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Interaction *in vivo* between the Two Matrix Attachment Regions Flanking a Single Chromatin Loop

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In interphase nuclei as in metaphase chromosomes, the genome is organized into topologically closed loop domains. Here, we have mapped the ends of the loop domain that contains the *Ifng* (interferon- γ) gene in primary and cultured murine T-lymphocytes. To determine whether the ends of the loop are located in close proximity to each other in the nuclear space, the 3C (chromosome conformation capture) technique, which detects protein-mediated DNA–DNA interactions, was utilized. A strong interaction was demonstrated between the two ends of the loop, which were close enough to become cross-linked *in vivo* in the presence of paraformaldehyde. Chromatin immunoprecipitation combined with the 3C technique demonstrated that topoisomerase II α and MeCP2, but not topoisomerase II β , heterochromatin-associated protein HP1 or CTCF, were involved in this interaction. The present findings have important implications in terms of mechanisms of illegitimate recombination that can result in chromosomal translocations and deletions.

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Introduction

DNA is compacted in different ways within the eukaryotic nucleus. At the lower level, DNA is packed into core histone-containing nucleosomes. With the adjunction of additional proteins, the resulting chromatin is further compacted into 30-nm fibers whose organization at an even higher level defines loop domains. After extraction of histones from metaphase chromosomes or interphase nuclei, these loops are visualized as anchored to a proteinaceous nucleoskeleton also referred to

as the nuclear matrix or nuclear scaffold.^{1,2} Chromatin loop domains vary in size, between 20 and 200 kb,^{3,4} with functionally related genes often organized into gene clusters within individual loops. Chromatin loop domains are dynamic: the organization of loops may change during development and differentiation.^{5–8}

Each chromatin loop is attached to the nuclear matrix via two scaffold/matrix attachment regions (S/MARs). S/MARs are DNA segments of 500 to 3000 bp (reviewed in Ref. 9) that may include DNA topoisomerase II (Topo II) binding sites¹⁰ as well as other sequence-specific motifs. In apoptotic processes, a long-range fragmentation of the genome occurs at S/MARs with chromatin loops excised through the enzymatic activities of Topo II or other endogenous nucleases.^{11–14}

It has been proposed that the excision of chromatin loops could provoke chromosomal translocations and deletions.^{15–19} Indeed, chromatin loop anchorage regions often coincide with nuclear recombination hot spots.^{18,20} Recombination, which may lead

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Abbreviations used: S/MAR, scaffold/matrix attachment regions; Topo II, topoisomerase II; BCR, breakpoint cluster region; 3C, chromosome conformation capture; Th, T helper; ChIP, chromatin immunoprecipitation; DNase, deoxyribonuclease.

