data

(A) Analysis of data presented in Figure 3. The distances between the center of the dots corresponding to the DNA hybridization signals and the nuclear lamina were measured using the Metamorph program (Universal Imaging Corp.). The results are expressed in arbitrary units with respect to the radii of the corresponding nuclei measured in the same manner by tracing a circle around the outside of the irregularly shaped nuclei. The number of nuclei examined was 251 for ct^6, 22°C; 203 for ct^6; $su(Hw)^V$, 22°C; and 431 for ct^6, 37°C.
(B) Analysis of data presented in Figure 6. The distances between two different hybridization signals were determined by measuring the distance between the centers of the dots and subtracting the sum of their radii. These distances were then divided by the radii of the corresponding nuclei. The number of nuclei examined was 318 for ct^6, 22°C; 240 for ct^6; $su(Hw)^V$, 22°C; and 341 for ct^6, 37°C. All distances were measured using the Metamorph program.

The Gypsy Insulator Causes Two Sequences from Separate Chromosomal Locations to Come Together at the Same Site in the Nucleus

A third prediction of the model proposed above is that two sequences normally separated in the genome should come together in the nucleus when a copy of the gypsy insulator is inserted in each of the two sequences. We chose two DNA sequences located in the X chromosome at subdivisions 4D and 7B to test this prediction. When these two sequences were used as probes for in situ hybridization using cells from the wild-type *Oregon R* strain, two distinct signals were observed (Figure 6A). The same experiment was then repeated using the ct^6 strain containing copies of the gypsy element at 4D and 7B (7B is the cytological location of the *ct* gene). In this case, the two hybridization signals clearly overlapped in most nuclei of diploid cells in interphase (Figure 6B). The same ct^6 strain containing the two gypsy insertions at 4D and 7B, but also carrying a null mutation in the *su(Hw)* gene, showed two distinct separate hybridization signals corresponding to each of the probes as in the wild-type *Oregon R* strain (Figure 6C).

These results were quantitated by measuring the ratio of the distance between hybridization signals to the radius of the corresponding nucleus. To account for the difference in sizes of FISH signals, the distance between hybridization dots was calculated by measuring the distance between the centers of the dots and subtracting the sum of the radii of each pair of dots. Figure 4B shows the results of this statistical analysis. A negative number indicates that the two hybridization signals partially or completely overlapped, whereas a value of zero indicates that the two hybridization signals were immediately adjacent to and touching each other. The results of this analysis show that, in flies carrying a wild-type *su(Hw)* gene, the two hybridization signals overlapped in almost 75% of the nuclei, whereas no overlap was observed in the signals present in nuclei of the $su(Hw)^V$ strain (p $\leq$ 0.00001); the same result was obtained for *Oregon R*. These results suggest that insulator sequences and their associated protein components cause two sequences located far apart in the genome to come together in the same nuclear location. This evidence strongly supports the hypothesis that individual gypsy insulator sites gather at specific nuclear locations to form the large aggregates observed in diploid nuclei during interphase.

The Nuclear Distribution of Gypsy Insulator Bodies Correlates with the Transcriptional State of the Cell

If the role of the gypsy insulator is to organize the chromatin fiber within the nucleus, this organization might be static and serve only a structural role or, alternatively, it might have a functional significance in a manner that is relevant to gene expression. In the latter case, there should be a correlation between the organization imposed by the gypsy insulator and the ability of genes to be transcribed. To address this issue, we determined the possible effects of heat shock on the subnuclear arrangement of gypsy insulator bodies. During the heat-shock response in *Drosophila*, there are dramatic changes in the transcriptional state of the cell. A small number of genes, the heat-shock genes, are turned on, while transcription of the rest of the genome is turned off (Ashburner and Bonner, 1979). One would then expect that if the organization of gypsy insulator bodies were important for transcription, these global changes in gene expression would be accompanied by alterations in their nuclear arrangement. Results from the analysis of the distribution of GFP-su(Hw) protein in live nuclei suggest that this is the case. Cells from larval imaginal discs showed the typical punctated pattern of gypsy insulator bodies (Figure 7A). After a 20-min heat shock at 37°C, the su(Hw) protein appeared to be distributed throughout the nucleus in a uniform pattern; the punctated distribution had disappeared, with only some cells showing one faint dot of su(Hw) protein (Figure 7B). To test whether this alteration is also true for the mod(mdg4) protein, we determined its distribution in fixed cells using antibodies. As was the case for su(Hw), the mod(mdg4) protein was distributed in a diffuse pattern after a brief heat shock, with some cells still displaying one gypsy insulator body (Figures 7C and 7D).

