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DNA Loop Anchorage Region Colocalizes With the Replication Origin Located Downstream to the Human Gene Encoding Lamin B2

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Abstract The recently developed procedure of topoisomerase II-mediated DNA loop excision has been used to analyze the topological organization of a human genome fragment containing the gene encoding lamin B2 and the ppv1 gene. A 3.5 kb long DNA loop anchorage/topoisomerase II cleavage region was found within the area under study. This region includes the end of the lamin B2 coding unit and an intergenic region where an origin of DNA replication was previously found. These observations further corroborate the hypothesis that DNA replication origins are located at or close to DNA loop anchorage regions. J. Cell. Biochem. 69:13–18, 1998. © 1998 Wiley-Liss, Inc.

Key words: nuclear matrix; replication origin; topoisomerase II-mediated DNA loop excision; DNA loop anchorage sites

The problem of spatial organization of replication units in cells of higher eukaryotes has been intensively studied over the past 15 years [reviewed by Van der Velden and Wanka, 1987; Razin, 1987; Dijkwell and Hamlin, 1995; Razin et al., 1995]. It was first reported by Buongiorno-Nardelli et al. [1982] that the average size of supercoiled DNA loop-domains in different eukaryotic cells correlated directly with the average size of replicons in these cells. This observation actually suggested that individual loops corresponded to replicons. It is obvious that this may happen only if a specific element of each replicon (and there are only two such elements, namely a replication origin and a termination site) is attached to the nuclear matrix. Analysis of DNA label distribution within DNA loops in pulse-chase experiments performed with synchronized cells made it possible to suppose replication origins to be permanently bound to the nuclear matrix [Aelen et al., 1983; Carry et al., 1986; Dijkwell et al., 1986]. In agreement with this supposition it has been found recently that cloned nuclear matrix DNA fragments from cells in G0 and G1 phases replicate at the very beginning of S phase and hence may be presumed to reside in proximity to replication origins [Brylawsky et al., 1993]. Independent evidence supporting the supposition that replication origins are permanently bound to the nuclear matrix was obtained in experiments on mapping the replication origin in the domain of chicken α-globin genes [Razin et al., 1986]. The origin was found within a 3 kb long DNA fragment of the upstream part of the domain which had been shown previously to be permanently attached to the nuclear matrix. Furthermore, the comparison in corenaturation experiments of unique DNA sequences surrounding the replication origins and permanent sites of DNA attachment to the nuclear matrix demonstrated that at least in chicken erythroid cells all origins were permanently attached to the nuclear matrix, i.e., were located at the DNA loop anchorage sites or very close to them [Razin et al., 1986].

Recently we have elaborated a novel approach for mapping DNA loop anchorage sites by topoisomerase II-mediated DNA loop exci-

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sion [Razin et al., 1993; Gromova et al., 1995; Iarovaia et al., 1996). In the present study this approach has been used for mapping the DNA loop anchorage region in the vicinity of the human gene encoding lamin B2. The loop anchorage region has been found to include the replication origin previously mapped in close proximity to the end of the lamin B2 coding unit. Hence, our data further corroborate the supposition that replication origins are located at (or close to) the basements of DNA loop-domains.

MATERIALS AND METHODS

Cell Culture

Human cells, line HL 60, were cultivated in RPMI 1640 medium supplemented with 10% foetal bovine serum and antibiotics.

Preparation of Agarose Blocks With Living Cells

The cells were collected by centrifugation and washed with PBS buffer. 10^7 cells were resuspended in 50 ml of PBS buffer and mixed with an equal volume of molten 1.5% low-melting agarose prepared in PBS buffer. The suspension was placed into a plug mold former and left at 0°C for 5 min.

Treatment of Living Cells With Dimethylepipodophyllotoxinthenylidene-β-D-glucoside (VM 26)

The agarose blocks with immobilized cells were incubated for 40 min at 37°C in RPMI medium supplemented with VM 26 (from 0 to 100 mg/ml, as specified in the figure legends). After incubation the agarose blocks were placed into the "stop-solution" (1% SDS, 0.4 M Na-EDTA (pH 8.0), 0.5 mg/ml proteinase K) and the digestion was carried out for 36 h at 55°C.