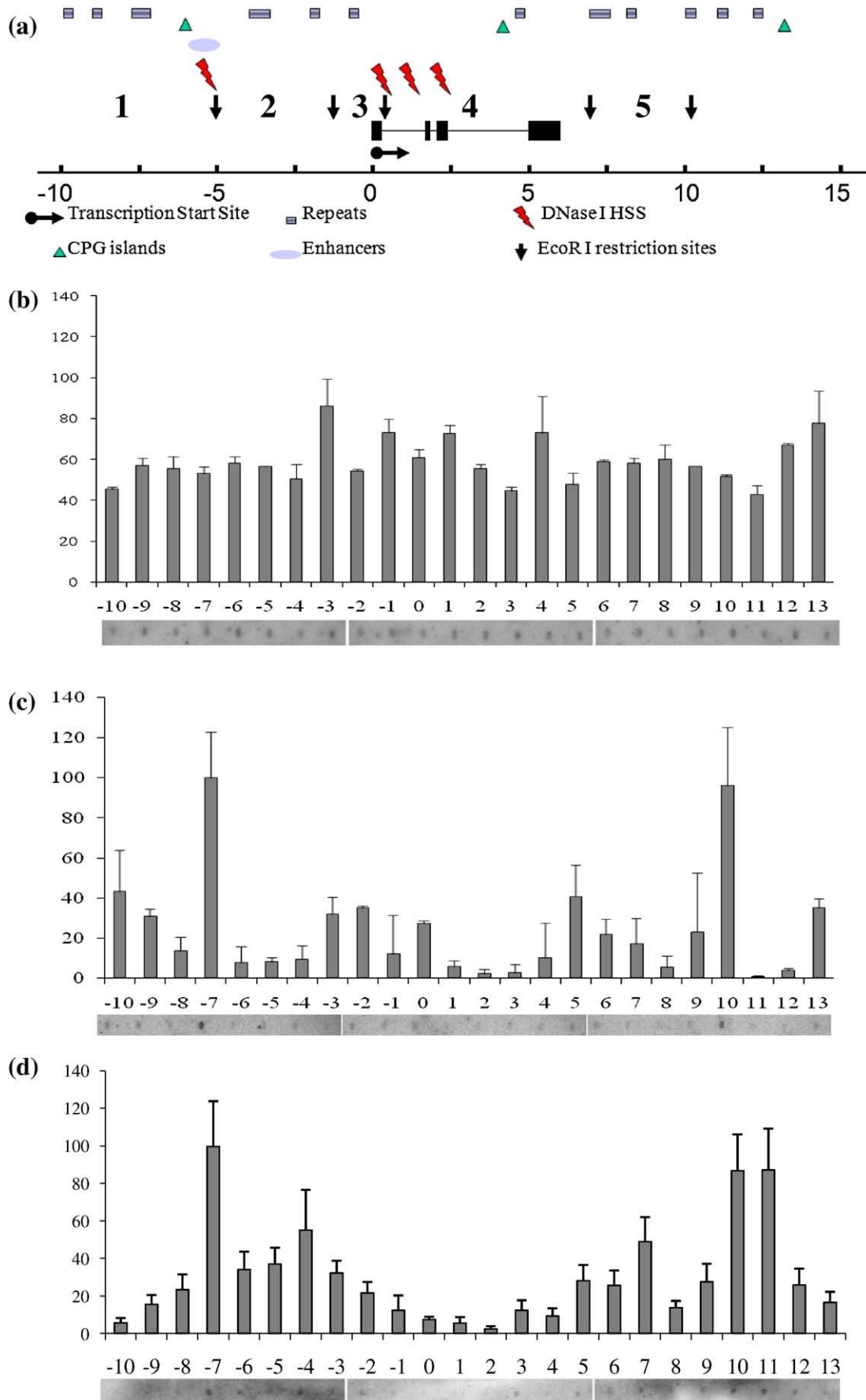


Fig. 1 (legend on next page)

to chromosome translocations and deletions, occur in some regions of the genome much more frequently than in others. Such regions are named breakpoint cluster regions (BCRs). Of several BCRs characterized in detail (see Refs. 16,21 and 22 for reviews), one is located within the deletion-prone type 1 interferon (*Ifn*) gene-containing locus on human chromosome 9p. In humans, various leukemias and glioma tumors are associated with chromosomal rearrangements whose breakpoints are located within or adjacent to the 450-kb-long *Ifn* gene cluster.^{23,24} Interestingly, these BCRs co-map with S/MARs within the region.²⁰

The presence of a Topo II activity in the nuclear matrix can provide a molecular basis for triggering illegitimate recombination resulting from deletion/recombination events between two DNA regions.^{16,25–29} For this kind of rearrangement to take place, physical proximity of the two DNA regions involved seems required. As previously proposed, the recombination event could take place at the bases of loop domains.¹⁵ So far, data supporting this hypothesis have been provided by electron microscopy and fluorescence in situ hybridization experiments performed on histone-depleted chromatin,^{1,18} techniques whose resolution is insufficient to be conclusive at the molecular level. A better resolution can now be achieved, however, using the chromosome conformation capture (3C) technique.³⁰

We have previously mapped the S/MARs in the *Ifn* gene domain at different stages of T helper (Th) cell differentiation, from stem cells to differentiated Th1 and Th2 lymphocytes, and demonstrated dynamic changes of the *Ifn* gene domain spatial arrangement, from random loop organization in stem cells to specified S/MARs in differentiated cells.³¹ Here, we have addressed the question of whether the two S/MARs located at the ends of a given chromatin loop physically interact with each other. We have investigated the chromatin loop that contains the *Ifn* gene in murine T-lymphocytes. We have first mapped the ends of the loop domain using a specifically designed genomic DNA array and we have used the 3C technique to demonstrate their physical proximity in the nuclear space. Chromatin immunoprecipitation (ChIP)-loop experiments^{32,33} provided indication as to possible molecular mechanisms leading to intrachromosomal rearrangements.