The alteration in the distribution of gypsy insulator bodies observed after temperature elevation could be
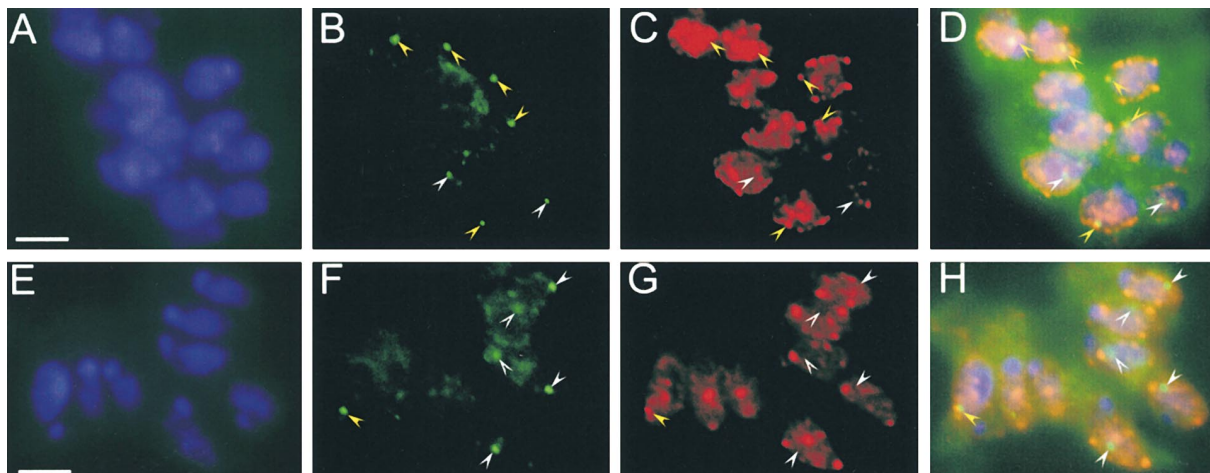
Figure 5. Nuclear Localization of DNA Sequences Containing or Lacking the Gypsy Insulator, and Insulator Proteins

A DNA probe containing the *bithorax* locus was used as hybridization probe on cells from imaginal discs of *Drosophila* third instar larvae from the *bx*^{34e} (A, B, C, and D) or *Oregon R* (E, F, G, and F) strains. The same samples were then incubated with antibodies against mod(mdg4). The location of the nuclei as visualized by DAPI staining is shown in (A) and (E). The DNA hybridization signal is shown in green (B and F), and the location of mod(mdg4) protein is shown in red (C and G). (D) and (H) depict the merged images of the DAPI, DNA, and protein localization data. Arrows indicate the location of DNA hybridization signals in (B), (D), (F), and (G). Yellow arrows indicate the location of hybridization signals that correspond to a dot of mod(mdg4) accumulation in (C) and (G), whereas white arrows indicate the location of hybridization signals that do not overlap with sites of mod(mdg4). In (C) and (G), the arrows indicate the corresponding regions of the nuclei where the DNA hybridization signals are located in (B) and (F).

caused by a decrease in the cellular levels of the su(Hw) and mod(mdg4) proteins; Western analyses showed that this was not the case and that the levels of the two proteins were the same before and after heat shock (data not shown). A second possibility was that the su(Hw) protein did not bind to its target sequence after heat shock and, as a consequence, both protein compo-

