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Fatal Connections: When DNA Ends Meet on the Nuclear Matrix

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Abstract A damaged nucleus has long been regarded simply as a ''bag of broken chromosomes,'' with the DNA free ends moving around and forming connections with randomly encountered partners. Recent evidence shows this picture to be fundamentally wrong. Chromosomes occupy specific nuclear domains within which only limited movement is possible. In a human diploid nucleus, 6.6×10^9 base pairs (bp) of DNA are compartmentalized into chromosomes in a way that allows stringent control of replication, differential gene expression, recombination and repair. Most of the chromatin is further organized into looped domains by the dynamic binding of tethered bases to a network of intranuclear proteins, the so-called nuclear scaffold or matrix. Thus, DNA movement is severely curtailed, which limits the number of sites where interchanges can occur. This intricate organizational arrangement may render the genome vulnerable to processes that interfere with DNA repair. Both lower and higher eukaryotic cells perform homologous recombination (HR) and illegitimate recombination (IR) as part of their survival strategies. The repair processes comprising IR must be understood in the context of DNA structural organization, which is fundamentally different in prokaryotic and eukaryotic genomes. In this paper we first review important cellular processes including recombination, DNA repair, and apoptosis, and describe the central elements involved. Then we review the different DNA targets of recombination, and present recent evidence implicating the nuclear matrix in processes which can induce either repair, translocation, deletion, or apoptosis. J. Cell. Biochem. Suppl. 35:3–22, 2000.

Key words: apoptosis; breakpoint cluster region (BCR); chromatin domains; DNA repair; DNA structure; fragile sites; interferon gene cluster; nuclear matrix; recombination hotspots; scaffold/matrix-attached regions (SAR, MAR); topoisomerases (TOPO I/II)

THE GAME: SALVAGE VIA ILLEGITIMATE RECOMBINATION

Several decades ago, it was noted that broken chromosomes can rejoin efficiently because of an apparent stickiness of the newly created ends [McClintock, 1938]. Related observations showed that transfected DNA molecules commonly are also covalently joined into multimers prior to their integration into a chromosome [Perucho et al., 1980]. More recent in vivo studies of DNA end joining in mammalian cells have revealed an unexpected diversity of possible mechanisms [reviews: Roth and Wilson, 1988, Meuth, 1989]. In cells, not only can blunt or complementary restriction enzyme-generated ends be ligated, but mismatched ends also can be efficiently joined by single-strand ligation. This can occur either in the absence of any homology, or after terminal pairing of usually fewer than five homologous nucleotides [Roth and Wilson, 1988]. Topoisomerase I was one of the first molecules to be implicated in this end-joining process [Bullock et al., 1985], but an increasing number of alternative or additional factors have since been suggested.

For the organism, the primary role of efficient end joining is to repair double-strand breaks.

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These would otherwise be lethal, as they would cause the loss of all genes telomeric to the breakpoint. DNA breakage repair by HR is very accurate, but has been demonstrated to play a central role in maintaining the integrity only of small genomes. In lower eukaryotes such as yeast and *Trypanosoma brucei*, HR dominates over IR. In organisms of only slightly higher complexity (viz. *Dictyostelium*), the ratio of HR to IR events is 1:5, while in mammalian cells it approaches 1:1000. This shift does not represent a greater efficiency of HR in lower eukaryotes but rather an increasing reliance on IR in more complex organisms [Capecchi, 1990]. It has been noted, however, that the choice of repair system can also be dictated by cell cycle progression and substrate structure [Liang et al., 1998].

Both types of somatic recombination are part of the repair machinery that has evolved to counteract the DNA damage caused by exposure to chemicals or radiation. Here the strategy of higher eukaryotes is to jam together the pieces of DNA in order to prevent the loss of genetic material. In this process deletions or insertions of small numbers of nucleotides at the site of joining are common concomitants. These usually do not have important consequences as they are likely to occur in "nonessential" DNA, which represents the majority of a higher eukaryotic genome. Moreover, somatic mutations may be only detrimental to the existing organism, and not its progeny, since DNA aberrations can be repaired using the homologous allele.

On another level, efficient end joining is a driving force for evolution. DNA breakage followed by end joining provides a mechanism by which exons can be shuffled, genes can be duplicated, and chromosomes can be rearranged. Occasionally, new arrangements of genes or gene families emerge and may be preserved in the gene pool. Thus, the DNA end-joining mechanism for repairing breaks, which initially served as a salvage pathway, has the potential to contribute enormously to evolutionary diversification.

THE PLAYERS: ENZYMES INVOLVED IN DNA BREAKAGE

Topoisomerases

DNA topoisomerases assist in controlling the level of DNA supercoiling in both prokaryotic and eukaryotic cells. Three major classes of eukaryotic topoisomerases have been identified, which control DNA superhelicity by processes that introduce transient breaks in either one (TOPO I, TOPO III) or both (TOPO II) strands of duplex DNA, then pass either a single- or double-stranded DNA through the break prior to its ligation. Because these enzymes affect DNA topology, they have been implicated in almost every aspect of DNA metabolism.

Eukaryotic TOPO II is an abundant, heterodimeric nuclear enzyme which cleaves both DNA strands in a process coupled to ATP hydrolysis. After an ionic complex is formed with Mg^{2+}, the TOPO II dimer becomes transiently bound to the 5′ phosphoryl end of the broken strand via covalent linkage to a specific tyrosine residue. Protein–protein interactions between the two subunits keep the strand ends in proximity, while a loop of duplex DNA is transported through the break before the ends are religated [reviews: Wang 1996; Withoff et al., 1996]. By this process TOPO II enzymes can both alter the superhelicity of DNA domains and decatenate intertwined DNA loops. These activities maintain the integrity of DNA during replication and during both mitotic and meiotic chromosome segregation. TOPO II enzymes also are thought to participate in DNA recombination events, including the processes underlying IR.

Paradoxically, both the absence and the malfunctioning of topoisomerases have been linked to genome instability. Inactivation of either the *top*2 or the *top*1 gene in yeast triggers hyperrecombination, leading to excision of rings from the rDNA gene cluster. When topoisomerase expression is (re-)initiated, these rings integrate back into the rDNA locus. These observations suggest that topoisomerases maintain DNA integrity during events where a plectonemic junction would otherwise trigger recombination, such as when supercoiling persists during transcription or replication.

Conversely, there are processes and drugs that stall the TOPO II enzyme at the point where the cleavable complex has formed and both DNA strands have been broken (Fig. 1A). The dimeric nature of TOPO II suggested that it could mediate illegitimate recombination via subunit exchange between the DNA-linked halves of two distinct enzyme molecules. This "subunit-exchange" mechanism now appears