Results

The *Ifn* gene domain is organized in a single loop in murine T cells

The murine *Ifn* gene-containing domain has been studied extensively over the past 20 years (reviewed in Ref. 34). Its most prominent features are summarized in Fig. 1a. Sites of DNA loop anchorage on the nuclear matrix have been determined previously through various approaches.^{35,37–39} Here, we have prepared matrix-associated DNA by extensive deoxyribonuclease (DNase) I treatment of isolated nuclei leading to the digestion of non-protein-associated DNA, with nuclei further extracted in high-salt-concentration buffer to remove histones and other soluble proteins along with their associated DNA. The remaining nucleoskeleton contains the regions of DNA loop anchorage to the nuclear matrix. This nuclear matrix DNA was purified, radiolabeled and used as a probe to examine the chromatin loop organization of the murine *Ifn* gene domain. We have recently developed a mapping approach based on hybridization of nuclear matrix DNA with arrays of DNA oligonucleotides,^{40,41} which allows for rapid and accurate examination of loop anchorage regions over large sequenced segments. The DNA sequences located at/or close to the loop anchorage sites are overrepresented in nuclear matrix DNA as compared to total DNA. That is why comparison of relative intensities of signal observed upon the hybridization of the total DNA and the nuclear matrix-associated DNA with the oligonucleotide arrays can be used to identify the position of the nuclear matrix attachment sites.

In contrast to total DNA (Fig. 1b), the nuclear matrix DNA purified from neutral Th cells produced a specific hybridization pattern (Fig. 1c). The strongest signals were restricted to two sites located at positions -7000 and $+10,500$ relative to the *Ifn* transcription start site (Fig. 2c), marking the borders of the chromatin loop domain. As seen in Fig. 1d, in the murine T-cell lymphoma cell line EL4, strong signals were also detected between these sites, suggesting that the borders of the loop domain are the same in these cells. At other positions the weaker signals were somewhat different. For example, signals at -2 , -3 and -4 are low for T cells and

Fig. 1. Mapping of nuclear matrix attachment sites in the murine *Ifn* gene domain using a DNA array technique. (a) Schematic representation of the 24-kb *Ifn* gene-containing domain on mouse chromosome 10. Positions of five EcoRI restriction sites from the transcription start site of the *Ifn* gene are indicated by arrows: site 0, $-12,245$; site 1, -5048 ; site 2, -1248 ; site 3, $+229$; site 4, $+6891$; site 5, $+10,264$. The numbers of corresponding EcoRI restriction fragments are shown between the arrows. The positions of the three CpG islands: site 1, -5755 to 5378 ; site 2, $+4418$ to 4687 ; site 3, $+13,644$ to $13,848$ and of the DNA repeats were determined using the WebGene software (<http://www.itb.cnr.it/webgene/>). (b–d) Total DNA and DNA remaining attached to the nuclear matrix of nuclei extracted from neutral Th cells and from the EL4 cell line were radiolabeled and hybridized with a DNA array covering the *Ifn* gene-containing domain. For each experiment, results shown were from one out of six arrays tested. Quantitation data represent hybridization of total DNA (b) or nuclear matrix DNA from neutral Th (c) and EL4 (d) cells normalized against a positive control (a ubiquitous MAR from the murine κ immunoglobulin gene locus.^{35,36} The results presented are averaged from three independent experiments, each carried out in duplicate.

high for EL4 cells. Interestingly, the -4 signal is located in the vicinity of the *Ifng* enhancer. Signals were also slightly different at 0, 6, 7 and 11. These differences may be due to the changes in transient interactions with the nuclear matrix occurring upon immortalization of EL4 cells. These interactions may also reflect heterogeneity of the Th cell population. Nevertheless, the two matrix attachment regions (MARs) located at positions -7000 and $+10,500$, which represented the borders of the chromatin loop domain, were the same in both cell lines. Therefore, we used EL4 cell line as a convenient source of material in further experiments. From these observations, it appears that in these murine T cells, the *Ifng* gene domain is organized into a single loop with a size of approximately 18 kb.