nents of the gypsy insulator fell off the chromosomes in cells subjected to temperature elevation. To test this possibility, we examined the distribution of su(Hw) and mod(mdg4) proteins on polytene chromosomes from salivary glands. Figures 7E through 7H show that the number and intensity of bands was the same for both proteins before and after heat shock, suggesting that
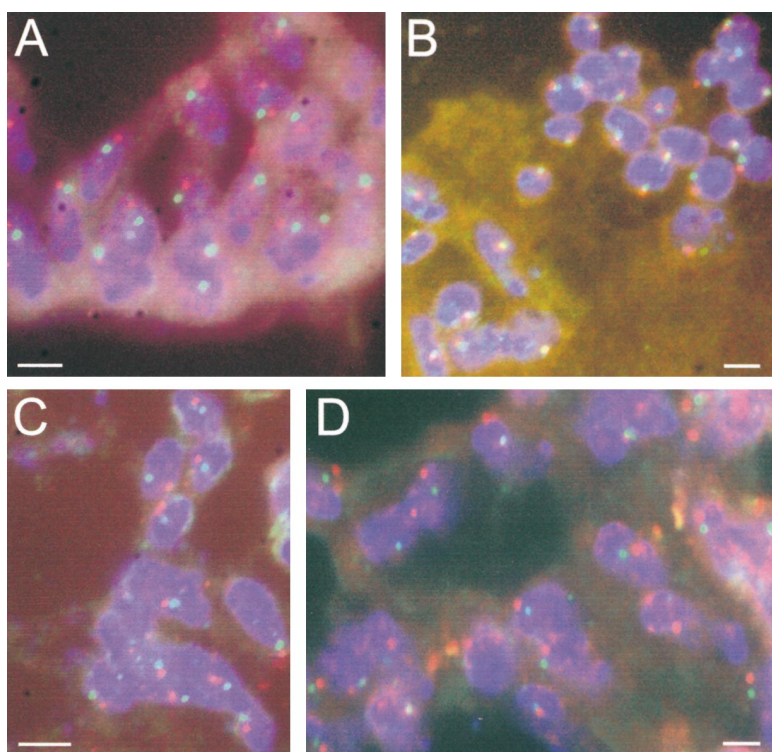

Figure 6. Effect of the Gypsy Insulator on the Nuclear Distribution of Two Different DNA Sequences

DNA sequences from the 4D and 7B polytene chromosomal subdivisions were employed as hybridization probes in FISH experiments using imaginal disc cells from *Oregon R* (A), *ct*^{6} (B), *ct*^{6}; *su(Hw)*^{V} (C), and *ct*^{6} subjected to a 20-min heat shock at 37°C (D). The *ct*^{6} strain contains copies of the gypsy element at chromosomal subdivisions 4D and 7B within the region spanned by the probe. Hybridization signals corresponding to sequences from the 4D region are in red, whereas signals corresponding to 7B sequences are in green.
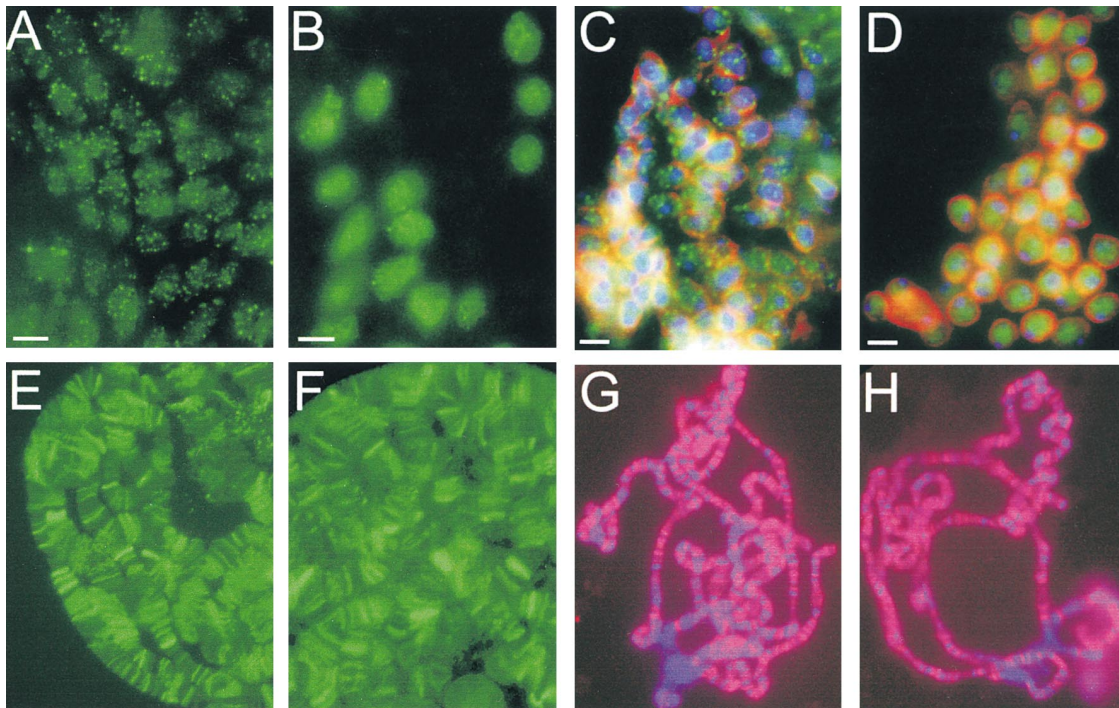
Figure 7. Effect of Heat Shock on the Chromosomal and Nuclear Distribution of Su(Hw) and Mod(Mdg4) Proteins