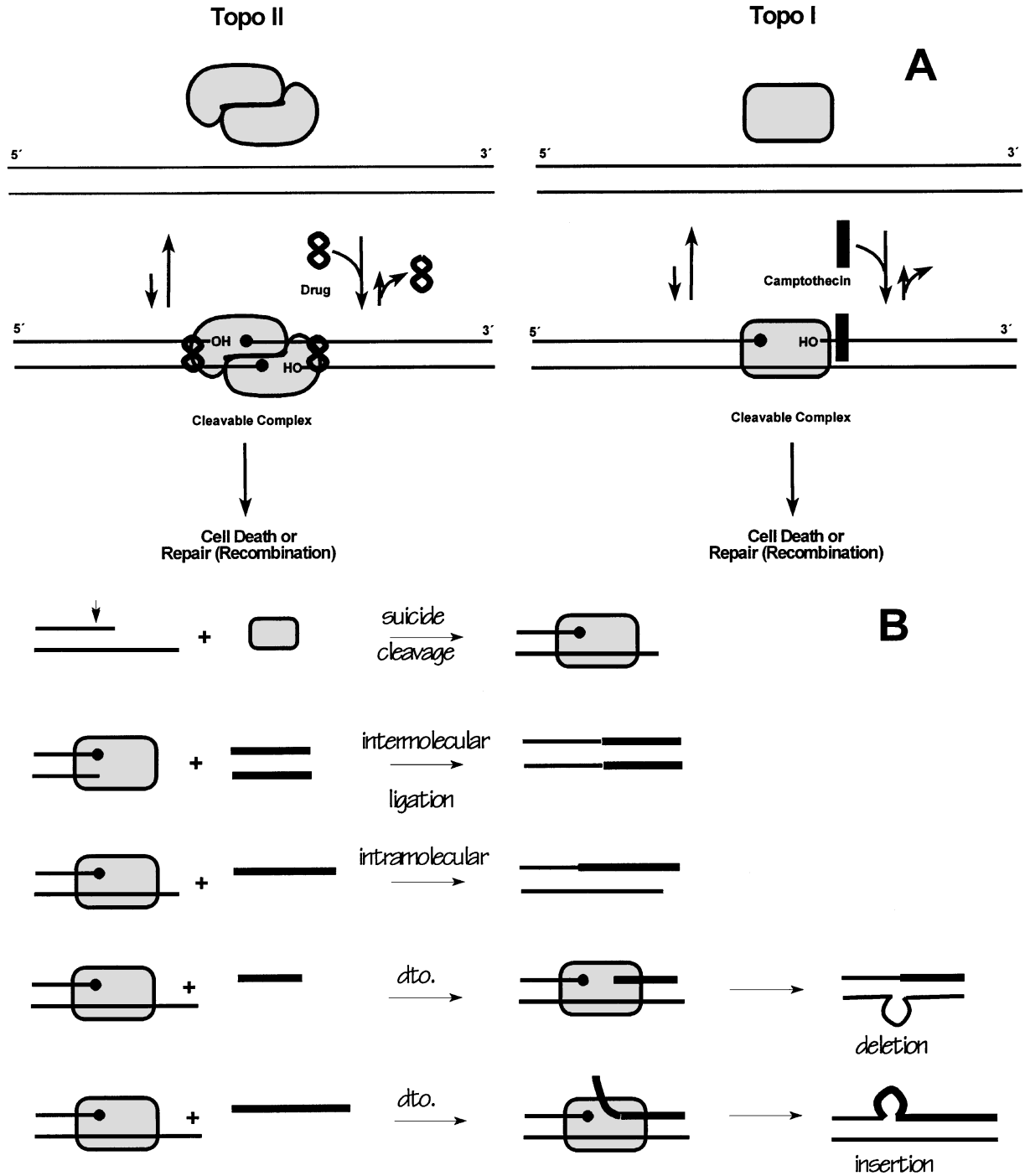


Fig. 1. Possible malfunctions of topoisomerases. (**A**) Interruption of the topoisomerase reaction cycle by a drug. A TOPO II drug stabilizes the covalent intermediate preventing the religation step. The free DNA ends are subject to recombination. Camptothecin binds to a stereospecific site at the interface of the TOPO I/DNA complex and generates a free end that cannot be religated [Svejstrup et al., 1991]. (**B**) DNA rearrangements catalyzed by TOPO I.

unlikely, as topoisomerase dimers are known to be highly stable. However, a DNA end generated by topoisomerase will be recombinogenic, even if it is not linked to a subunit. If the attached protein were removed, perhaps by a cellular repair system, recombinogenic ends would be exposed [Wang, 1996].

TOPO I acts in a similar fashion to TOPO II, although it only cleaves a single strand, and the enzyme monomer is transiently linked to the 3′ end of the broken DNA (Fig. 1A). Model studies have associated TOPO I with many forms of rearrangements in situations where a faulty reaction partner becomes available for ligation reaction. Hence this enzyme could be the source of multiple erroneous rearrangements if wrong ends come into proximity (Fig. 1B), in line with earlier hypotheses. While TOPO II is frequently associated either with regulatory regions that contain a DNAse I hypersensitive site (HS) or with domain boundaries (S/MARs, see "THE PLAYGROUND: THE NUCLEAR MATRIX") and [Withoff et al., 1996], it is TOPO I that is most abundant in transcribed regions, where its action provides the principal way to relieve transcription-induced torsional stress. This view is supported by UV crosslinking studies showing that TOPO I becomes recruited to a transcribed gene but not to its flanking sequences. Both enzymes are attracted most strongly to plectonemic structures, so it is uncertain whether the "consensus sequences" that have been proposed for these enzymes from in vitro assays involving linear DNA substrates play any role in the living cell [Käs and Laemmli, 1992; Timsit et al., 1998].

Both TOPO I and II are essential for DNA function and cell survival. Recent studies have identified these proteins as the cellular targets of several anticancer drugs. These drugs stabilize covalent intermediates between topoisomerases and DNA, and thereby stimulate chromosomal deletions and rearrangements [Wang, 1996]. Topoisomerase II inhibitors (anthracyclines, epipodophyllotoxins, etc.) are active against several types of tumors, although they often induce the development of multidrug resistance or therapy-related secondary tumors. Unlike TOPO II, TOPO I is not a cell cycle-dependent enzyme, and thus may be a more desirable target for anticancer drug development. The most prominent TOPO I inhibitor, camptothecin, has shown activity against a broad range of tumors, and is not a substrate for multi-drug-resistance-associated proteins. Used for centuries in traditional Chinese medicine, this drug was rediscovered in the 1950s during a search for compounds that could be used for steroid synthesis. Whether TOPO I inhibitors trigger IR processes in vivo similar to those depicted in Figure 1B, and the extent to which the temporary existence of unligated DNA ends provokes mis-ligation, are questions that remain to be investigated.

Apoptotic Nucleases

Apoptosis occurs in three steps. In the first step, an endonuclease is activated that is constitutively bound to chromatin domain borders. This enzyme introduces single-strand breaks at the initial nuclear concentration of nuclear Mg^{2+} [Walker et al., 1997]. In the second step a reaction mediated by accumulating Ca^{2+} causes further DNA degradation at other classes of sites. Under these ionic conditions the domain-bound endonuclease produces double-stranded breaks without having to dissociate ("single-hit" kinetics). The third step involves internucleosomal DNA cleavage at linker regions within DNA loops, which generates the prototypical DNA ladder. This stage still is Ca^{2+}-dependent and also requires proteolysis, as it can be blocked by a variety of serine protease inhibitors [Walker et al., 1997].

Apoptosis is characterized by several unique morphological nuclear changes, including chromatin condensation and nuclear fragmentation. Because TOPO II acts both to maintain higher order chromatin structures and to generate free ends during an abortive reaction cycle (Fig. 1A), a role for this enzyme in apoptosis has been proposed [Roy et al., 1992; Oberhammer et al. 1993]. However, apoptosis also proceeds in cells that are continuously exposed to a TOPO II inhibitor, suggesting that other mechanisms are more important for DNA fragmentation.

Both DNAse I and DNAse II have been suggested as the nuclease that triggers apoptosis. Although neither of these is a nuclear enzyme, DNAse I antibodies are able to neutralize the apoptotic nuclease, suggesting some kind of relationship. These observations support the current view that apoptosis is triggered by the action of a family of cysteine proteases called caspases on a protein complex that has DNAse I-like characteristics. The central component of this complex is the DNA fragmentation factor (DFF) which is a heterodimeric protein composed of 45 (DFF45) and 40 kDa (DFF40) subunits. The 45 kDa subunit is a specific molecular chaperone and an inhibitor for the nuclease activity of DFF40. DFF45 can be cleaved either by caspase-3 or -7 to set free the nuclease activity of DFF40. Histone H1 binding to DFF associates this enzyme with DNA, and stimulates its nuclease activity by increasing its

k_{cat}, and decreasing its K_m-values [Liu et al., 1999].

THE RULES: REPAIR MECHANISMS OR APOPTOSIS

More than 80% of human cancers appear to arise from deficiencies in repair pathways, despite the large amount of time and energy cells invest in DNA repair. Radiation, mutagens, free radicals, and topoisomerase inhibitors are among the many agents that cause DNA damage. There also are agents which activate signaling pathways that disrupt chromatin, and can lead to apoptosis if allowed to proceed to completion. *Excision repair* is a sophisticated process that targets the repair machinery to lesions in the actively transcribed strand. The initial steps require only three proteins in *E. coli* but in mammals at least 30 proteins are needed, including those that sense open chromatin structures [reviews: Naegeli, 1995; Boulikas, 1996]. Other mechanisms perform *mismatch repair* of newly replicated DNA, a process in which DNA glycosylases directly remove the damaged base from deoxyribose.

The first step of any repair pathway is recognition of a lesion by specific proteins, which interact with other repair factors in a multiprotein complex. Examples include HMG-box nuclear proteins that sense non-B structures such as cruciform or platinum adducts, p53 that recognizes strand breaks or mismatches and induces cell cycle arrest, and poly(ADP)ribosylase protein (PARP), which is involved both in DNA excision repair and in DNA breakage and rejoining. PARP starts its reaction cycle by binding to a DNA lesion via zinc fingers, then activating poly(ADP-ribose) synthesis. This causes automodification and leads to a modification of other chromatin components. These processes cause transient dissociations of histones and permit repair proteins (helicases, TOPO I) to gain DNA access.

Most significant in the present context, mammals also have highly active mechanisms for repairing potentially lethal chromosomal *double-strand breaks* (DSBs). The predominant pathway for joining any particular pair of DNA ends depends on the structural features of their termini [Roth and Wilson, 1986]. It has recently been shown that an endonuclease-generated DSB introduced into one of two direct repeats can stimulate HR by as much as three or four orders of magnitude in mammalian DNA, suggesting that under these circumstances homology-directed repair may occur [Liang et al., 1998]. However, DSBs are more typically repaired by IR processes. Most of the junctions that form when single strands from the two participating DNA ends can be abutted (i.e., a 5′ extension opposite a 3′ extension, or either extension opposite a blunt end) appear to arise from direct single-strand ligation. Short-sequence pairing is one explanation for the frequent occurrence of up to five nucleotides of homology at IR junctions in mammalian cells [Roth and Wilson, 1986 and below], although pairing between such short homologies alone is not sufficient to hold the duplex together. If, for instance, TOPO I were attached to one DNA end, it could perform an intramolecular rejoining reaction with another free end (Fig. 1B). Although such an intermediate requires no additional stabilization, this process could nevertheless profit from short homologies.