A 3C analysis reveals the existence of tight interactions between the ends of the loop containing the *Ifng* gene domain

To determine whether the ends of the DNA loop that includes the *Ifng* gene domain are located in close proximity to each other, we then used the 3C technique to study protein-mediated DNA-DNA interactions. We thus explored the 25-kb, including the *Ifng* gene, domain. Genomic DNA from neutral CD4⁺ Th cells was subjected *in vivo* to paraformaldehyde to cross-link regions of genomic DNA in close proximity to each other. Non-cross-linked DNA was used as a control. Cross-linked and control DNA templates were then subjected to EcoRI digestion. The positions of the five EcoRI sites analyzed within the *Ifng* gene domain are reported in Fig. 1. Cross-linked templates were re-ligated at low DNA

concentration to favor intramolecular ligation, while control templates were re-ligated at very high DNA concentration to favor intermolecular interactions. The efficiency of EcoRI digestion was monitored by PCR with primers designed for each restriction site and used in pairwise homologous (5+5) and non-homologous (5+1, 5+2, 5+3, 5+4) combinations as described in Materials and Methods.

The frequency of interaction between restriction fragments was analyzed. The ratio between the amounts of PCR products obtained from cross-linked or control non-cross-linked templates was calculated to estimate their relative proximity in the nuclear space. All results were reported to a reference (100 arbitrary units) obtained using a pair of primers positioned in opposite directions at either end of restriction fragment 5 and designed to readily PCR-amplify following self-circularization of the fragment. As seen in Fig. 2, a very strong interaction was demonstrated between fragments 5 and 1 with PCR amplification occurring as readily as in the 5+5 control. This was particularly interesting, since the two fragments correspond to the two ends of the *Ifng* gene-containing loop (Fig. 1). It is of note that lower levels of interactions were also detected between fragment 5 and the central part (fragment 3) of the loop. This could reveal weaker transient interactions with the nuclear matrix. From these data, we conclude that the two ends of the DNA loop are located in very close proximity to each other *in vivo*, at least in neutral T-helper lymphocytes that do not express the *Ifng* gene. Next we have verified that the strong interaction between the loop ends was conserved in the EL4 cells, and indeed, the 5+1 interaction was almost as strong as in the 5+5 control (Fig. 2).

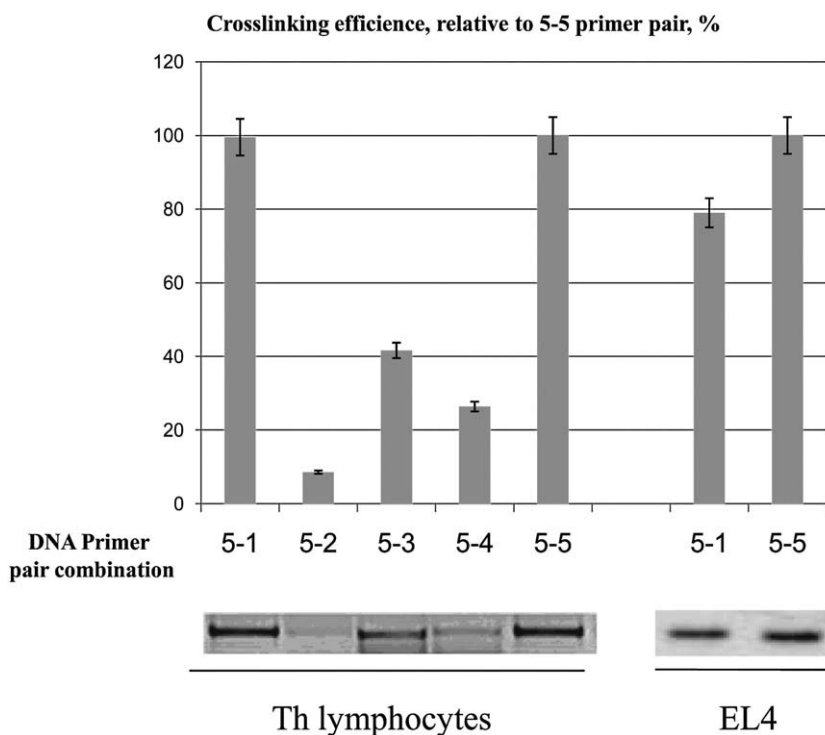


Fig. 2. Specific interactions as detected using the 3C technique on genomic DNA cross-linked *in vivo*. Cross-linked templates were used for PCR amplification at low DNA concentration using the indicated primer pairs. Note the strong interaction between fragments 1 and 5, each containing one end of the loop.