Samples were visualized by fluorescence microscopy, either directly using live cells (A, B, E, and F) or after fixation using Texas red and fluorescein isothiocyanate-conjugated antibodies (C, D, G, and H).

(A) Live imaginal disc cells from a strain expressing a GFP-su(Hw) fusion protein grown at 22°C.

(B) Live imaginal disc cells from the same strain, but subjected to heat shock for 20 min at 37°C.

(C) Localization of mod(mdg4) (green) and lamin (red) proteins in fixed cells from the same strain grown at 22°C; DNA stained with DAPI is shown in blue.

(D) Localization of mod(mdg4) (green) and lamin (red) proteins in fixed cells from larvae heat shocked at 37°C for 20 min; DNA stained with DAPI is shown in blue.

(E) Polytene chromosomes from an intact salivary gland nucleus of a strain expressing GFP-su(Hw) protein and grown at 22°C.

(F) Same as (E) but the larvae were incubated at 37°C for 20 min.

(G) Localization of mod(mdg4) (red) on polytene chromosomes from a squashed salivary gland obtained from a strain grown at 22°C; DAPI-stained DNA is shown in blue.

(H) Localization of mod(mdg4) (red) on polytene chromosomes from larvae incubated at 37°C for 20 min.

the alterations observed in diploid nuclei were not caused by disruption of protein–DNA or su(Hw)-mod(mdg4) interactions at individual insulator sites.

Alterations in the Pattern of Gypsy Insulator Bodies Induced by Heat Shock Are Accompanied by Changes in the Nuclear Distribution of DNA

One possible explanation for the observed alteration in the pattern of gypsy insulator bodies after heat shock was that the interactions among individual sites that give rise to the rosette structures represented in Figure 2 were disrupted, causing their disorganization. If this were the case, one would expect that changes observed at the protein level would then correlate with alterations in the arrangement of DNA sequences containing the gypsy insulator. To test this hypothesis, we examined the nuclear distribution of a DNA sequence located at subdivision 4D in the ct^6 strain carrying a gypsy element at this chromosomal position, and we compared the subnuclear location of this sequence in non-heat-shocked cells versus cells subjected to a 37°C heat shock for 20 min. Whereas in imaginal disc cells from flies grown at 22°C the 4D sequence was located prefer-

entially in the nuclear periphery (Figure 3C), in cells subjected to a brief heat shock, the sequence was located preferentially inside of the nucleus (Figure 3E). The distance between the nuclear lamina and the FISH signal was measured and the distribution is shown in Figure 4A. Before heat shock, 50% of the nuclei showed a FISH signal at a distance of less than 0.1 units from the periphery. This distance increased to almost 0.5 in cells subjected to heat shock. In addition, heat-shocked cells showed a wide range in the distribution of these distances, compared to control cells. The difference in the subnuclear distribution of the DNA sequence between control and heat-shocked cells was highly significant ($p \leq 0.0001$). This result suggests that the changes observed in the localization of su(Hw) and mod(mdg4) proteins are accompanied by changes in the localization of the DNA to which these proteins are bound. Because the proteins stay bound to the DNA after heat shock, the only explanation for this observation is that the rosette structure formed by the gypsy insulator was disrupted as a consequence or pre-requisite for the heat-shock response.

