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Chromatin loops, illegitimate recombination, and genome evolution

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Chromosomal rearrangements frequently occur at specific places (''hot spots'') in the genome. These recombination hot spots are usually separated by 50–100 kb regions of DNA that are rarely involved in rearrangements. It is quite likely that there is a correlation between the above-mentioned distances and the average size of DNA loops fixed at the nuclear matrix. Recent studies have demonstrated that DNA loop anchorage regions can be fairly long and can harbor DNA recombination hot spots. We previously proposed that chromosomal DNA loops may constitute the basic units of genome organization in higher eukaryotes. In this review, we consider recombination between DNA loop anchorage regions as a possible source of genome evolution.

Keywords: DNA loops; MAR; NHEJ; nuclear matrix; topoisomerase II

Introduction: compartmentalization of the genome in eukaryotic cell nuclei

It is well known that DNA compaction in eukaryotic cell nuclei is based on a hierarchical formation of several chromatin structures such as: (i) nucleosomes, which are the basic repeat elements of chromatin, consisting of a DNA chain and core histones; (ii) 30-nm fibers; (iii) chromosomal DNA loops; and (iv) chromosome territories. The latter were discovered in the early 1990s, when techniques that permitted the visualization of individual chromosomes in interphase cell nuclei were developed.[1,2] The domain that separates chromosome territories is called the interchromosome domain.[2] It has been proposed that this chromatin-free domain is maintained by electrostatic repulsion of negatively charged surfaces of neighboring chromosomes. For many years, proponents of the above model favored it over the nuclear matrix model, which was first proposed in the middle 1970s and postulates that the eukaryotic genome is organized into DNA loops bound to a high salt-insoluble proteinaceous structure called the nuclear matrix, also known as the nuclear scaffold, nuclear cage, nuclear skeleton, or nuclear ghost[3] (for details, see Box 1). In 2001, an updated model of chromosome territories was suggested. In this model, chromosome territories are subdivided into ''1 Mbp chromatin domains'' built up as a rosette of small DNA loops approximately 100 kbp long that are in contact with the interchromosome domain.[4,5] This model is not perfect because it cannot provide an explanation of experimental data obtained in recent years. In particular, it is difficult to explain how individual genomic regions within the nuclei move directionally. This movement occurs when actively transcribed genes are relocated out of a chromosomal territory.[6–8] Another example of such movement is the active nuclear repositioning of DNA damaged by α-particles.[9] The ''motors'' that mediate this repositioning should either be fixed somewhere, in order to pull the target chromatin domain connected to the motor *via* some filament, or they should move together with the bound chromatin region along a skeletal filament. In either scenario, some kind of nuclear skeleton is necessary. Furthermore, the presence of specific recombination hot spots coinciding with topoisomerase II-sensitive DNA sites[10–12] can only be explained by the nuclear matrix model.

In conjunction with the concept of chromosome territories, we suggest that the ''100 kbp chromatin domain,'' the most fundamental chromosome organizational level with respect to gene regulation, is formed by the anchoring of loop basements at the nuclear matrix. The presence of the nuclear matrix as a basis for anchoring chromosomal DNA loops can easily explain the biochemical data accumulated over several decades.[13] The supposition that DNA loop-domains are anchored to the nuclear matrix can easily be combined with the model postulating that DNA loops are organized into rosettes (Fig. 1).

In the present review, we will focus on the level of DNA loops considered to be the major structural units of chromatin that determine the functional independence of distinct gene clusters. Thirty years ago, scientists studying the organization of chromosomes were fascinated by the electron microscopy

Abbreviations: BCR, breakpoint cluster region; DSB, double-strand DNA breaks; MAR, matrix associated region; NHEJ, non-homologous end joining.

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Nuclear matrix.