Topo II α and MeCP2 may mediate the interaction between loop ends

We then looked for proteins that would mediate the interaction between matrix attachment regions (MARs) flanking the *Irfng* gene-containing DNA loop. For this purpose, we used a combination of ChIP and 3C methods referred to as the ChIP-loop assay.^{32,33} Cross-linked chromatin was purified from EL4 cells and digested with EcoRI. An aliquot was subjected to the usual 3C assay, while the rest was immunoprecipitated using antibodies to different nuclear proteins potentially involved in the interaction. These included Topo II α and β , the latter being preferentially localized in the nucleolus, the hetero-

chromatin-associated protein HP1, CTCF, a protein that binds to insulators, and MeCP2, a protein that binds to methylated DNA. CENP-A, a histone variant specifically present in pericentromeric chromatin, was used as a negative control. In each immunoprecipitation, the precipitated material was ligated, and the ligation frequency between different restriction fragments was estimated by real-time PCR.⁴² Virtually no intramolecular ligation was observed between fragments 1 and 5 when antibodies to CENP-A, CTCF or Topo II β were used as immunoprecipitating agents. This was in sharp contrast with the cross-ligation demonstrated between fragments 1 and 5 when antibodies were directed to either Topo II α or MeCP2 in the immunoprecipita-

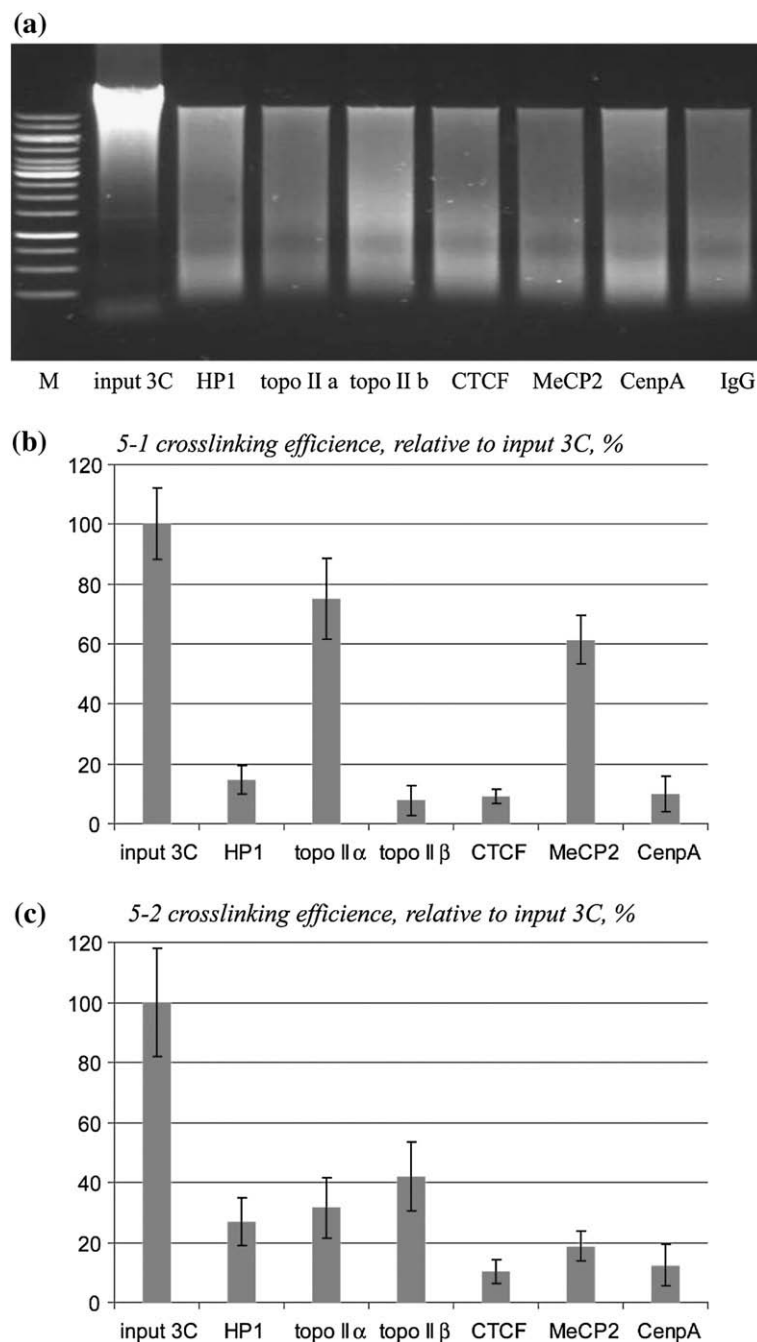


Fig. 3. Presence of Topo II α and MeCP2 at loop ends demonstrated by the ChIP-loop assay. Chromatin cross-linked in EL4 cells was immunoprecipitated using antibodies against various chromatin-associated proteins. (a) DNA gel electrophoresis of "input 3C" and ChIP-loop templates immunoprecipitated using the indicated antibodies. M, mass ruler molecular weight marker (Fermentas, Lithuania). The immunoprecipitated templates were ligated and subjected to quantitative PCR using the 5+1 (b, loop ends) or 5+2 (c, control) primer pairs. The strength of the signals was calculated relative to the "input" (non-immunoprecipitated) cross-linked template subjected to the usual 3C assay. Results presented correspond to the average of four independent PCR reactions.

tion step (Fig. 3b). Interestingly, very little of the above proteins was found in the control intramolecular complexes formed between fragments 5 and 2 (Fig. 3c), suggesting that the presence of Topo II α and MeCP2 is indeed specific for the interaction between the two ends of the loop.

Discussion

In many cases, non-homologous recombination events take place between DNA segments mapping close to MARs. One facilitating factor for such illegitimate recombination events would reside in a very close physical proximity of the involved DNA sequences in the nuclear space. In the present study, we have addressed the question of whether the MARs located at the two ends of a given chromatin domain would physically interact with each other. As a model, we have examined the chromatin loop containing the *Ifng* gene domain. We have first mapped the two MARs that flank the domain in neutral Th lymphocytes and in cells from the T-cell lymphoma cell line EL4. These were chosen as convenient models as both types of T cells were demonstrated to have their *Ifng* gene contained within a small DNA loop 18 kb in size (Fig. 1).