The presence of "filler DNA" was first observed at junctions between V, D, and J gene segments in immune system rearrangements [Landau et al., 1987]. Here, filler DNA is formed by the template-independent addition of nucleotides to broken 3′ ends by a cell type-specific terminal transferase. Approximately 10% of junctions generated in nonlymphoid cells also contain extra nucleotides [Roth and Wilson, 1988]. The observation that mammalian cells contain a pool of oligonucleotides with a length distribution similar to that of filler DNA [Plesner et al., 1987] indicates that endogenous oligonucleotides may be an important source for filler sequences. These oligonucleotides can be attached to the ends either by direct ligation or by pairing of short homologies followed by repair.

Presence of mini-direct repeats, the use of ssDNA as filler, and the filling-in of protrusions are the defining properties of "*Error-prone repair*" (EPR) mechanisms which are usually not the first choice for a cell, and are only applied in the absence of alternatives. Prominent situations for the preferential use of EPR arise from the presence of two simultaneous breaks in a highly fragile stretch of DNA (a so-called Breakpoint Cluster Region, BCR; see "THE *MLL* GENE AND ITS RECOMBINATION PARTNERS"):

- two blunt-end breaks may cause deletions; if coupled to a translocation, the sequence is missing in both derivative chromosomes;

- two staggered double-strand breaks which produce sticky ends may cause an inversion; if a translocation is triggered, the inverted fragment will be found in one of the partners;
- two staggered single-strand cuts forming 5′ overhangs trigger a fill-up reaction and thereby a sequence duplication;
- two staggered single-strand cuts forming 3′ overhangs will cause the (partial) deletion of the protruding ends.

Apoptotic degradation of genomic DNA in mammalian cells is one of the processes which causes the excision of large DNA fragments, ranging in size from 50 kbp to more than 300 kbp. These fragments probably represent entire chromosomal DNA domains as the DNA fragmentation patterns that arise during apoptosis or after TOPO II cleavage of chromatin are surprisingly similar or even identical [Iarovaia et al., 1995; Lagarkova et al., 1995; and "THE PLAYGROUND: THE NUCLEAR MATRIX"). A very prominent hypersensitive site in the *MLL*-BCR of both humans and mice (called $HS^{TOP\ II}$ in Table I and "THE *MLL* GENE AND ITS RECOMBINATION PARTNERS") and an analogous site in the AML1 locus (human chromosome 21q22) are both cleaved following TOPO II inhibition and likewise by apoptotic stimuli [Stanulla et al., 1997]. Therefore it has been proposed that apoptosis, if interrupted at early stages, may be followed by repair processes that would allow the cell to escape from programed cell death.

THE TARGETS

Hotspots of Recombination

Increasing evidence suggests a role for chromatin structure in the responses of eukaryotic cells to carcinogens and to ionizing radiation [review: Roti Roti et al., 1993]. An important initial step in the carcinogenic process is the attack of DNA by the ultimate carcinogen. Transcriptionally active chromatin is more susceptible to carcinogens than is inactive chromatin, possibly because single-stranded DNA is much more prone to damage compared to double-stranded DNA ("THE PLAYGROUND: THE NUCLEAR MATRIX") and [Boulikas, 1996"].

These relationships have been extensively studied using haloacetaldehydes such as chloroacetaldehyde (CAA), which arises from the metabolic activation of vinyl chloride (VC), a known human and rodent carcinogen. This che-

TABLE I. Genomic Rearrangements Correlated With S/MARs or S/MAR-Associated Features[a]

Gene	Deletion (kb)	BCR (kb)	Structure	Alu	L1	S/MAR	HSS	TopoII Cons.	TopoII Cut	V(D)J	Ref
A. Translocations											
MLL (11q23)		8.3		++	++	2	$1HS^{TOPII}$	6+1	1	neg.	1,2
× *AF9* (9p22)		15 and 7		++		2	$1HS^{TOPII}$	1		neg.	1
× *AF4* (4q21)		6, 9 and 5		++		2					2
11q23			PAL			ATRR					3
× 22q11			PAL			ATRR					3
bcr (22q11)		5.8 (CML) 25 (ALL)					$3HS^{TOPII}$			+	4
× *abl* (9)											4
c-myc (8q24)						1		+		+	5
× *IgH* (14q21)										+	4
× *Igκ* (2)										+	5
× *bcl-2* (18q21)		0.15								+	4
mouse *Ig-κ* (6)		0.01				1		+	+		6
× *pvt-1* (15)											6
B. Deletions											
α-Thalassemia (16p13)	0.9		DR, IR	AA							4
β-Thalassemia (11p15.5)	0.6,1.4,4.2	IVS2				1	+				6
β-Thalassemia (11p15.5)	1.4		DR,IR	A	L1						4
LDL receptor (19p13.2)	0.8–7.8	IVS15	DR,IR	AA		1					4
DMD locus (Xp21)		IVS7 (110)		L1	(A)	1			1		7
IFN (I) family (9p21)	<1000	450				36	multiple	multiple			8
Ring chromosome 21				L1		1		+			6
Rabbit Ig-κ ΔS/MAR	0.2					1		+			6
Lysosomal acid α-Glucosidase (GSDII)	9			AA							9

PAL, palindrome; ATRR, AT-rich region; AA, Alu elements at both sides of a deletion; L1, LINE element; DR, direct repeat; IR, inverse repeat. References: 1, Strissel et al., 2000; 2, Marschalek et al., 1997; 3, Kurahashi et al., 2000; 4, Meuth, 1989; 5, Hörtnagel et al., 1995; 6, Sperry et al., 1989; 7, McNaughton et al., 1997; 8, Strissel et al., 1998b; 9, Huie et al., 1999.

mical conversion starts with the generation by P_{450} of chloroethylene oxide (CEO), which is either hydrolyzed or rearranged to form CAA. Haloacetaldehydes react with adenines and cytosines once these nucleobases have been exposed in single-stranded regions of DNA, converting them to their respective fluorescent etheno-derivatives [Bode et al., 1992]. The sites that are targeted by CAA in the living cell are also reactive in vitro when placed in negatively superhelical plasmids. Another ultimate chemical carcinogen, *N*-acetoxy-2-acetylaminofluorene, detects the same non-B DNA structures that react with CAA in supercoiled plasmid. DNA [Kohwi-Shigematsu et al., 1988].

Recently, Pourquier et al. [1998] applied purified mammalian TOPO I and oligonucleotides to study cleavage and religation in the presence of 1,*N*-6-ethenoadenosine (EA) adducts located immediately downstream from a unique TOPO I site. EA markedly enhanced cleavage complexes when it was incorporated at the $+1$ position of this site which was ascribed to a reduction in the rate of the religation step. These results show that the carcinogenic EA adduct can efficiently trap human TOPO I reaction intermediates, thereby mimicking camptothecin (Fig. 1B). Chiang et al. [1997] demonstrated for a human lymphoblastic cell line that CAA caused deletions at 45% of its reaction sites. These observations show that in the living cell cutting and rejoining of DNA can occur in places where etheno-derivatives prevent the reassociation of single strands.

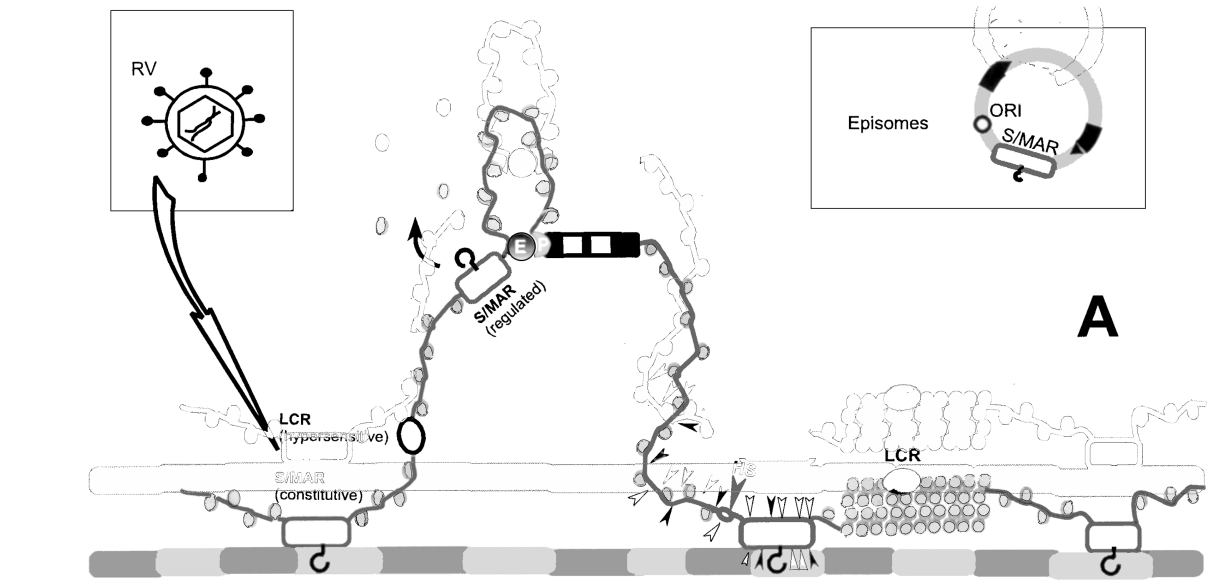
Similar conclusions emerge from the study of Legault et al. [1997] who assessed the susceptibility of supercoiled plasmids containing a base-unpairing region (BUR) to DNA damage either by depurination or by Fe^{3+}-bleomycin. Single strand-specific S1 nuclease was used in combination with 5′-end-labeling to detect either single-strand breaks or gaps after cleavage of abasic sites. The DNA cleavage patterns showed (i) a highly preferential co-localization with S1 hypersensitive sites and (ii) a surprisingly similar localization of DNA damage induced by quite different types of clastogenic (crack-provoking) reagents.