The nuclear matrix is an operationally defined skeletal structure underlying the nucleus.[90–92] Although the nature of the nuclear matrix is still unclear and the existence of this structure has been questioned over the years (for review, see Refs[93–96]), it is likely that nuclear compartmentalization depends on a specific structure.[97–99] For this reason, the idea of the nuclear matrix has survived over the last 30 years. According to the classical definition,[90] the nuclear matrix is composed of nuclear lamina, the residual nucleolus, and the so-called internal nuclear matrix, which is a filamentous network that connects the nuclear lamina and the residual nucleolus. The structure of the nuclear lamina has been well characterized, but data concerning the structural biochemistry of the internal nuclear matrix remain controversial. Many internal matrix proteins have been identified,[100–102] but far less is known about how these proteins assemble to make the fibers, filaments, and other assemblies of the internal nuclear matrix.[103,104] The critics of the nuclear matrix model usually highlight the fact that experimental procedures used for chromatin removal from isolated nuclei, such as high salt extraction, may cause aggregation of proteins.[5,93,95] In this context, it is revealing that identical elements have been identified in nuclear matrices prepared using different experimental procedures.[105] Furthermore, protein fibrils that have properties similar to those of internal nuclear matrix fibrils have been observed in unfractionated cell nuclei,[106] as well as in the nuclei of living cells.[107]

(EM) pictures of histone-depleted metaphase chromosomes published by Laemmli's group.[14] These pictures showed that, at least in metaphase chromosomes, DNA was organized into large loops 50–100 kb long attached to a scaffolding structure composed of non-histone proteins.[15] The presence of similar loops in interphase nuclei was predicted from the dependence of sedimentation properties of the so-called nucleoids (histone-depleted nuclei) on the concentration of intercalating agents in the medium. In this way, nuclear DNA was shown to be organized in a number of independent topological domains.[3]

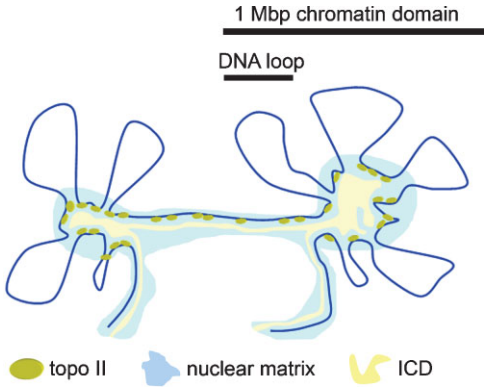

Figure 1. Spatial organization of chromatin loops. The ~1 Mbp chromatin domain is built up as a rosettes of 20–100 kbp chromatin fibers, anchored to the nuclear matrix, which may be in contact with interchromatin domain, ICD (see the text for further discussion).

Genomic DNA is partitioned into loops in a specific fashion

Since the first observation of large DNA loops in metaphase chromosomes and interphase nuclei, many attempts have been made to determine whether the organization of chromosomal DNA into loops is specific or random. Several approaches have been developed to map the bases of DNA loops (for review, see Refs[13,16,17]). The most popular experimental approach presently used to study the specificity of the interaction between DNA and the nuclear matrix was developed by Garrard's group.[18] Using this technique, these researchers showed that isolated nuclear matrices—nuclei depleted of both DNA and histones—contain specific affinity sites that can detain a subset of sequences present in total DNA. The DNA sequence elements detained selectively by isolated nuclear matrices in the presence of a non-specific competitor were called matrix-associated regions or MARs. Binding of these DNA sequences to nuclear matrices was not tissue- or even species-specific.[19] In prior work independent of that of Garrard's group, Laemmli and coworkers[20] used a different protocol that allowed them to isolate the so-called scaffold attachment regions (SARs). Although it was assumed for some time that SARs interact with the nuclear matrix *in vivo*, later studies showed that they most likely form complexes with the nuclear matrix during nuclease treatment of histone-depleted nuclei.[17,21] It should be highlighted that complexes of MARs/SARs with the nuclear matrix are unstable to high salt extraction, and there is no evidence that MARs/SARs normally interact with the nuclear matrix *in vivo*.[22]

We have developed a new procedure for mapping DNA loop anchorage sites. Instead of isolating the so-called "nuclear matrix DNA," we have devised a way to cleave the genome at the loop anchorage sites and then to characterize individual loops.[23–26] In this method, DNA topoisomerase II is used to cleave DNA in the loop anchorage regions; this enzyme is one of the major components of the nuclear matrix.[27] The topoisomerase II-mediated DNA loop excision protocol has been used successfully to map the organization into loops of large genomic areas, including the human *dystrophin* gene.[28] Importantly, DNA loops and loop anchorage regions identified in this study have been directly visualized *via* hybridization of the corresponding BAC probes with the so-called nuclear halos.[28,29] Thus, using the

topoisomerase II-mediated DNA loop excision technique, we have shown for the first time that DNA loops mapped biochemically correspond to the DNA loops seen under a fluorescence microscope in histone-depleted nuclei. Clearly, this result strongly supports the validity of the radial loop model of the eukaryotic chromosome organization. Indeed, the protocol of DNA loop mapping by topoisomerase II-mediated DNA loop excision was based entirely on this model, as well as on the presence of DNA topoisomerase II in the nuclear matrix.