Next, we have used the 3C technique to demonstrate that indeed, the MARs flanking the *Ifng* gene-containing loop are located close enough to be cross-linked by paraformaldehyde. Results obtained from a combination of the 3C technique and various immunoprecipitating antibodies in ChIP-loop assays allow us to conclude that at least two proteins, namely, Topo II α and MeCP2, are associated with the two MARs. In contrast, we found no evidence for the presence of Topo II β , CTCF or HP1 at the basis of the loop. MeCP2 is a protein whose binding to methylated DNA has inhibitory function on gene transcription during normal brain development. This function is lost due to mutations in MeCP2 causing the Rett syndrome.^{32,43} MeCP2 is also a component of the nuclear matrix.⁴⁴ From our findings, we propose that MeCP2 could be involved in the association between the ends of the *Ifng* gene-containing DNA loop. Interestingly, the existence of a CpG island within the same EcoRI restriction fragment as the S/MAR located at -7000 (Fig. 1a) could account for the recruitment of MeCP2. It thus appears that MeCP2 could play a role in holding together the two ends of the loop. Thus, we provide here the first hard biochemical evidence that the nuclear matrix attach-

ment sites (MARs) are located in close contact within the nuclear space.

Our results also confirm and further extend previous observations indicating that Topo II plays an important role in DNA loop anchorage to the nuclear matrix.⁴⁵ They additionally support a hypothesis postulating that the ends of DNA loops constitute preferential sites for illegitimate recombination that may be mediated or triggered by the cleavage activity of this enzyme.^{15,16} It is also important to emphasise that S/MARs are likely to favor recombination. Indeed, transfected S/MAR-containing constructs integrate at much higher copy numbers than their S/MAR-free counterparts.⁴⁶ Cellular S/MARs have been found to be prominent integration sites for proviruses and also for certain transgene constructs.^{47,48} On the other hand, Topo II is one of the major components of the nuclear matrix and it has long been suggested that it can promote recombination between DNA sequences interacting with the nuclear matrix.^{15,16} One of the activities associated with this enzyme is intramolecular ligation, i.e., Topo II is capable of joining two non-homologous ends of DNA.⁴⁹ Even more important may be the fact that stalled Topo II complexes are recognized as double-stranded DNA breaks by cellular repair systems and trigger non-homologous end joining repair. This non-homologous repair may be therefore the main cause of chromosomal rearrangements occurring in response to inhibitors that affect Topo II activities.^{25,29} Whatever is the particular mechanism of illegitimate recombination stimulated by inhibition of Topo II, it is well established that Topo II cleavage sites are often located close to recombination-prone regions.²¹ A close proximity of DNA loop ends and the presence of Topo II at the bases of the loop may therefore result in deletions of genomic DNA via loop excision (Fig. 4), a hypothesis that will require further studies.