Extraction of Permeabilized Cells With 2M NaCl and Treatment With VM26

The agarose blocks with embedded cells were incubated in a buffer containing 0.5% Nonidet P40 (NP 40), 2M NaCl, 2 mM Na-EDTA, 1 mM phenylmethanesulphonylfluoride (PMSF), and 20 mM Tris-HCl (pH 7.5). The incubation was carried out for 1 h at 4°C with gentle agitation. The blocks were then washed three times (30 min each) at 4°C in a buffer containing 20 mM Tris-HCl (pH 7.5), 0.1 mM Na-EDTA, 50 mM KCl, and 10 mM $MgCl_2$. The blocks were then placed in the same solution supplemented with VM 26 and were incubated for 40 min at 37°C. After incubation the blocks were placed in the stop-buffer and protein digestion was carried out as described above.

Treatment of Purified DNA With DNA-Topoisomerase II

Two µg of DNA were incubated with 500 ng of purified topoisomerase II for 15 min at 37°C in 150 ml of reaction mixture containing 20 mM Tris-HCl (pH 7.6), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 5% glicerol, and 0–15 mg/ml of VM-26, as specified in the legend to Figure 3. The reaction was terminated by addition of SDS up to 1% and EDTA up to 100 mM. This was followed by digestion of proteins with proteinase K (0.5 mg/ml) for 5 h at 55°C and purification of DNA by phenol-detergent extraction procedure.

Electrophoresis, Digestion of DNA With Restriction Endonucleases, Southern Transfer, and Hybridization

Pulsed field gel electrophoresis (PFGE) in 1% low melting agarose was carried out in a Bio-Rad CHEF DRIII system in 0.5X TBE buffer at 14°C for 14–22 h at voltage gradient 6 V/cm with the switch time ramped linearly from 30 to 90 s. Concatemers of lambda phage DNA were used as markers. DNA fragments ranging in size from 50 to 150 kb were cut from the gel. After digestion of DNA with restriction endonucleases, which was carried out directly in a low-melting agarose, the samples were loaded on 0.8% agarose gel and the second separation of DNA by conventional agarose gel electrophoresis was carried out in order to resolve fragments ranging in size from 0.5 to 25 kb. Alkaline transfer of DNA to Hybond N+ membranes (Amersham) was carried out as described in the manufacturer's manual. Prehybridization (1 h) and hybridization (5 h) were carried out at 60°C in a RapidHyb solution (Amersham). After hybridization the filters were washed two times (30 min each) at 60°C in 2X SSC-0.5% SDS solution and one time (30 min) at 60°C in 0.1X SSC-0.5% SDS solution. The filters were then exposed to the Kodak RX film at -70°C with an intensifying screen (Dupont).

PCR Amplification

PCR amplification of SB12 probe was performed as described by Biamonti et al. [1992].

RESULTS AND DISCUSSION

Excision of DNA Loop-Domains From the Genome of Human HL60 Cells by Topoisomerase II Mediated DNA Cleavage at Matrix Attachment Sites

In order to check whether the topoisomerase II-mediated DNA loop excision protocol may be applied for mapping the DNA loop organization in HL60 cells a series of preliminary experiments was carried out. One can see (Fig. 1) that incubation of high-salt-extracted cells in a medium containing VM-26 results in the excision of large DNA fragments ranging in size from 50 to more than 700 kb. No such fragments were released in the control experiment when VM-26 was not present in the incubation medium. In this experiment all DNA remained at the start (note that the ~700 kb band seen in lanes 2–6 is a compression artefact and it may contain fragments considerably larger than 700 kb). It is evident that the size distribution of the released fragments depends on the concentration of VM-26 in the incubation medium. At higher VM-26 concentrations, the sizes of the released fragments were relatively smaller. In order to verify the specificity of DNA cleavage at the loop basements, the Southern analysis was carried out. After transfer to a nylon membrane, the DNA fragments were hybridized to a probe representing the coding sequences for 28S rRNA. A typical result is shown in Figure 1, lane 6. It is evident that topoisomerase II cleaves the nucleolar genes into bands with the size divisible by the size of rDNA repeat in human cells (45 kb). As has been shown previously [Razin et al., 1993] this cleavage pattern originates as a result of topoisomerase II-mediated DNA cleavage within the loop anchorage sites in the nontranscribed spacers. Although the size of nucleolar DNA loops is equal to the size of a repeated unit, only a portion of anchorage sites is cleaved under our experimental conditions. That is why most of the bands seen in Figure 1, lane 6 represent oligomers of DNA loops.