To further test this hypothesis, we examined the distri-

bution of two different gypsy-containing DNA sequences, present at chromosomal subdivisions 4D and 7B, before and after heat shock. Before heat shock, these two sequences were located in close proximity within the nucleus and often overlapped (Figure 6B). After a brief heat shock at 37°C, the two sequences appeared far apart within the nucleus (Figure 6D). Analysis of data obtained from these experiments indicates that both the distance and the range increased after heat shock (p ≤ 0.0001; Figure 4B). These results support the conclusion that the global changes in transcription observed during the heat-shock response were accompanied by a reshuffle of the DNA within the nucleus as a consequence of alterations in the chromatin organization imposed by the gypsy insulator.

Discussion

Models to explain the molecular mechanisms of insulator action are based on their idiosyncratic effects on gene expression. Some of these effects can be explained by assuming that insulators interfere with the propagation of a directional signal from the enhancer to the promoter. This signal could be the looping of intervening DNA; the tracking of a transcription factor along the DNA toward the promoter; the topological state of the DNA; or some form of chromatin alteration that affects the primary structure of the chromatin fiber, such as histone modifications or changes in nucleosome condensation or spacing. Other properties of insulators point to an involvement of these sequences in the establishment or maintenance of higher-order chromatin organization. For example, the ability of insulators to buffer the expression of transgenes against chromosomal position effects (Kellum and Schedl, 1991; Roseman et al., 1993) and their location at the boundaries between active and inactive chromatin (Udvardly and Schedl, 1985; Prioleau et al., 1999) are both indicative of a role in the establishment of higher-order chromatin domains. In addition, the observed interactions between proteins involved in chromatin dynamics, such as enhancers/suppressors of position-effect variegation and trxG/PcG proteins, and the protein components of some insulators are also suggestive of a role for these sequences in higher-order chromatin organization (Gerasimova and Corces, 1998). The results presented here support the involvement of insulators in nuclear organization and suggest that this organization is the primary cause of their effects on gene expression by subsequently affecting the transmission of signals from the enhancer to the promoter.

We have previously shown that the su(Hw) and mod(mdg4) proteins are components of the gypsy insulator and localize at approximately 500 sites on polytene chromosomes from salivary glands of third instar larvae (Gerasimova and Corces, 1998). The observed distribution of insulator proteins in polytene chromosomes suggests that they should bind at more or less regular intervals in the chromosomes of diploid cells in interphase. Given the large number of sites and their regular distribution, one would expect to observe a diffuse homogeneous scattering of insulator sites in the nuclei of interphase diploid cells. Surprisingly, this is not the case

and, instead, gypsy insulator proteins accumulate at a small number of nuclear locations. This has led to the suggestion that each of the locations where su(Hw) and mod(mdg4) proteins accumulate in the nucleus is made up of several individual sites that come together, perhaps through interactions among protein components of the insulator. Interestingly, the locations in the nucleus where individual insulator sites appear to aggregate are not random. Analysis of the distribution of gypsy insulator bodies in 3D reconstructions of nuclei from diploid cells in interphase indicate that, although not all the aggregate sites are present in the nuclear periphery, approximately 75% of them are present immediately adjacent to the location of the nuclear lamina. This finding suggests that the formation of gypsy insulator bodies perhaps requires a substrate for attachment, and the physical attachment might play a role in the mechanism by which insulators affect enhancer-promoter interactions. The nuclear lamina itself might serve as a substrate for attachment, perhaps through interactions between lamin and protein components of the insulator. The nature of the substrate involved in the attachment of the aggregate sites found in the interior of the nucleus is unknown, but it is interesting that a lamin network has also been detected in the inside of the nucleus (Neri et al., 1999). This attachment might impose a topological or physical constraint on the DNA that interferes with the transmission of a signal from an enhancer located in one domain to a promoter located in an adjacent one. The preferential aggregation of insulator sites at the nuclear periphery and the possibility that this targeting might take place through interactions with the nuclear lamina led to the idea that the gypsy insulator might be equivalent to matrix attachment regions/scaffold attachment regions (MARs/SARs; Gerasimova and Corces, 1998). This hypothesis is directly supported by the finding of MAR activity within the DNA sequences containing the gypsy insulator (Nabirochkin et al., 1998).