DNA loop anchorage sites harbor recombination hot spots

Several years ago, we proposed a hypothetical model postulating that recombination between DNA loop anchorage sites may cause long-range DNA rearrangements and thereby provide starting material for the genome evolution.[30] This model was based on several indirect findings, such as (i) a correlation between DNA loop sizes and average distances between recombination hot spots,[31,32] (ii) frequent occurrence of MAR elements and topoisomerase II cleavage sites at recombination junctions,[33,34] and (iii) intrinsic intermolecular DNA ligation activity of eukaryotic DNA topoisomerase II.[35] In order to test this model, it was first necessary to check whether recombination hot spots, also known as chromosomal breakpoint cluster regions (BCRs), colocalize with DNA loop anchorage sites. The domain-loop map of the human *dystrophin* gene strongly suggests that this may be the case. Indeed, the major recombination hot spots present in this gene[36] reside within the DNA loop anchorage area.[28] Because the most frequent rearrangements of the *dystrophin* gene are deletions of different sizes, we did not wish to include this case in our study. Rather, we focused on examining whether the BCRs participating in chromosomal translocations are located at the nuclear matrix. Many genes involved in translocations associated with secondary (treatment-related) leukemias have been identified. The recombination junctions within these genes have been mapped and characterized. They are not randomly distributed within genes participating in reciprocal translocations, but rather they are clustered within one or several relatively short regions (BCRs) (for review, see Ref.[37]). In our studies, we investigated the association between the nuclear matrix and two BCRs present in the *AML-1* and *ETO* genes, which are frequently involved in reciprocal t(8;21) translocations. Both BCRs studied were found to reside at the nuclear matrix, in contrast to other parts of the genes.[38] Similar observations were made when several other recombination hot spots were studied.[25,39,40] Thus, it seems that recombination hot spots are generally located at the nuclear matrix.

Inhibition of DNA topoisomerase II in living cells stimulates illegitimate recombination

Topoisomerase II can carry out DNA recombination *in vitro*,[35,41] and drug-induced inhibition of topoisomerase II activity can either mediate or stimulate illegitimate recombination in cultured cells.[42,43] In order to study the quantitative aspects of the recombinogenic activity of DNA topoisomerase II, we developed a special plasmid model (Fig. 2). The test construct is based on a plasmid containing an inducible marker gene *ccdB* (“killer” gene), expressed under the control of the *lac* gene promoter. The product encoded by *ccdB* is lethal for bacterial cells. The plasmid also contains the SV-40 replication origin, allowing it to replicate in T-antigen-producing Cos-1 cells. We expected that illegitimate recombination between DNA sequences flanking the *ccdB* gene would result in the loss of this gene