Materials and Methods

Murine cell cultures

Cell cultures were performed by standard methods essentially as described.⁵⁰ Briefly, single-cell suspensions were prepared from the spleen of 4- to 6-week-old mice. Red blood cells were removed by hypotonic lysis. CD4⁺ T cells were purified by negative selection using magnetic beads (Genome Therapeutics, Waltham, MA). The average purity of CD4⁺ cells was 90% as determined by flow cytometry.

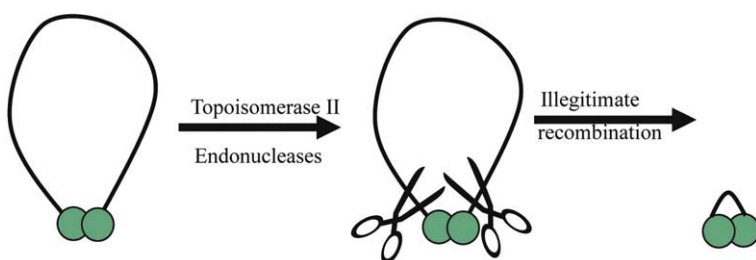


Fig. 4. Schematic model of illegitimate recombination based on DNA breaks generated by Topo II/endonucleases in the immediate proximity of the S/MARs at the two ends of a chromatin loop.

Tissue culture plates were coated with anti-CD3 monoclonal antibody (mAb) (10 µg/ml, 145-2C11 clone, American Type Culture Collection) overnight at 4°C and thoroughly washed with Hanks' balanced salt solution. Purified CD4⁺ T cells (1 × 10⁶/ml) were stimulated with plate-bound anti-CD3 mAb and syngeneic irradiated spleen cells at a density of 1 × 10⁶ cells per milliliter in RPMI 1640 medium containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 U/ml), 2 mM L-glutamine, and 5 × 10⁻⁵ M 2-mercaptoethanol. The stimulation with anti-CD3 leads to an intermediary step in differentiation of Th cells (potentiation for transcription), but does not result in activation of *Ifng* gene, which requires additional interleukin-12 stimulation.⁵⁰

Cells were cultured for 5 days under neutral conditions (no cytokine additives) and harvested for analysis of chromatin conformation.

EL4 cultured murine T-cell lymphoma line (a kind gift of Prof. L. Zitvogel) was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 5 mM 2-mercaptoethanol, and antibiotics.

Purification of nuclei and nuclear matrices

Nuclei were purified from murine cells as described elsewhere.⁵¹ Nuclear matrices were prepared by treatment of the isolated nuclei with DNase I followed by extraction with 2 M NaCl essentially as described.⁵¹

The size distribution of the loop domain attachment sites (the matrix-bound DNA fragments) was in the range of 200–1000 bp. The fraction of the loop domain attachment sites constituted 2–5% of the total DNA.

Nuclear matrices were digested with proteinase K and extracted with phenol–chloroform. Isolated nuclear matrix DNA was treated with ribonuclease A and, after labelling, used as a probe in a Southern/dot blot with the DNA array covering the 25 kb of the murine *Ifng* gene-containing domain. Nuclear matrices obtained from three independent experiments were used for hybridizations. Each labelling/hybridization experiment was carried out in duplicate. Similar amounts of DNA were taken for the labelling reaction.

DNA array

DNA array consisted of 24 oligonucleotides (25- to 35-mer) spaced 1 kb apart (see [Supplementary Data](#)). The oligonucleotides had a similar melting temperature. Prior to hybridization, the oligonucleotides were analyzed *in silico* to avoid repetitive DNA sequences. The oligonucleotides were slot-blotted onto Zeta-probe GT filters in 0.4 M NaOH and fixed by baking at 80 °C for 30 min. Each filter contained the array in duplicate. The hybridization was carried out at 60 °C in the modified Church buffer [0.5 M phosphate buffer (pH 7.2), 7% SDS, 10 mM EDTA (ethylenediaminetetraacetic acid)] overnight. The blot was washed subsequently in 2 × SSC and 0.1% SDS two times for 5 min, then in 1 × SSC and 0.1% SDS two times for 10 min. The blots were exposed using Kodak PhosphorScreens and analyzed on a Fuji FLA-3000 PhosphorImager for 3–72 h. All experiments were done in triplicate.