The nonuniform distribution of gypsy insulator bodies inside of the nucleus allowed us to test the hypothesis that these sites indeed correspond to several individual insulators coming together in a single nuclear location. The model was tested with insulator sequences carried by the gypsy retrovirus, but it is likely that the conclusions are also applicable to putative endogenous insulators. As predicted by this hypothesis, a DNA sequence usually located randomly with respect to the periphery versus interior compartments in the nucleus became preferentially located in the periphery when a copy of the gypsy insulator was present in this sequence. The correspondence between the presence of the insulator and the peripheral localization was not 100%, as one would expect from the location of 25% of gypsy insulator bodies in the interior of the nucleus. The idea that insulator bodies result from the coalescence of individual gypsy insulators was also supported by the overlap in the nucleus between a DNA sequence containing the gypsy insulator and sites in the nucleus where insulator bodies were present. The strongest evidence in support of a role for the gypsy insulator in the organization of the chromatin fiber within the nucleus through the aggregation of individual insulator sites came from the analysis of the localization of two specific DNA sequences. The two sequences utilized in the experiments de-

scribed above were located at 4D and 7B in the X chromosome. Because the X chromosome contains approximately 21,600 kb of DNA (Adams et al., 2000), each chromosomal division would contain approximately 1,000 kb of DNA. Therefore, these two sequences were separated by approximately 3,000 kb of DNA, and they appeared as two distinct hybridization signals in nuclei of diploid cells. Nevertheless, the presence of insulator sites in these two sequences in cells containing copies of gypsy at 4D and 7B caused them to associate into a single nuclear location.

The organization imposed by the gypsy insulators on the chromatin fiber might explain the effect of these sequences on the silencing effects of Polycomb response elements (PREs). The gypsy element is able to block silencing caused by interactions among PREs located on the same chromosome, but is not able to block silencing due to PREs located in different chromosomes (Sigrist and Pirrotta, 1997). These results could be explained if the rosette organization imposed by gypsy insulators on a specific chromosome interfered with other types of intrachromosomal interactions mediated by PREs, whereas the gypsy-induced organization still allowed interchromosomal interactions between these elements. An important question arising from the role of insulators in nuclear organization is whether this organization is static and has a mostly structural role, or is dynamic and has a direct functional significance. If the latter were the case, one would expect a correlation between the pattern of nuclear organization of insulator bodies and the transcriptional state of the cell. Changes in transcription that take place during the heat-shock response in *Drosophila* represent an ideal situation to address this question because the alterations in gene expression induced by temperature elevation are global, affecting the whole genome (Ashburner and Bonner, 1979). If the gypsy insulator plays a role in establishing higher-order chromatin domains, and this organization of the chromatin fiber is important for transcription, it should then be possible to detect alterations in the pattern of insulator bodies within the nucleus concomitant with changes in gene expression induced by heat shock. Indeed a dramatic modification in the distribution of su(Hw) and mod(mdg4) proteins can be observed in the nuclei of heat-shocked cells. The normal punctated pattern disappears and these two proteins are present diffusely throughout the nucleus. The observed changes are not due to effects of heat shock on su(Hw) and mod(mdg4) levels, or their ability to interact with DNA or with each other, because both proteins are still present in polytene chromosomes at normal levels after temperature elevation. The most likely explanation for the observed changes is a reorganization in the higher-order chromatin structure imposed by the gypsy insulator. This conclusion is strongly supported by the changes observed in the localization of gypsy-containing DNA. Sequences containing the gypsy insulator and located in the nuclear periphery before heat shock moved toward the center of the nucleus after temperature elevation. Similarly, two insulator-containing DNA sequences, normally located in close proximity, moved apart when cells were subjected to a brief heat shock. These results suggest a correlation between the transcriptional changes taking place during the heat-shock response and alterations in the nuclear arrangement of the DNA determined by the gypsy insulator, supporting a role for insulators in the organization of the chromatin fiber in a manner that has functional significance in the control of gene expression.