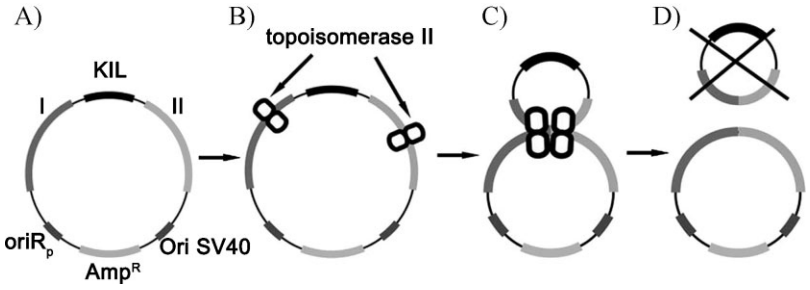

Figure 2. Principles of the test system for studying illegitimate recombination. **A**: The test plasmid construct, containing an inducible “killer” gene (*KIL*) flanked by two different target sequences (I and II). The plasmid also contains a bacterial selection marker (Amp^R) and two replication origins, prokaryotic (OriR$_P$) and eukaryotic (SV-40 ori). **B**: Topoisomerase II binds to the regions flanking the *KIL* gene and forms cleavable complexes in the DNA. **C**: Two molecules of topoisomerase II interact with each other according to the subunit exchange model. **D**: Exchange of topoisomerase II subunits results in illegitimate recombination, which gives rise to two circular molecules. One of these does not contain the plasmid origin of replication and therefore cannot survive in bacterial cells, whereas the other lacks the killer marker and is thus resistant to IPTG. Note that our assay detects only those recombination events that eliminate the killer gene.

and therefore the appearance of deletion forms of the construct able to grow on a medium containing the *lac* gene inducer IPTG. The construct was transfected into Cos-1 cells, which were then treated with different inhibitors of DNA topoisomerase II. After different time periods, the plasmid DNA was isolated and used to transform competent *E. coli* cells that were then grown on a medium with or without IPTG. This experimental design permitted us to calculate the percentage of recombination events resulting in the loss of the *ccdB* gene. The experiments described above clearly demonstrated that inhibition of DNA topoisomerase II activity during DNA replication strongly stimulated the process of illegitimate DNA recombination.[44] At the same time, the ability of DNA topoisomerase II to mediate DNA transfer directly (*i.e.*, *via* subunit exchange[45]) was estimated to be very low.

In cells treated with topoisomerase II poisons, double-strand DNA breaks (DSB) are introduced predominantly at sites where the DNA loops are anchored to the nuclear matrix

Anticancer drugs have long been thought to target primarily topoisomerase II in the nuclear matrix.[25,46–48] However, only indirect evidence supports this view. To further study the role of the nuclear matrix in the repair of topoisomerase II-induced DSB, we investigated the nuclear distribution of γH2AX foci; these foci indicate the presence of DSB.[49] The so-called *in situ* nuclear matrices[50] were prepared under mild conditions from cells treated with VP16. To avoid solubilization of γH2AX itself, the cleaved-off pieces of DNA loops were extracted from DNase I-treated permeabilized cells with a 0.5 M NaCl solution. This extraction permits the solubilization of chromatin because of H1 removal, but it does not remove core histones from DNA. When *in situ* matrices prepared from VP16-treated cells were stained for γH2AX and examined under a fluorescence microscope, it became clear that most, if not all, nucleosomes containing γH2AX remained at the nuclear matrix, whereas more than 80% of nuclear DNA was excluded (Fig. 3).[51] This observation may be due to the assembly of repair complexes at the nuclear matrices.[52] Another attractive possibility is that when the activity of topoisomerase II is suppressed, DSB are introduced preferentially at sites of DNA attachment to the nuclear matrix by insoluble DNA topoisomerase II, as was proposed previously.[25,48] To distinguish between the two possibilities, we repeated the above experiment, but we used γ-rays to induce DSB in the DNA of living cells. We found that following the removal of approximately 80% of the chromatin, only a minor portion of γH2AX foci detected in cells exposed to γ-irradiation remained associated with the *in situ* nuclear matrices (Fig. 3).[51] Thus, we concluded that upon suppression of DNA topoisomerase II activity, DSB are introduced in the regions of DNA attachment to the nuclear matrix. At the same time, the results of this experiment strongly argue against an active role of the nuclear matrix in the assembly of protein complexes involved in DSB repair. Indeed, if the matrix were involved in this assembly, the γH2AX foci would be concentrated at the matrix independently of the nature of the agent used to induce DSB. Importantly, in cells treated with topoisomerase II poisons, the nuclear matrix-bound γH2AX foci were found to be distributed throughout the entire nuclear volume. These results correlate well with previous observations that DNA topoisomerase II is part of the internal nuclear matrix that covers the internal nuclear space connecting the nuclear lamina with nucleoli.[53,54]