Analysis of chromatin conformations by the 3C assay

The 3C technique was applied to determine the frequencies of interaction between different regions in the *Ifng* gene domain *in vivo*. The 3C assay was performed

essentially as previously described.^{30,52} Harvested cells were resuspended in cell lysis buffer with protease inhibitors, homogenized with a B-dounce homogenizer on ice and centrifuged to pellet the nuclei. The suspension was diluted to a final concentration of 1 × 10⁸ nuclei per milliliter. To cross-link proteins and DNA, paraformaldehyde (Sigma) was added directly to isolated intact nuclei to achieve a final concentration of 1% in the 3C buffer. Glycine (0.125 M final concentration) was added after 10 min to stop the reaction. To remove non-cross-linked proteins from DNA, SDS (Sigma) was added to a final concentration of 0.1% for 10 min. Before EcoRI digestion, Triton X-100 (Sigma) was added to a final concentration of 1% to sequester SDS.

EcoRI (New England Biolabs) restriction enzyme digestion was optimized and monitored by PCR using primer pairs designed specifically for each of five EcoRI restriction sites. After digestion of chromatin, 1.6% SDS was added for 20 min at 65 °C to inactivate EcoRI. Optimal conditions for intramolecular ligation of cross-linked fragments (T4 ligase, New England Biolabs) were established by using DNA at different concentrations and monitoring products of ligation by PCR. To find the linear range for cross-linked template, samples were serially diluted after ligation. Cross-linking was reversed by overnight incubation of samples with proteinase K at 65 °C, followed by phenol–chloroform purification of DNA. Control templates were produced by omitting the paraformaldehyde cross-linking step. Purified DNA was digested with EcoRI and randomly ligated without dilution. Cross-linked and control DNA preparations were subjected to PCR amplification with site-specific primer pairs and quantitative analysis.

Primer pairs for PCR analysis were designed to span each of five chosen EcoRI restriction sites. Forward and reverse primers were used with cross-linked and control templates in pairwise homologous (5+5) and non-homologous (1+5, 2+5, 3+5, 4+5) combinations. Primer sequences are available on demand. All PCR products were resolved by electrophoresis on 2% agarose gels containing ethidium bromide and quantified using the Stratagene Image Analyzer (Stratagene, La Jolla, CA). All the necessary controls have been done in accordance with the 3C protocol (data not shown) (Ref. 52, no. 2134). Samples were run in duplicate, experiments were repeated three times and the results were averaged.

ChIP-loop assay

Formaldehyde-cross-linked chromatin was also subjected to a ChIP-loop assay.³² Briefly, DNA–protein complexes were digested with EcoRI, and an aliquot was subjected to the 3C protocol as described above. The rest was precleared for 6 h at 4 °C with rabbit immunoglobulins (Sigma) and protein G magnetic beads (ChIP-IT Express kit, Active Motif, USA) and aliquoted. The aliquots were incubated overnight at 4 °C with protein G magnetic beads (ChIP-IT Express kit, Active Motif) and rabbit polyclonal antibody against HP1, Topo IIβ, CTCF, CENP-A (Santa Cruz Biotechnology, Santa Cruz, CA), DNA Topo IIα, MeCP2 (Abcam, UK), or normal rabbit immunoglobulins (Sigma, USA). The beads were then washed, resuspended in 200 µl of ligation buffer, and DNA ligated and purified as in usual 3C experiments.

Real time PCR was performed with 5+1 and 5+2 primer pairs using a TaqMan probe annealing between primer 5 and EcoRI site 5, as described elsewhere.⁴² A calibration curve for determination of relative amounts of DNA in

immunoprecipitated templates was generated by carrying out real-time PCR on a series of twofold dilutions of "input 3C" template that was not precipitated but subjected to the usual 3C assay as described above. A PCR signal from templates precipitated with normal rabbit immunoglobulins (approximately 25% of the total signal) was subtracted from PCR signals from templates precipitated with specific antibodies. Each PCR reaction was carried out in quadruplicate and the corresponding results were averaged.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.jmb.2008.12.022](https://doi.org/10.1016/j.jmb.2008.12.022)

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