These findings raise the question of exactly which elements in the genomes of higher eukaryotes are subject to damage and recombination. Some reports hint at the importance of switch and variable diversity-joining region recombination sequences. These regions represent a specialized case in which the immunoglobulin or T-cell receptor gene regions in B or T cells are processed by the recombinases of the immune system. Burkitt lymphomas, for example, arise from reciprocal translocations between chromosomes 8 and either 2, 14 or 22, that join the *c-myc* gene to an immunoglobulin gene. Non-Burkitt lymphomas frequently contain translocations between the Cμ enhancer of the IgH gene and the *bcl*-2 (B-cell leukemia/lymphoma) gene. These breakpoint hotspot regions can be as small as 30–100 bp. Therefore, the faulty use of normal conserved recombination sites is one important factor in certain types of translocations [review: Meuth, 1989].

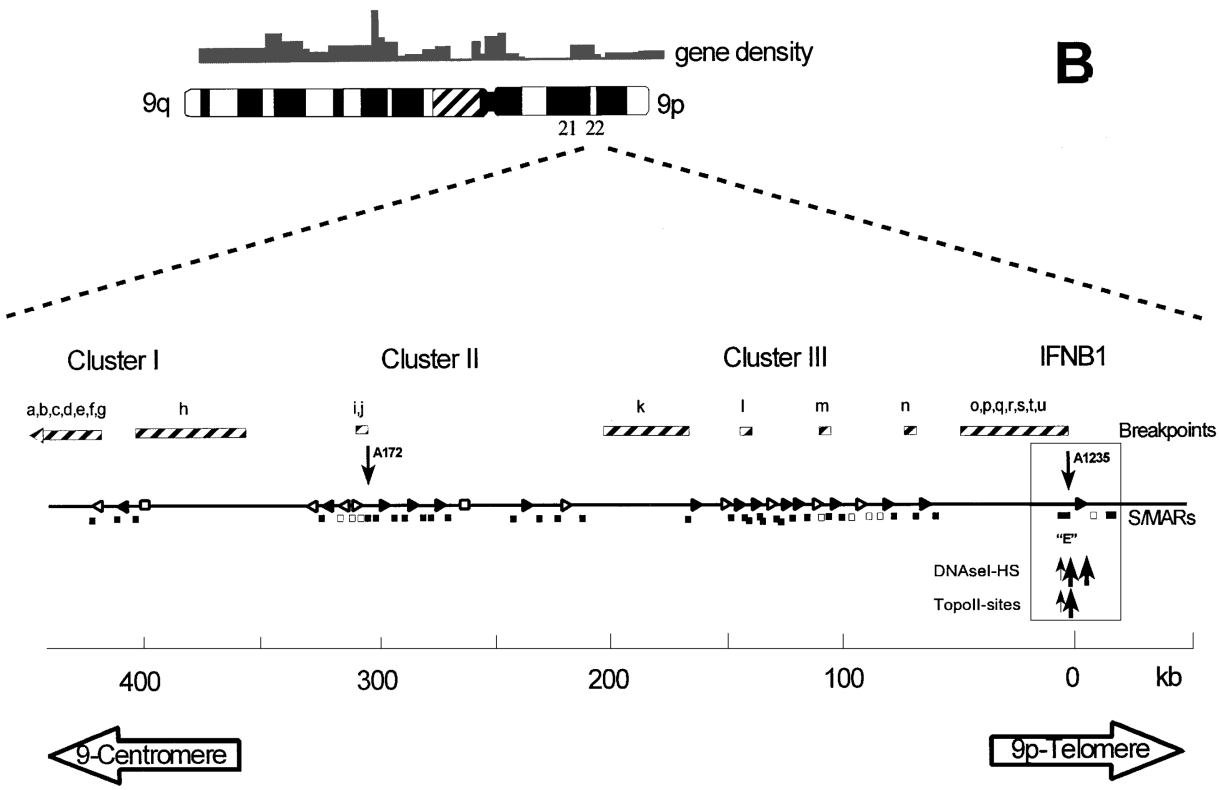
LINES AND SINES

Recent reports show that remnants of reverse transcribed genes are very common in the genome, due to their presence in retroelements. Retroelements are subdivided into two classes, retrotransposons that have long terminal repeats (LTRs) and retroposons that do not have LTRs. Retroposons are highly abundant in the human genome, and presumably also in those of most other mammals. One class of retroposons, the long interspersed nuclear elements (LINES) constitute about 5% of the total human genome. There are about 100,000 copies of LINES, some of which are full length and encode a functional reverse transcriptase. Another class of retroposons, the short interspersed nuclear elements (SINES), are related to LINES but have many deletions. SINES represent an even larger proportion of the human genome (500,000 copies). They too can modify gene expression by their movement, amplification, and re-insertion into genes and regulatory sequences, but to do so they need to "borrow" reverse transcriptase, from other sources.

Retroelements are involved in shaping the genome, determining its size and organization, and directing its evolution. The human prototype SINES, the Alu repeat, is roughly 300 nucleotides in length. Alu elements have been classified into subgroups I–IV, of which only type IV members are transcribed. An RNA polymerase III start site is located within this element, and can direct gene expression in response to cellular insults such as viral infections or exposure to carcinogens. This expression may facilitate recombination with other Alus or their flanking regions.



Scaffold: - ***Lamins***;
- ***Matrins*** (ARBP/meCP2, Calmodulin, **DNA-Pol β**, **HAT, HDA, HMG 1/2,** Nucleolin, NuMa, **PARP**, SAF-A/hnRNP, SAF-B, **Topo II**, **ssDNA-Binders**);
- specific transcription factors and transcriptional mudulators.



Alu family repeat sequences sometimes are located at chromosomal breakpoints, particularly in germ-cell-derived (inherited) mutations where many breaks localize to Pol III promoter regions [review: Meuth, 1989]. Alus are found at or near the breakpoints of most deletions responsible for familial hypercholerestemia; the LDL receptor gene region is especially rich in these repeats. Alus also are associated with a significant number of deletions in the α- and β-globin regions. The largest deletion in the α gene cluster results from a homologous crossover between two Alu family members 62 kbp apart that causes the loss of a complete loop of packaged DNA (see "THE PLAYGROUND: THE NUCLEAR MATRIX"). In contrast, most other exchanges in the α- and β- globin gene regions involve a non-Alu sequence at one of the breakpoints.

During evolution, SINES have continuously screened mammalian genomic chromatin for the most attractive integration sites. It is possible that SINES might not themselves be favorable recombinogenic targets, but rather indicators for the presence of such features at the sites of their integration. So we must also consider the nature of these preferred sites.

Scaffold/Matrix-Attached Regions (S/MARs)

The 25 million nucleosomes in a mammalian nucleus are organized into about 60,000 chromatin loops by periodic attachments to the nuclear scaffold or matrix at positions separated on average by 70 kbp [Gasser and Laemmli, 1987; Vogelstein et al., 1980 and Fig. 2A]. A number of assays have been developed to search for the DNA sequences that mediate chromosomal loop attachment, the so-called "scaffold- or matrix-attached regions" (S/MARs). Significantly, all these assays identify the same fundamental class of anchorage sequences [Kay and Bode, 1995, and references therein].

S/MARs map to non-random locations in the genome. They occur in the flanks of transcribed regions, in introns, centromeres and telomeres, and also at gene breakpoint cluster regions [reviews: Bode et al. 1995; Boulikas 1995; and below]. S/MARs harbor binding sites for essential nuclear structural proteins [Fig. 2; review: Bode et al., 2000]. Due to their unique chromatin structure, they are necessary for correct chromosomal replication and transcription [Boulikas, 1995], condensation [Strick and Laemmli, 1995], and recombination [Sperry et al., 1989 and below].