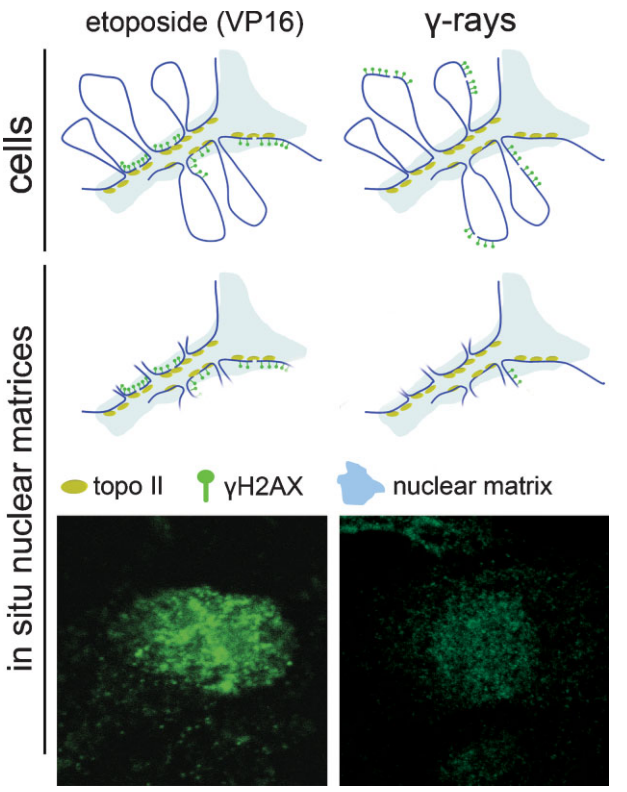

Figure 3. Topoisomerase II-specific drugs, in contrast to γ-rays, induce DSB at the sites of DNA loop anchorage to the nuclear matrix (see the text for further discussion).

DSB induced by DNA topoisomerase II are repaired by error-prone non-homologous end joining (NHEJ) that may cause translocations and other recombination events

In our experiments with the plasmid construct, we have shown that treatment of cells with topoisomerase II poisons

stimulates illegitimate recombination (see above, Fig. 2). In addition, it is well documented that chemotherapy using topoisomerase II-specific drugs can cause secondary leukemias associated with different chromosomal rearrangements.[10,55] Detailed analysis of such translocation junctions has suggested that the main cause of recombination events is the repair of DSB by NHEJ (Box 2).[10] Known NHEJ proteins include Ku, DNA–protein kinase (DNA–PK_{cs}), DNA ligase IV, and its cofactor XRCC4.[56–59] We analyzed the intracellular distribution of Ku, DNA–PK_{cs}, and DNA ligase IV to find out whether these proteins colocalize with γH2AX foci located at the nuclear matrix in cells treated with topoisomerase II poisons. Almost perfect colocalization was observed in the case of Ku70, and partial colocalization was observed in the case of DNA–PK_{cs} and DNA ligase IV.[51] These results strongly suggest that topoisomerase II-induced DNA scissions are repaired at the nuclear matrix by NHEJ. It should be emphasized that Ku antigen, DNA–PK_{cs}, and DNA ligase IV are present in cell nuclei regardless of the presence of DSB, in contrast to γH2AX. This finding may reflect the fact that all three proteins have functions in addition to those in NHEJ, which is why one would not expect to observe complete colocalization of all three proteins with the γH2AX foci concentrated at the nuclear matrix. Furthermore, the above-mentioned colocalization was clearly visible only after extraction of the majority of soluble proteins during preparation of *in situ* nuclear matrices.[51] A clear role for NHEJ in the repair of DNA damage induced by agents targeting topoisomerase II was independently demonstrated by other authors who used quite different experimental approaches.[60,61]

Non-homologous end joining.

NHEJ is regarded as the dominant mechanism for DSB repair in vertebrates, especially during the G0 and G1 phases of the cell cycle.[108] NHEJ is also known to be essential for repairing DSB arising during V(D)J recombination.[109] In the course of repair, the subunits of the Ku70/Ku80 heterodimer bind free DNA ends at DSB, and DNA–PK_{cs} is subsequently recruited to the DSB. Because chemical damage to the DNA prevents the rejoining of most breaks, additional proteins are required to remove the damage and to fill any remaining gaps. With helicase and exonuclease activities, the Rad50–Mre11–Nbs1 complex may function in NHEJ by processing DNA ends.[108] Once processed, the targeted free ends are ligated by DNA ligase IV in conjunction with XRCC4.[110]