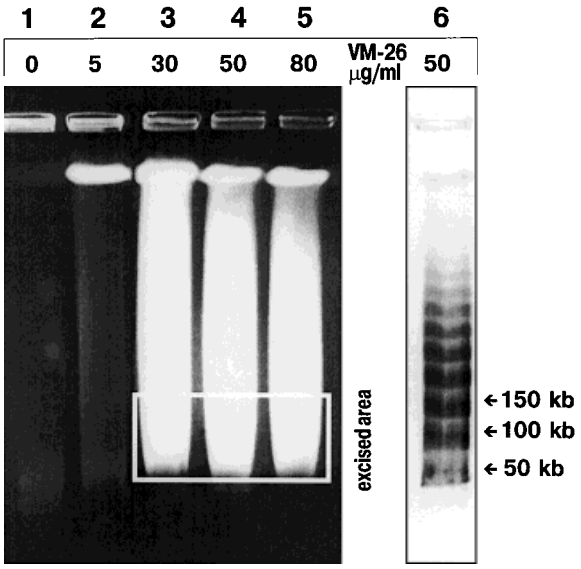

Fig. 1. Separation of excised DNA loops by Pulsed Field Gel Electrophoresis. **Lanes 1–5:** Electrophoretic separation of large DNA fragments excised from the genome of human HL60 cells as a result of inhibition of endogenous topoisomerase II (ethidium bromide staining). The concentration of VM-26 used in each case is indicated above the lanes. The white rectangle indicates the area which was cut from the gel for further analysis. **Lane 6:** The result of hybridization of the 28S rRNA probe to the material transferred to a nylon filter after electrophoretical separation of DNA sample identical to this shown in slot 4 (autoradiograph). The arrows to the right of lane 6 show the positions of molecular weight markers (phage l DNA concatemers).

Mapping of a DNA Loop Anchorage/Topoisomerase II Cleavage Region Downstream to the Gene Encoding Lamin B2

It was found previously that a short intergenic region located between the gene encoding lamin B2 and the ppv1 gene contained an origin of DNA replication [Biamonti et al., 1992; Giacca et al., 1994]. With the aim to find out if this region is anchored to the nuclear matrix we checked whether the ends of DNA fragments excised from the genome by topoisomerase II-mediated DNA cleavage could be mapped within this region. In order to enrich the DNA samples in the ends of individual loops, the products of topoisomerase II-mediated cleavage ranging in size from 50 to 150 kb (Fig. 1, lanes 3–5, white rectangle) were cut from the gel after PFGE, and DNA was eluted. This DNA was then additionally digested with the Bam HI restriction enzyme. After digestion, the fragments were separated by conventional agarose gel electrophoresis and transferred to a nylon filter. The design of the indirect end-labeling experiment is shown in Figure 2A. The probe SB12 complementary to the end of the 12 kb Bam HI fragment was described previously [Giacca et al., 1994]. We analyzed in parallel the products of topoisomerase II-mediated DNA cleavage carried out in vivo (in living cells) and in high salt extracted nuclei. In both cases the topoisomer-