S/MARs do not have clear consensus sequences associated with them. Although prototype elements consist of AT-rich regions several hundred base pairs in length, over-all base composition seems not to be the primary determinant of their activity. Instead, binding activity appears to require a pattern of AT-richness that confers a propensity for local strand-unpairing under torsional strain [Benham et al., 1997; Tsutsui, 1998; Michalowski et al., 1999]. The fact that S/MARs coincide with easily melted DNA sequences accords with their established involvement in DNA replication [Baiker et al. 2000]. Both chemical and enzymatic probes have been applied to show that this strand separation potential is utilized in the living cell for anchoring a chromatin domain to the matrix and that DNA accessibility is modulated at times of transcriptional activity [reviews Bode et al., 1995, 1996, 1998]. These properties both topologically separate each domain from its neighbors, and provide a means by which trans-

Fig. 2. Domain organization of eukaryotic genomes. (**A**) Constitutive scaffold/matrix attached regions (S/MARs) subdivide the eukaryotic genome into loops of 4–200 kbp (average: 70 kbp). S/MARs associate with ubiquitous components of the nuclear matrix (lamins and matrins), among these components involved in recombination and repair (TOPO II, DNA-Polβ, PARP and single-strand binding proteins). They can also accommodate specific transcription factors [Stein et al., 1998, 1999]. S/MARs are recombinogenic elements and are thereby a preferred target for the entry of retroelements and retroposons [Mielke et al., 1996]. Certain regions at S/MARs are particularly vulnerable by DNA damaging events (arrowheads) among these agents are TOPO II-specific drugs (open arrowheads) with a specificity for HS^{TOPII} sites (large solid arrowhead). The nuclear matrix has multiple roles in guiding transcription (e.g., the support of enhancers), DNA repair, and replication. A S/MAR is able to recruit the replication apparatus to the origin of replication as demonstrated by an episomal vector which maintains its extrachromosomal status due to nuclear matrix contacts [Baiker et al., 2000]. (**B**) Organization by S/MARs of the type 1 interferon gene cluster on the short arm of human chromosome 9. All genes (solid triangles) but few pseudogenes (light triangles or rectangles) are organized by associated S/MAR elements (solid rectangles below the line indicate high-affinity S/MAR elements). Translocation A1235 occurs within a prominent breakpoint cluster (events o–u) adjacent to the IFNB1 upstream S/MAR. Other breakpoints have been symbolized by lettering (a–n) or an arrow (A172). All breakpoints appear to be associated with intragenic S/MAR regions [Strissel et al., 1998].

criptional events within a given domain become augmented [Bode et al., 2000].

The strand separation potential of S/MARs can be computationally predicted from the base sequence, and is usually displayed as a stress-induced duplex destabilization (SIDD) profile [Benham et al., 1997; Bode et al., 1998, 2000 and Fig. 3]. The SIDD profile of a sequence displays the predicted free energy G(x) needed to guarantee separation of the base pair at each position x along the DNA sequence, under the assumed level of torsional tension.

Since single-stranded DNA is known to be much more sensitive to damaging agents than is double-stranded DNA, S/MARs have been postulated to be hypersensitive to DNA breaking events [Legault et al., 1997]. The energy stored in a single-stranded region could be retrieved by the formation of nearby cruciforms or slippage structures. These alternate structures and single-stranded regions themselves may be recognizable features to DNAses, topoisomerases or other structure-converting enzymes. In fact, S/MARs have been shown to possess significant potential for alternate secondary structure formation and they also contain naturally curved sequences, which promote base-unpairing under superhelical stress [Mielke et al., 1996].

Owing to these structural properties, S/MARs are expected to be recombinogenic structures. This expectation is supported by several observations. First, some transfected S/MAR constructs integrate at much higher copy numbers than their S/MAR-free counterparts [Bode et al., 1996]. Second, endogenous cellular S/MARs are the dominant (possibly exclusive) integration sites for proviruses and perhaps also for certain other transgene constructs [Bode et al., 1995; Mielke et al., 1996; Baer et al., 2000]. The integration of retroposons may obey the same rules that govern retroviral integration, and therefore the location of these elements may simply be a marker for the presence of S/MARs (See "LINES and SINES").

Although this is not a general property of Alu sequences, some of the elements can function as S/MARs themselves, provided they contain a sufficiently high number of ATTA, ATTTA, homeodomain or transcription-factor-binding sites. S/MAR function may also be associated with those Alu sequences that have origins of replication (supporting) function in primate cells [review: Boulikas, 1995].

THE PLAYGROUND: THE NUCLEAR MATRIX

An increasing number of ubiquitous proteins is being identified that recognizes the structural features of S/MARs. These include lamins and matrins, recently characterized components of the nuclear network [Bode et al., 2000]. Proteins with the potential to be important in recombination, translocation or integration have been included in Figure 2A. All known functional attachment points appear to be able to bind TOPO II, which is a prominent component of the matrix in dividing cells (see "TOPOISOMERASES"). In this regard we have to distinguish two genetically and biochemically distinct topoisomerase II proteins, TOPO IIα and TOPO IIβ. TOPO IIα localizes to the nucleus and is mostly expressed at the G2/M boundary of the cell cycle. In contrast, TOPO IIβ shows no cell cycle variation and is found in the nucleolus. Active heterodimers with largely unknown properties have also been identified [Biersack et al., 1996]. These observations together suggest that S/MARs may become sequestered by this protein at the bases of chromosome loops during metaphase. In the balance of this review references to TOPO II will refer to the α-isoform.

Razin and colleagues have developed a procedure by which chromosomal DNA loops are excised by TOPO II-mediated DNA cleavage at matrix attachment regions. This procedure is now routinely used to probe the occupancy of S/MARs in various cell types and organisms [Iarovaia et al., 1995 and references therein]. In proliferating lymphocytes, these loops are likewise excised from the genome by either endogenous TOPO II or by an exogenously added single-strand-specific nuclease. In non-dividing lymphocytes these procedures generated no specific pattern of long-range fragmentation suggesting proliferation arrest not only to correlate with decreased TOPO II activity, but also with a change in DNA packaging. Similar or even identical cleavage patterns are generated at early stages of apoptosis [Lagarkova et al., 1995], initiated by the proteolysis of distinct matrix proteins [Gohrig et al., 1997, Dynlacht et al., 1999], and the subsequent generation of single-strand cuts in the DNA (see "APOPTOTIC NUCLEASES").

In contrast to TOPO II, TOPO I is usually not regarded as an intrinsic matrix component. This topoisomerase, however, becomes associated with the matrix in a facultative manner

during transcription and DNA repair. Among the multitude of enzymes involved in repair, only poly(ADP-ribose) polymerase and DNA polymerase β have been preferentially localized in the matrix (Fig. 2A). A number of other repair enzymes such as endonucleases, DNA helicases or ssDNA binding proteins are likely to be additional components of the nuclear matrix, which has a proven abundance of ssDNA binding sites [reviews: Bode et al., 1995, 1996, 1998, 2000]. Thus the matrix is viewed as a dynamic nuclear microenvironment which not only hosts transcription and replication complexes, but also proteins involved in DNA repair. This architecture most likely plays a role in the preferential repair of active over inactive genes ("THE RULES: REPAIR MECHANISMS OR APOPTOSIS" and [Boulikas 1996]).

One critical question is: what facilitates chromosomal translocations to occur between two widely separated chromosomes which do not share any obvious homologies. To enable broken DNA strands to interact, contact must be established between them. The probability of such an interaction depends on distance, search volume, metabolic activity, and time. It is still a matter of contention how these requirements are satisfied after DNA damage. In this review we have tied all the molecular players (enzymes involved in breaking and repair) and the DNA targets (LINES, SINES, S/MARs) to the nuclear matrix, which is the site we propose where damaged ends meet to be repaired. This model has gained much support over the years and to prove its predictions several well-characterized examples will be discussed. Before we evaluate recently investigated, representative translocation and deletion events in "TWO SCENARIOS—ONE SET OF RULES", we will briefly summarize the existing evidence identifying S/MARs as possible hotspots of recombination [Meuth, 1989; Sperry et al., 1989 and Table I].

The human β-globin locus covers 70 kb of DNA on the short arm of chromosome 11. More than 20 deletions have been identified in this region with sizes ranging from 619 bp to more than 100 kbp. One group of γδβ-thalassemias is characterized by deletion events, all of which are of similar size (5–6 kb) and have endpoints within rather narrow regions. This endpoint clustering suggested that these deletions result from the juxtaposition of distant sequences by their anchoring to the nuclear matrix, with the deletion excising a complete domain [Vanin et al., 1983]. In this picture, the observed staggered positions of the 5′ and 3′ endpoints are explained by a "sliding frame" as sequences move through the anchorage point, for instance during replication. While in these cases the attachment region cannot be pinpointed, another group of β-thalassemias have a deletion with one end in the IVS-2 sequence of the β-globin gene [Anand et al., 1988], at a site where we have previously localized a strong S/MAR sequence [Klehr and Bode, 1988]. Another example is the formation of a human ring chromosome 21, which correlates with a S/MAR at one of the breakpoints [Sperry et al., 1989]. The murine immunoglobulin κ-S/MAR contains both recombination hotspots and several TOPO II binding sites resulting in a number of translocations, some of which correlate with plasmacytomas [Shapiro and Weigert, 1987; Sperry et al., 1989]. In some other cases the amplification of selectable loci has also been ascribed to the associated S/MARs and these findings have lent further support to their function as hotspots for IR [Meuth, 1989].

TWO SCENARIOS—ONE SET OF RULES?