Experimental Procedures

Construction of Transgenic Flies, DNA Isolation, and Antibody Preparation

Flies were maintained in standard medium and grown at 22.5°C and 75% relative humidity. The ct^6 strain containing gypsy insertions at chromosomal subdivisions 4D and 7B was obtained in crosses with a strain carrying a mutation in the *flamenco* gene, which causes mobilization of the gypsy element (Song et al., 1994). The strain expressing a GFP-su(Hw) protein was made by P element-mediated transformation. A plasmid containing the su(Hw) gene present in a 5.3 kb Xba I-Kpn I fragment was altered by introducing a Not I site at the ATG initiation codon of the su(Hw) protein; the complete GFP coding region was then inserted into the Not I site such that the resulting plasmid encodes a GFP-su(Hw) fusion protein containing the complete su(Hw) coding region. The GFP-su(Hw) transgenic strain was then crossed to a stock carrying the $su(Hw)^V$ null allele; the GFP-su(Hw) fusion protein is functional and rescues the mutant phenotype of the null allele. DNA from P1 clones was isolated as described by Sternberg, 1990. Antibodies to su(Hw) and mod(mdg4) proteins were prepared as previously described (Gerasimova and Corces, 1998). Antibodies against msl-2 were obtained from Dr. Mitzi Kuroda. Monoclonal antibodies against lamin were obtained from Dr. Harry Saumweber.

In Situ Hybridization and Immunolocalization

DNA probes for FISH experiments were isolated from P1 phages obtained from the *Drosophila* Berkeley Genome Project. The P1 clones used in these experiments include DS01156, which maps to the chromosomal interval 4D1–4D2; DS00188, which maps to 7B1–7B6; and DS00471, which maps to 89E1–89E2 (Hartl et al., 1994). The precise location of the DNA sequences contained in these P1 clones was confirmed by carrying out in situ hybridization experiments to polytene chromosomes of the ct^6 and bx^{34e} strains using the P1 clone and gypsy element as probes simultaneously (data not shown).

Probes for in situ hybridization were labeled by nick translation using biotin-16-dUTP or digoxigenin-11-dUTP. Imaginal discs were fixed in 2% paraformaldehyde for 25 min and incubated in 45% acetic acid for 5 min on polylysine-treated slides covered with siliconized coverslips. Slides were frozen in liquid nitrogen for 30 min, treated with 200 mg/ml RNase in phosphate-buffered saline (PBS) for 1 hr at room temperature, subjected to an ethanol series, and air dried. The labeled probe was applied to the slide in 2X SSC (150 mM NaCl, 15 mM sodium citrate [pH 7.0]), 10% dextran sulfate, 50% formamide, 400 μg/ml salmon sperm DNA, covered with a coverslip, and sealed with rubber cement. DNA was denatured by incubating the slides at 97°C for 5 min. The slides were then incubated for 12–15 hr at 37°C, washed twice in 50% formamide, 2X SSC at 37°C, and rinsed with PBS. For FISH-only experiments, the slides were incubated with streptavidin-fluorescein or rhodamine-conjugated antidigoxigenin antibodies for 1.5 hr at room temperature, washed three times with PBS, incubated with DAPI, and rinsed with PBS. After drying and application of Vectashield, the slides were visualized under UV light with a Zeiss microscope. When the experiment involved simultaneous FISH and immunolocalization steps, the slides were incubated in antibody dilution buffer (130 mM NaCl, 7 mM sodium phosphate [pH 7.4], 0.1% Triton X-100, 1% bovine serum albumin) for 1.5 hr at room temperature after the incubation with the DNA probe, changing the buffer three times. Incubation with the primary antibody was carried out overnight at 4°C, the slides washed three times with PBS, and incubated with streptavidin-fluorescein or the secondary antibody for 1.5 hr at room temperature. After washing three times with PBS and incubation with DAPI, the slides were visualized as described above.

Microscopy and Image Analysis

Images were recorded using a Photometrix CH350 cooled CCD camera and a Zeiss Axiophot microscope equipped with fluorescence capability. Distances between hybridization signals in nuclei were measured using the Metamorph program (Universal Imaging Corporation). Measurements were normalized with respect to the radius of the nucleus to compensate for differences between preparations. When nuclei had an ovoid shape, we used the longest radius to avoid biasing the results. Student t test and chi square analysis were done using Statistica 4.0 (Statsoft Inc.).

Images for 3D reconstruction were collected using a Photometrix CH350 camera and a Zeiss Axiovert S100 2TV microscope. Data were collected moving the sample through the focal plane of the lens at 0.1 μm intervals. Data stacks were deconvolved using Deltavision's software package based on the empirical point-spread function developed by Agard et al. 1989.

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