If the repair of topoisomerase II-induced DSB by the error-prone NHEJ mechanism was indeed the main cause of chromosomal translocations, one would expect the components of the NHEJ repair system to be preferentially bound to the known BCRs in cells treated with topoisomerase II poisons. Studies of secondary leukemias led to the extensive characterization of a number of chromosomal BCRs frequently involved in reciprocal translocations.[10,37] As described previously, BCRs frequently contain MAR elements and *in vivo* topoisomerase II cleavage sites.[10,62] We selected a particular genomic site within the BCR of the human *AML1* gene, which was reported to contain a strong site for topoisomerase II-mediated DNA cleavage in cultured Jurkat cells, which are a T cell leukemia/lymphoma cell line.[11] We analyzed the association of this particular region with the known components of NHEJ (Ku80 and DNA–PK_{cs}) and with Rad52, which is involved in the homologous recombination pathway, in Jurkat cells either treated or not treated with VP16. Using chromatin immunoprecipitation (ChIP), we showed that after exposing cells to VP16, Ku80, and DNA–PK_{cs} localized to the region under study, whereas Rad52 did not.[63] Thus, in this particular case, the role of NHEJ in chromosomal translocations appears to be quite clear. The observations made for other systems suggest that this role is not an exception, but rather a common way to generate chromosomal translocations.[64,65]

Breakage first or meeting first? Is the nuclear matrix a dynamic structure?

As discussed in the previous sections, the incorrect joining of cleaved DNA chains is likely to be the main cause of translocations occurring in cells treated with topoisomerase II poisons.[51,64–66] It seems obvious that broken DNA chains of different chromosomes can be incorrectly joined only when they are close to each other. In this context, it is important to remember that in eukaryotic cell nuclei, different chromosomes occupy specific non-overlapping spaces known as chromosome territories.[4] Furthermore, each chromosome occupies a specific nuclear layer, with gene-rich chromosomes being located close to the nuclear center and gene-poor chromosomes close to the nuclear periphery.[4,67,68] In some cases, the translocation partners are located close to each other in the nuclear volume,[69] but this is not a general rule. The specific radial distribution of chromosome territories places certain restrictions on the mobility of most genes and consequently on the probability of translocations between different chromosomes.[70] Nevertheless, in some cases, well-known translocation partners are present in chromosomes that occupy distinct nuclear layers, for example, the translocation t(8;21)(q22;q22), one of the frequent chromosome translocations in acute myeloid leukemia.[11] Chromosome 21, bearing the *AML1* gene, is located relatively close to the nuclear center, whereas chromosome 8, bearing the *ETO* gene, occupies a more peripheral nuclear position.[67] To

explain the possibility of translocation despite the distance between the chromosomal regions involved, it was proposed that broken DNA ends can migrate over large distances within nuclei. Such migration has indeed been observed in nuclei irradiated with α-particles,[9] as well as in nuclei treated with inhibitors of DNA topoisomerase II.[71] Furthermore, indirect evidence suggests that in both cases actin–myosin motors actively power the movement of broken chromosome ends.[9,71]

In conjunction with these results, it is interesting to discuss what happens to the nuclear matrix during migration of broken DNA ends. As described in the previous section, in cells exposed to topoisomerase II poisons, DSB are induced preferentially at sites of DNA attachment to the nuclear matrix. Furthermore, the nuclear matrix plays an essential role in maintaining nuclear positions of chromosomal territories.[72] To this end, it is important to emphasize that the internal nuclear matrix covers the whole nuclear space, and that is why genes located on different chromosomes can be cleaved by topoisomerase II of the nuclear matrix. Movement of the broken DNA ends, which are fixed at the nuclear matrix, is possible only when the nuclear matrix itself undergoes spatial reorganization. Together, these results highlight the dynamic nature of the nuclear matrix. Therefore, it is important to remember that the dynamic properties of the nuclear matrix were postulated twenty years ago in the tensegrity model of Pienta and Coffey.[73]

Conclusions: recombination between the loop ends is a means of rapid genome evolution