Although each tumor has its own anomalies, certain recurrent properties suggest the regular involvement of oncogenes or tumor suppressor genes in their development. An early oncogene example is the reciprocal translocation t(9;22)(q34;q11). The resulting "Philadelphia chromosome" is found in the cells of virtually all patients with chronic myelogenous leukemia (CML), and also in approximately 25% of acute lymphoblastic leukemia (ALL). The chromosome is formed as a consequence of a reciprocal translocation in which a small fragment of chromosome 9 is fused to the broken end of chromosome 22. Breakpoints on chr 9 appear to be scattered throughout the *c-abl* gene, while for chr22 they occur within the *bcr* gene which for CML patients involves a 5.8 kbp area known as the major breakpoint cluster region (BCR). For ALL, patient breakpoints locate in a 20 kb region within *bcr* intron 1. Located on chromosome 9q34, the *c-abl* protooncogene encodes a nonreceptor protein-tyrosine kinase. The translocations give raise to a new 210 kDa fusion protein (CML), or a 190 kDa fusion protein (ALL), respectively, which have markedly increased auto-phosphorylating activity relative to normal c-Abl. These fusion proteins

transform transfected cells, and induce leukemia in transgenic mice [Huettner et al., 2000].

In Burkitt's lymphoma, *c-myc* is translocated from its normal chromosomal location into juxtaposition with immunoglobulin genes, where it comes under the transcriptional control of a cell type-specific immunoglobulin gene enhancer. Continuous *c-myc* expression causes cellular transformation. An analogous site in the mouse genome is involved in murine myelomas. These translocations are usually regarded as rare variations on the normal rearrangement events that occur in lymphoid cells. Altogether, *c-abl* and *c-myc* are only two in an increasing number of proto-oncogenes that have been found at points of chromosomal translocation in human tumor cells.

Internal deletions or the loss of part of a chromosome are an alternative cause for abnormal development. A classic example is retinoblastoma, a disease which led to the identification of the first known tumor-suppressor gene product, pRB. Children who inherit a single defective copy of *rb*, often seen as a small deletion on chromosome 13, show changes in their retinal development.

We note that translocations which lead to synthesis of oncogenic fusion proteins must be rather precisely positioned in order to preserve the relevant exons and reading frames. This consideration has led to two hypotheses for their origin, which need not be mutually exclusive: (1) *The selection hypothesis* proposes that initial rearrangements occur *without a local preference*. In this hypothesis breakpoints cluster at specific BCRs because functions of the resulting fusion gene product confer a proliferative advantage to the cell. (2) *The hotspot hypothesis* states that chromosomal rearrangements *preferentially occur at BCRs*. This instability suffices to explain a clustering, irrespective of the nature of the resulting proteins.

Deletion events that lead to inactivation of a tumor suppressor do not have to be precisely located, since cancerous growth may result from the loss or dysregulation of a tumor suppressor gene, or a mutation-induced malfunctioning of its protein product. Therefore, this type of rearrangements may be more closely related to the existence of recombinational hotspots. Recent paradigms for both classes of carcinogenic events are translocations initiating in the *MLL* breakpoint cluster region on human chromosome 11 on one hand, and deletions involving the type I interferon (*IFN-*) gene cluster on human chromosome 9, both of which will be discussed below.

THE *MLL* GENE AND ITS RECOMBINATION PARTNERS

A gene on human chromosome 11q23 is involved in more than 40 chromosomal translocations, and is suspected of participating in the generation of many highly malignant leukemias. The gene is referred to as *ALL*-1 (Acute Lymphoblastic Leukemia), *MLL* (Mixed Lineage Leukemia) or *HRX* (Human trithoRaX). Both its cDNA and the genomic region have been sequenced.

The MLL protein is an important regulator of embryonal and hematopoietic development [Hanson et al., 1999 and references therein]. In resting PBMCs it is distributed in a speckled pattern across the nucleus, with an increased density at the nuclear envelope. But in early and late metaphase it becomes associated with chromatin [Ennas et al., 1997]. Several domains of the human MLL protein have DNA-binding and transcription activation properties. The N-terminal region contains minor groove binding motifs like SPKK, and three AT hooks similar to those in HMG I/Y. Motifs with homology to cysteine methyltransferase and a bipartite Zn^{2+}-finger are located further downstream [Ennas et al., 1997]. Present information indicates that the human *MLL* locus encodes a member of the regulatory trithorax family, with the potential to act in a tissue-specific manner [Bernard and Berger, 1995].

Translocations involving *MLL* have been observed in many malignant diseases of the hematopoietic system. These translocations lead to a recombination with *MLL* partner genes that encode either nuclear or cytosolic proteins. Some of the resulting fusion proteins are associated with specific leukemia types. For example, *MLL/AF9* is found mainly in acute de novo myeloid leukemia (AML), whereas *MLL/AF4* is prevalent in acute de novo B-lymphoblastic leukemia (ALL) in infants. In addition, depending on the drugs and the total drug doses, between 1 and 15% of cancer patients treated with TOPO II inhibitors for a variety of tumors develop therapy-related leukemia (t-AML) and rarely t-ALL. Of 11q23 abnormalities, over 75% are based on *MLL* gene rearrangements which occur within an

8.3 kbp breakpoint cluster region (BCR; see below).

AF4 (*ALL*-1 Fused chromosome 4) is the *MLL* partner gene located on 4q band 21. It encodes a protein of more than 1200 amino acids which contains a putative NLS and a nucleoside triphosphate binding motif, but has little homology to other known factors. Remarkably, exons of both *MLL* and *AF4* terminate in phase, so a fusion mRNA will contain ORFs with in-frame transitions between protein-coding sequences from both chromosomes. *AF9* (*ALL*-1 Fused chromosome 9) is another partner gene of *MLL* that is located on 9p22. *AF9* encodes a 568 amino acid protein which is rich in proline and serine, and also contains a NLS. It is highly homologous to *ENL*, yet another recombination partner on 19p13, and contains a short C-terminal region similar to that of RNA polymerase II. The *MLL*/*AF9* fusion mRNAs have the same in-phase character as was seen above with the *AF4* partner gene. In addition to these translocations, *MLL* also recombines with itself resulting in partial gene duplications of exons 2–6 or 2–8, the majority involving Alu/Alu homologous recombination [Bernard and Berger, 1995]. These duplications are found in patients with acute myeloid leukemia and result in a MLL fusion protein with a duplication of the amino terminus including the AT hooks.

Although the precise functions of the fusion proteins are not known, the chimaeras could, in principle, alter the normal function of MLL in several ways. A new transcription factor could be formed by the fusion of MLL DNA binding motifs with a currently unidentified transactivation domain from the protein partner. Alternatively, a fusion protein could exert a dominant negative effect by either competing for normal MLL targets or by sequestering the MLL protein. One possible common feature of these partners is the formation of a dimerization domain that could cause leukemic transformation by altering transcriptional regulation [Bernard and Berger, 1995].

Cytogenetic and molecular studies of human tumors have consistently demonstrated that the rearrangements correlating with tumorigenesis are highly nonrandom. While certain chromosomal translocations involved in the origin of leukemias and lymphomas are due to malfunctions of the recombinatorial machinery of immunoglobulin and T-cell receptor-genes, this mechanism can be ruled out for t(4;11)(q21;q23) translocations [Gillert et al., 1999; Reichel et al., 1999] and it is also unlikely for t(9;11)(q21;p22) [Strissel et al., 2000]. At the break sites duplications, deletions, and inversions of less than a few hundred nucleotides are commonly observed. DNA repair is therefore probably initiated by several strand breaks on both participating chromosomes followed by nonhomologous end joining-processes (See "THE RULES: REPAIR MECHANISMS OR APOPTOSIS").

Even though MLL is encoded by at least 37 exons spread over approximately 120 kb [Nilson et al., 1996; Marschalek et al., 1997], most translocations occur in the 8.3 kbp BCR between exons 8 and 14. An in vivo TOPO II cleavage site and a DNase I hypersensitive site (HS) colocalize near exon 12 between nucleotides 6800–7000 within a high affinity S/MAR [Aplan, 1996; Strissel, 1998a]. The majority of de novo leukemia patient breakpoints map to the 5′ half of the 8.3 kb *MLL* BCR. Although this region contains a series of Alu repeats, the associated translocations cannot be explained by Alu/Alu recombination. In contrast, t-AML and de novo infant leukemia t(4;11) DNA breakpoints are more frequently found in the 3′ half of the MLL BCR. Chemotherapeutic agents such as VP16 and natural bioflavonoids have been implicated in t-AML and de novo infant leukemia, respectively [Strick et al., 2000]. Both substances target cellular TOPO II and cleave the *MLL*-BCR in the same manner. The fact that patient breakpoints localize to the BCR, and the finding of only one single unique fragile site mapping within the entire gene supports the idea that the biological role of the transforming proteins (selection hypothesis) cannot be the sole reason for explaining such a clustering of translocation events. While this fragile site is sensitive both to DNase I and TOPO II (HS^{TOPII}) it also responds to a wide range of apoptotic stimuli [Stanulla et al., 1997] suggesting that the origin of this hotspot region is causally related to the higher order chromatin structure of the *MLL*-BCR.