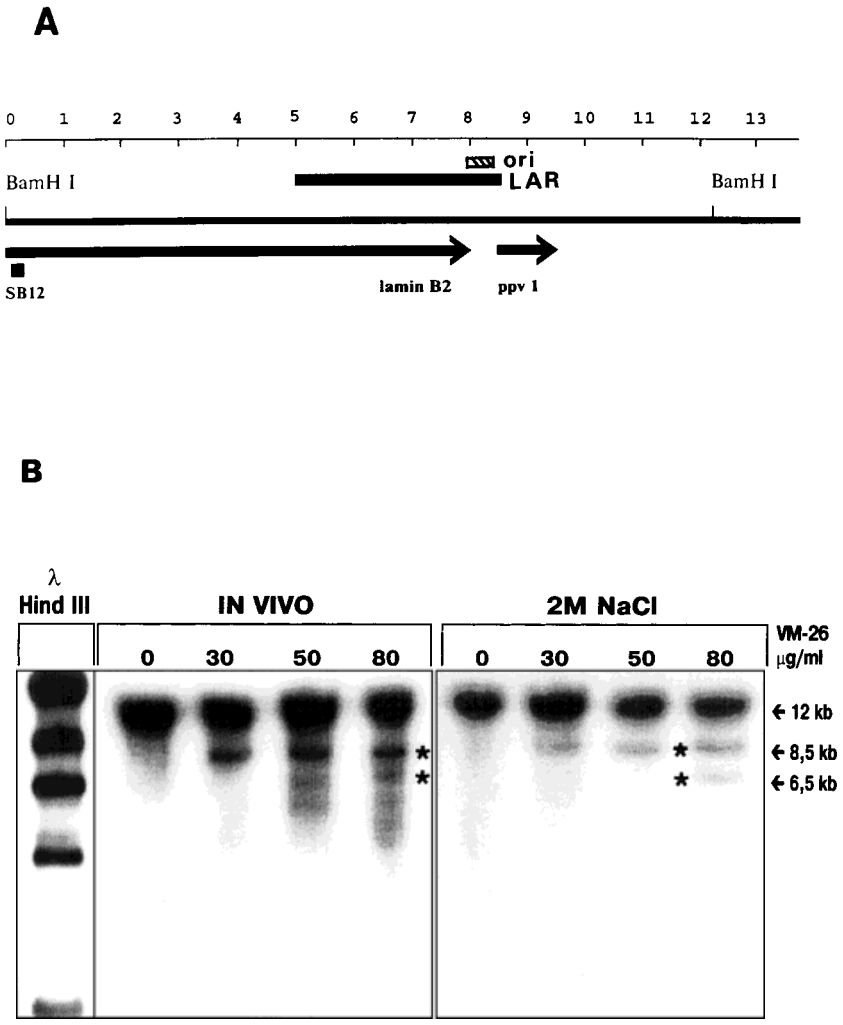

Fig. 2. Mapping of sites of preferential DNA cleavage by nuclear matrix-bound topoisomerase II within the 12.2 kb BamHI fragment including the intergenic spacer between the gene encoding lamin B2 and the ppv1 gene. **A:** A map of the area under study. The scale is in kb. The gene encoding lamin B2 and the ppv1 gene are shown by horizontal arrows (which indicate also the direction of transcription). The position of the probe SB12 is shown by the black box below the map. The topoisomerase II cleavage/DNA loop anchorage region (LAR) mapped in the present work is shown by the black bar above the map. The position of the replication origin (ori) is shown by hatched rectangle above the map. **B:** Indirect end labeling of the products of DNA cleavage by nuclear matrix-bound topoisomerase II. DNA samples from cells or high salt-extracted nuclei incubated with VM-26 were additionally digested with BamHI, separated by conventional agarose gel electrophoresis, transferred to a nylon filter and hybridized with the probe SB12. The concentrations of VM-26 used to stimulate the topoisomerase II-mediated DNA cleavage are indicated above the lanes. Additional bands generated by DNA cleavage with endogenous topoisomerase II are marked by asterisks. The molecular weights of the two most prominent fragments generated by topoisomerase II in high salt-extracted nuclei are indicated by arrows at the right side of the photo. The separation of molecular weight markers (fragments of λ phage DNA obtained by digestion with Hind III restriction endonuclease) is shown at the left side of the autoradiograph.

ase II cleavage sites were present within the area under study, as followed from the patterns of DNA fragments recognized by the SB12 probe. Indeed, in the samples taken both from cells and high salt-extracted nuclei incubated in a medium containing VM-26 this probe recognized the full sized 12 kb BamHI-BamHI fragment and several additional bands. The latter clearly represented the products of topoisomerase II-mediated DNA cleavage as they were not present in the control lanes loaded with DNA from cells untreated with VM-26. In agreement with our previous observations [Gromova et al., 1995] the patterns of topoisomerase II-mediated DNA cleavage in living cells and in high salt extracted nuclei were not exactly the same.