In previous work, we proposed that chromosomal DNA loops may constitute the basic units of genome organization and evolution in higher eukaryotes.[30] The data accumulated over the last 8 years strongly support our hypothesis. Indeed, several experimental model systems have shown that DNA loop anchorage regions harbor recombination hot spots. Additional experimental evidence argues that these convergent findings are not merely a coincidence, but that they reflect the intrinsic properties of matrix attachment regions, where DNA is in permanent contact with the insoluble DNA topoisomerase II of the nuclear matrix. Although most of the available data examine translocations that result from the inhibition of DNA topoisomerase II activity by exogenous agents (anticancer drugs), it is quite possible that the same effect may be produced by some environmental factors or even by certain metabolites. Regardless of the initiating event, the resulting formation of DNA topoisomerase II stalled complexes on DNA and the subsequent repair of DSB enhance the probability that DNA ends will be joined incorrectly, and thus the probability of translocations. It is interesting to analyze the possible evolutionary consequences of these translocations. At present, only translocations associated with secondary leukemias have been extensively studied. Many translocation partners have been identified and junction regions characterized.[10,37] BCRs are usually located within introns, but they may be fairly long and may include several exons and introns. The most interesting conclusion can follow if the positions of BCRs are projected to mRNA and further to the amino acid chain. This kind of analysis demonstrates that BCRs are usually located between important functional domains of the protein. Recombination between BCRs, which are located in DNA loop anchorage regions, will likely generate a variety of proteins with new combinations of functional domains. Thus, recombination between DNA loop anchorage sites may provide a mechanism for rapid genomic evolution (Fig. 4).

We realize that only changes in the genome of germ cells are significant for evolution. However, it is evident that correct meiotic recombination will not generate novel genes. Furthermore, translocation breakpoints do not coincide with meiotic recombination hot spots and are therefore unlikely to be the result of meiotic programmed DSB.[74] On the other hand, there is ample evidence that the non-homologous end-joining repair system operates during the zygotic cell cycle and also during spermiogenesis, at the stage of elongating spermatids (ES).[75,76] The sperm nuclear matrix organizes DNA into large loops and plays essential roles in the paternal pronucleus.[77–79] A precise analysis of the distribution of matrix attachment sites within the domain of chicken α-globin genes has demonstrated that positions of the most important sites, the so-called "permanent attachment sites,"[80] are conserved between somatic and sperm cells.[81] Moreover, it is well-documented that DNA topoisomerase II plays a critical role in triggering DSB formation in spermatids.[77,82–85] Under certain conditions, DNA in sperm can be cleaved into loop-sized (50 kb) DNA fragments, and DNA topoisomerase IIB

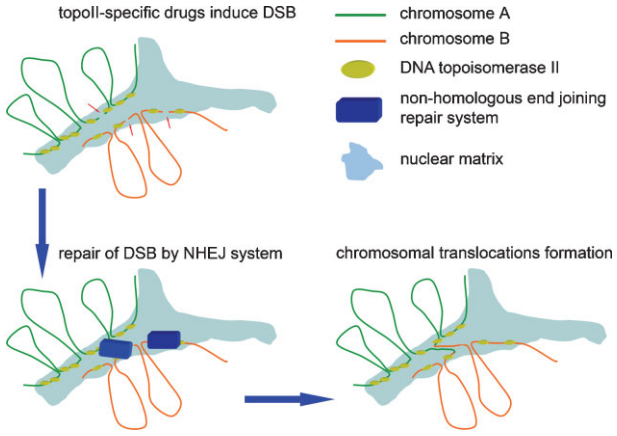

Figure 4. Repair of topoisomerase II-induced DSB can lead to chromosomal translocations (see the text for further discussion).

plays an essential role in this cleavage.[86] This finding strongly suggests that in sperm cell nuclei, topoisomerase II is located at the bases of DNA loops. Thus, the organization of the genomic DNA into loop-domains is likely to be the same in somatic and germ cells. Consequently, an illegitimate recombination between matrix-attachment regions mediated by the NHEJ repair system is likely to cause translocation events during spermiogenesis. This assumption is supported by evidence suggesting the presence of an active NHEJ system in spermatids.[76,87–89] Another window for chromosome rearrangements in germ cells is the first (zygotic) cell cycle. Unresolved DSB of parental chromatin can be repaired by the NHEJ system during the zygotic cell cycle.[75] In summary, we would like to emphasize that organisms have several means of accumulating *de novo* mutations that can be vertically transferred.

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