We have recently shown that a 4.5 kb S/MAR adjacent to the centromeric half of the MLL BCR has low affinity for the matrix, and a 5.5 kb S/MAR within the telomere-proximal portion of this BCR has high matrix affinity [Strissel-Broeker et al., 1996; Strissel et al., 2000]:

$$^{4.5}\mathrm{S/MAR} - \left[^{8.3}\mathrm{BCR}^{5.5}\mathbf{S/MAR}^{\mathrm{HS/TOPII}}\right]$$

In our initial study six out of eight t-AML breakpoints coincided with the strong S/MAR that harbors the HS^{TOPII} site, and the other two breakpoints localized to the region between the S/MARs. In striking contrast, *MLL* breakpoints in 23 of 31 de novo leukemia patients were found in the BCR *between* the S/MARs, but only eight breaks coincided with the strong 5.5 kb element. That is, although 75% of t-AML breakpoints coincide with the strong scaffold attached region, only 26% of de novo breakpoints occur there. A more comprehensive subsequent study localized 14 breakpoints to $^{5.5}$S/MAR, 50 breakpoints between the S/MARs ("within the domain") and 16 breakpoints to a region beyond the telomeric end of $^{5.5}S/MAR^{HS/TOPII}$ [RM, unpublished].

It was intriguing to find that these properties are not unique to the MLL-BCR, but recur at the BCRs of partner genes in a highly similar fashion. This suggests a model (indicated in Fig. 2A and described below) in which the S/MAR is a mediator, but not the center, for these types of IR. The *MLL* partner gene *AF9* region contains two BCRs which are associated with two strong S/MARs and a HS^{TOPII} site in the following form:

$$^{6.2}\mathbf{S/MAR1} - {}^{15}\mathrm{BCR1} - {}^{4.6}\mathbf{S/MAR2} - {}^{7}\mathrm{BCR2}^{HS/TOPII}$$

In this case, four de novo breakpoints and two t-AML breakpoints mapped "within the domain" to BCR1, while two other de novo breakpoints are closely associated with the HS^{TOPII} region in BCR2.

Breakpoints at *AF4*, the second *MLL* partner gene considered here, occur over a 50 kb region. The halo-mapping ("in vivo") procedure introduced by Mirkovich et al. [1984] suggests that this entire region may be associated with the scaffold. When the same area is analyzed by a specialized scaffold reassociation ("in vitro") approach, the presence of two high-affinity regions becomes apparent. This configuration is symbolized as follows:

$$^{16}\mathrm{BCR1} - {}^{2}\mathbf{S/MAR1} - {}^{20}\mathrm{BCR2} - {}^{2}\mathbf{S/MAR2} - {}^{4}\mathrm{BCR3}$$

Here, 35 breaks were mapped to BCR 1, 40 to BCR 2, and 15 to BCR 3; no break occurs in the center of S/MAR affinity. A possible age-related modulation in the mechanisms of chromosomal translocation is suggested by the observation that ALL-patients below and above 1 year of age had different distributions of breakpoints in two specific subregions of the BCR. Considered together, these results permit the following general conclusions:

1. The association of breakage/reunion events with S/MARs and TOPO II sites is strong. However,
2. a tight S/MAR–matrix interaction will protect DNA from breaking unless there is an endogenous TOPO II site; for *MLL* this site is preferentially used in therapy-related forms of leukemia.
3. There is a preferential localization of breaks within small chromatin regions near S/MARs or between two adjacent S/MARs (*MLL*, *AF4*, *AF9*); long S/MAR-free regions are not targets for the enzymes that are involved in breakage and repair.

This raises the question of how S/MARs can be protective against breakage/repair events, yet induce these events to occur preferentially in their vicinity. For MLL, the majority of 11q23 translocations are concentrated in the centromeric part of the BCR. Although transcribed Alus represent only a small (0.1%) fraction of all Alus, four of eight elements in this region show strong transcription by Pol III and this transcription is unidirectional [Marschalek et al., 1997]. This suggests the possibility that transcription could induce torsional stress which can be released by locally clustered breaks, possibly followed by translocation events. TOPO II could be involved in this process, since superhelical regions or the associated secondary structures facilitate association with the matrix whether or not they are part of a S/MARs [Kay and Bode, 1994]. It is also possible that, while initial binding of TOPO II occurs in the S/MAR, the enzyme scans the adjacent regions until it encounters a strong recognition feature which triggers the ultimate cut.

Alternatively or additionally, DNA damage may gain relevance at times of DNA replication. Carri et al. [1986] have proposed a mechanism by which both the origin of replication and replication termination sites coincide with S/MARs, a suggestion that has been supported by subsequent experimentation. During replication, DNA is reeled through positions adjacent to the attachment sites, where it becomes particularly vulnerable due to its single-stranded character. According to Vanin et al. [1983], such a process could lead to the deletion of loop-sized

pieces and such a model is supported by the finding that the 5′breakpoints were located the same distance apart and in the same order along the DNA as the respective 3′ breakpoints. Interestingly, a related observation has also been made in case of the *MLL/AF4* t(4;11) translocation events: the closer a break was located to the centromere on chr 11, the more the corresponding break approached the telomere of chr 4 and vice versa, suggesting the requirement for a defined spatial positioning of the participating chromosomes [Gillert et al., 1999]. Since the number of replication clusters in a nucleus is limited [Berezney et al., 1995], it is possible that the synchronized replication of the respective regions of both chromosomes at the same nuclear matrix site could lead to breakage/reunion events which have analogous positions along the two breakpoint cluster regions.

Deletion Breakpoints in the Interferon Locus

The complex repetitive type I IFN gene family maps to the short arm of human chromosome 9 within a 450 kb region at position p21–p22. It consists of one β gene (*IFNB1*), 13 α–genes ($IFNA_n$), one α-pseudogene (*IFNAP*), one ω-gene (*IFNW*), six ω-pseudogenes (*IFNWP*) and four additional pseudogenes. The coding regions but not the intergenic regions have a high (80–90%) homology to each other. In *Homo sapiens*, the single B1 gene diverged separately from the family of *IFNA* and *IFNW* genes. Since all functional members are flanked by S/MARs, it is reasonable that the primordial gene may have initiated this configuration which then promoted gene duplication and inversion events due to the recombinogenic nature of its flanks [Strissel et al., 1998a, 1998b]. Such a process could have created several subclusters by sequential duplication and an inversion event as discussed by Diaz [1995]. Within this locus, most pseudogenes appear to have lost their associated S/MARs during evolution [Benham et al., 1997; Strissel et al., 1998b] suggesting that, even though the base sequences of S/MAR may diverge, their locations and the attributes that confer their activities are maintained by selection pressure: only a loss of gene function allows truly random mutagenesis and the concomitant decline of strand separation potential [Benham et al., 1997].

One of the most common genetic abnormalities in human tumor cells is the deletion of the short arm of chromosome 9. These 9p deletions range from 200 kbp to over 1 Mbp, many of which include the *IFN* genes and sequences toward the centromere. Tumor suppressor genes have been identified in the minimum common deleted region at 9p21, most prominently INK4a/b (Inhibiting cdK4) and ARF (alternate reading frame). Recent observations indicate that the 9p21 region may harbor at least two additional tumor suppressor loci [see, e.g., Simons et al., 1999]. In normal cells, $p16^{INK4a}$ interferes with the binding of D-type cyclins to cdk4/6 kinase, which causes pRB to remain in its hypophosphorylated form whereby E2F-dependent genes stay repressed and the cell cycle is blocked at the G1/S checkpoint. So far unique for mammals, $p14^{ARF}$ (human) or $p19^{ARF}$ (murine) arise from reading frames overlapping with INK4a: the transcripts for INK4a and ARF have different first exons but share exons 2 and 3. Since INK4a and ARF proteins are encoded by distinct exon 2 reading frames, they share no common sequences and ARF has a different target as it regulates cell cycle progression via the p53 ubiquitinylation/degradation pathway [reviewed in Sharpless et al., 1999]. The analogous deletion events are even more common in mice: it is a well-known fact that murine cells lines become easily immortalized which is a consequence of losing the *Cdkn2a/b/ifna/b* region in the middle of chromosome 4.

Figure 3A summarizes tumor breakpoints which map within the *IFN* gene cluster: Among these, the A172 glioma breakpoint directly flanks a S/MAR 3′ to *IFNP11*, and the A1235 glioma breakpoint is found adjacent to the *IFNB1* 5′S/MAR. Interestingly, the A172 breakpoint junction is the result of a recombination event between the S/MAR and a LINE, which may have been mediated by AT-tracts [Pomykala et al., 1994; Strissel et al., 1998a, b]. The A1235 breakpoint resulted from a complex inversion/deletion event involving *IFNB1* [Strissel et al., 1998b]. For one of the breakpoint junction regions, mapping 300 kbp away, a S/MAR was found suggesting a S/MAR recombination event [Strissel et al., 1998b]. At least seven more breakpoints points (o-u in Fig. 3A) cluster in the 50 kbp region between *IFNB1* and *IFNW1* as determined by Southern analysis but we have to await cloning of the deletion breakpoint junctions to describe their precise correlation with the *IFNB1* 5′ bordering element.