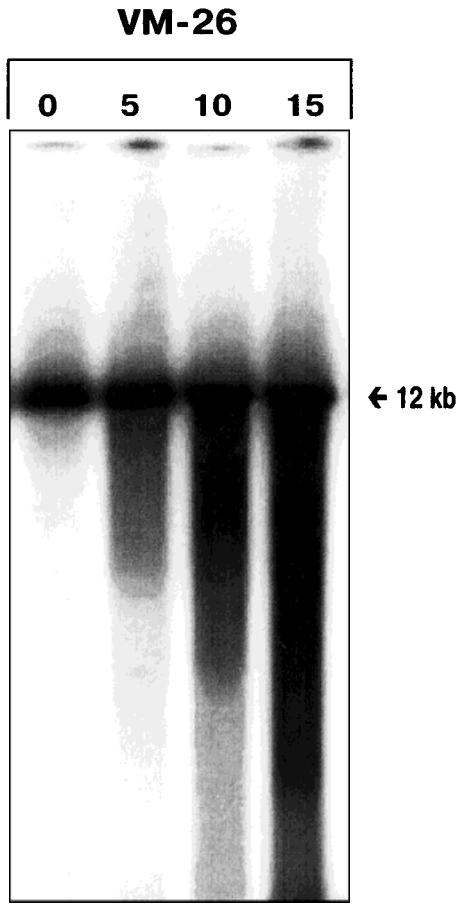

Fig. 3. Analysis of specificity of cleavage of the DNA segment under study upon incubation of naked DNA with purified topoisomerase II in presence of 0, 5, 10, or 15 mg/ml of VM-26. After cleavage by topoisomerase II all samples were digested with Bam HI, separated by agarose gel electrophoresis, transferred to nylon filter and hybridized with ^{32}P-labelled SB12 probe. Note absence of any specific bands with the exception of full-sized 12 kb Bam HI fragment recognized by SB12 probe.

However, the main cleavage product of 8.5 kb was detected in both cases. In high salt extracted nuclei only one more prominent cleavage product of 6.5 kb was detected. In living cells cleavages occurred within an area located between 5 and 8.5 kb from the left end of the full-sized BamHI fragment recognized by the SB12 probe. It is important that no specific bands (with the exeption of the ful-sized BAM HI fragment) was observed within the area under study when free DNA was cleaved by topoisomerase II in solution, then digested with BamHI and subjected to Southern analysis with the SB12 probe (Fig. 3). Hence, the specific bands identified in experiments with living cells or high salt-extracted nuclei could not originate due to the limited sequence specificity of free DNA cleavage by topoisomerase II. Taken together the above data indicate that an approximately 3.5 kb long DNA loop anchorage/topoisomerase II cleavage region is located within the 12 kb BamHI fragment under study at a distance of 5 kb from the left end of the fragment. This region includes the previously mapped replication origin (Fig. 2A).

CONCLUSIONS

It should be stressed that in spite of all data mentioned in Introduction the spatial organization of replication units in cells of higher eukaryotes still constitutes a matter of discussion because contradictory data are also available [reviewed by Dijkwel and Hamlin, 1995]. The above contradictions were partially due to the absence of a reliable procedure for mapping DNA loop anchorage sites [reviewed by Razin et al., 1995]. Many scientists still consider so-called Matrix Attachment Regions (MARs) as sites of DNA loop anchorage to the nuclear matrix. Although some MARs colocalize with replication origins and even seem to constitute an essential part of origins at least in yeast cells [Amati and Gasser 1988, 1990], MARs can be found also close to replication termination sites [for example in DHFR amplicon see Dijkwel and Hamlin, 1995] as well as iside replicons. In frames of the present discussion it is important to stress that there is no experimental evidence indicating that all MARs are involved in the DNA loop anchorage to the nuclear matrix. Our recent data suggest that at best MARs may constitute potential attachment sites [Iarovaia et al., 1996]. In contrast, there are good reasons to beleive that topoisomerase II-mediated DNA loop excision approach permits to identify the DNA loop anchorage regions (LARs) pre-existing in living cells [Razin et al., 1993; Gromova et al., 1995]. The present study demonstrating a colocalization of a replication origin with LAR in the intergenic spacer between the gene encoding lamin B2 and the ppv1 gene further supports the conclusion that replication origins are located at DNA loop anchorage sites.

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