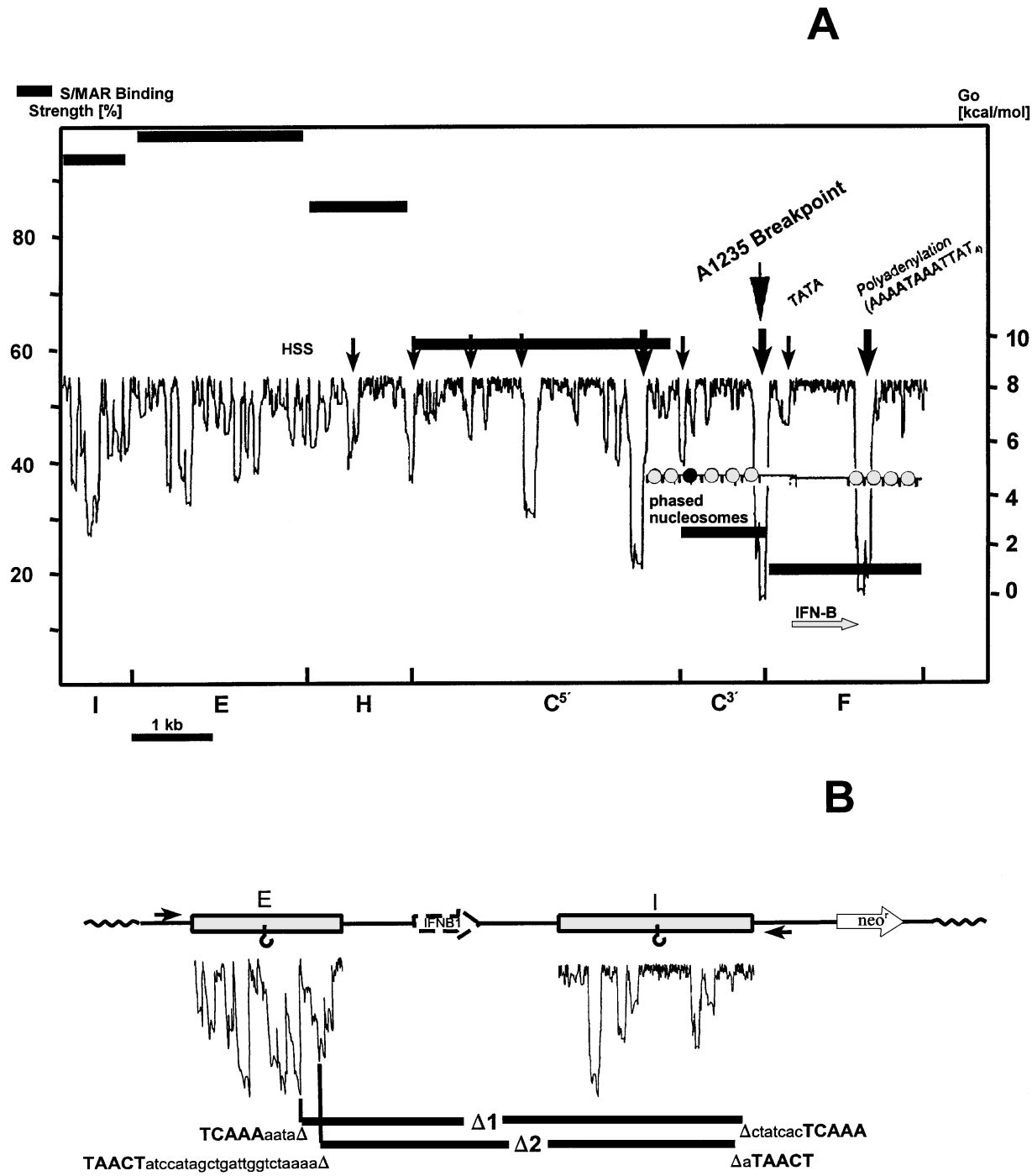


Fig. 3. High-resolution stress-induced-duplex-destabilization profile (SIDD-Maps) of *IFNB1*-associated S/MAR elements. A stabilization energy of 0 kcal/mol indicates that bases get apart at the respective superhelical density. (**A**) SIDD-map of the *IFNB1* upstream region (*Eco*RI-fragments F and $C^{3'}$) and its associated S/MAR (fragments $C^{5'}$, H, E and I; the position of fragment ''E'' is also indicated in Fig. 2B). S/MAR binding strength is indicated by the position of black vertical bars above the respective restriction fragment. DNAseI hypersensitive sites and HS^{TOPII} are shown (arrows); the most prominent destabilized site within fragment $C^{3'}$ correlates with the A1235 deletion breakpoint. (**B**): An artificial minidomain, constructed from the center regions of *IFNB1* upstream and downstream S/MARs. After transfer and stable anchoring of the construct in a host cell, deletions can be monitored by the preferential amplification of the resulting fragments by PCR. Deletions occur in association with S/MARs but not adjacent to the transcription unit (see text). Courtesy of Michaela Iber, with permission.

Figure 3B describes the results of a high resolution structural analysis for the upstream border of the *IFNB1* chromatin domain, and summarizes work presented in several previous publications [reviewed in Bode et al., 1995, 1996, 1998, 2000]. The maximum S/MAR potential is seen to reside in the *Eco*RI fragments I, E, and H, and the S/MAR border is found between fragments $C^{5'}$ and $C^{3'}$ where there is a drop in S/MAR strength. We have shown previously that S/MAR activity is tightly associated with the presence of regularly spaced, closely apposed minima in a SIDD profile [Benham et al., 1997; Bode et al., 1998, 2000]. We note that, although the pronounced but isolated SIDD minima in fragments $C^{5'}$, $C^{3'}$ and F do not constitute S/MARs per se (note the weak binding potential of fragments $C^{3'}$ and F), they do coincide with DNAse I hypersensitive sites. The SIDD minimum in $C^{3'}$ not only represents an in vivo TOPOII recognition site (i.e., a HS^{TOPII} site in the above nomenclature) but it also coincides with the A1235 breakpoint, which could be localized with nucleotide resolution [Pomykala et al., 1994]. Therefore, these deletion events are in full agreement with the conclusions derived from the *MLL* translocations: breakpoints are preferentially found at S/MAR borders, which are frequently marked by a constitutive DNAseI hypersensitive site [review: Bode, 1995]. These sites are well suited to accommodate superhelical stress by undergoing transitions to alternate structures that could create TOPO II recognition features. These results also suggest the value of SIDD-type analyses, not only for localization of domain borders but also for localizing regulatory elements within domains which, by virtue of their strand separation potential, represent potential substrates for cutting and rejoining activities.

The predictions of this model have been tested by a construct in which an *IFN*-minidomain has been composed from major portions of the *IFNB1* upstream and downstream S/MARs [Klehr et al., 1991; Benham et al., 1997]. In this context, both the inducible expression of the *IFNB1* gene and the constitutive expression of a reporter gene are augmented by one to two orders of magnitude. PCR primers have been positioned at the extreme termini of this minidomain in order to monitor deletion events as they occur during long-term cultivation. This system generated two major deletion patterns which initiated at a destabilized site in the upstream S/MAR element "E" and terminated at the 3′ flank of the downstream S/MAR element "I". These deletions were clean in the sense that no filler DNA was added, but they were characterized by short 5 bp direct repeats at the respective termini which typically occur in IR events. While these results are compatible with the postulated indirect role of S/MARs for the selection of breakpoints, they also show that the process may profit from sequence repetitions that could stabilize a recombination intermediate. The whole spectrum of requirements will be more fully understood once such a model domain has been supplemented by HS^{TOPII} sites and other elements that have been implicated in translocation and deletion events, as described throughout this article.

THE FUTURE

The forces driving IR have been reviewed several times. These reviews have focused either on the principles underlying random integration [cf. Roth and Wilson, 1988], or on the forces that govern chromosomal rearrangement [cf. Meuth, 1989]. It is generally agreed that multiple mechanisms are involved. This provokes the question of whether "there is any order to the chaos" [Meuth, 1989]. In the postgenomic era more and more sequence information is becoming available. This, together with powerful techniques to determine the localization and nature of nuclear compartments, will shed increasing light on the mechanisms of translocation, integration, and recombination, and give a sense of the multiplicity of principles that are actually involved.

The model developed here was initially based on existing evidence for the involvement of structures that compartmentalize the genome through interactions with the nuclear skeleton [Sperry et al., 1989]. Information regarding the nature of a variety of translocation and deletion events showed that the original version of this model required further refinement. All these analyses agree in that strong nuclear matrix-S/MAR contacts protect the attached DNA region from breakage, but are at the same time involved in aligning chromosomes that participate in IR. This has focused attention on S/MAR-associated DNA structural features and on elements within small chromatin loops that would respond to the topological changes associated with transcription or replication. Today we are

in the position to predict both, S/MAR activities and the localization of associated hypersensitive sites, by computer-assisted sequence analyses. These procedures will enable the design of specific experiments to further elucidate the relative role of the structural elements involved in translocation and deletion.

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