Vol. 213, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 4, 1995 Pages 282-288

TOPOISOMERASE-II-MEDIATED DNA CLEAVAGE WITHIN THE HUMAN RIBOSOMAL GENES

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Received June 24, 1995

SUMMARY: The interaction of topoisomerase II (topo II) with human ribosomal DNA (rDNA) was investigated *in vivo* using the antitumoral drug VM26, a specific inhibitor of topo II, that stabilizes the transient cleavable complex. rDNA-protein complexes isolated from nucleoli of TG cells were analyzed for double strand breaks with probes that covered almost all intergenic transcribed spacer (IGS) and all transcribed sequences of tandem repeat ribosomal DNA genes. Preferential cleavage sites were present in only a part of nucleolar rDNA, i.e., the transcribed region. Proteins, purified from the same complexes, were analyzed by Western-blot and stained by an antiserum against both topo II forms, showing the presence of topo IIβ.

In the regulation of the higher order structure of chromatin and chromosomes, a number of non-histone proteins are implicated, of these one well defined is topoisomerase II (topo II), an enzyme that catalyzes strand-passing of double stranded DNA in an ATP-dependent mechanism (reviewed in 1). Topo II changes DNA topology forming a transient DNA-protein complex with a monomer of topo II molecule covalently linked to the 5' end of each strand (2).

Topo II plays an important structural role as component of nuclear matrix or chromosome scaffolds (3, 4,) and its activity is necessary during replication for resolution of intertwined strands of neosynthetized DNA (5). Few data are available on the involvement of topo II in the control of transcription-dependent torsional stress (6). An analysis of topo II-mediated DNA cleavage of the c-*myc* gene in tumor cells indicates the presence of DNA double-strand breaks at specific positions corresponding to important regulatory region of the gene (7) and in *hsp*70 genes specific cleavage sites occurred at both 3' and 5' ends (8).

Two distinct forms of topo II, α and ß, recently identified in mammalian cells, have a monomer molecular weight of 170 kDa and 180 kDa respectively and differ in their catalytic properties. The two

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0006-291X/95 $12.00

forms are independently regulated: the level of the topo IIß is approximately constant during the cell cycle, whereas the α form is expressed predominantly in the late S and G_2/M phases (9, 10). It has been reported that the α form is localized primarily in the nucleoplasm, while the β form is present mainly in the nucleolus (11).

On the basis of the different properties and localization, the α and ß forms may have different functions in DNA and RNA synthesis. In TG human tumor cells the inhibition of enzyme activity by VM26 reduced [^{3}H]uridine incorporation into ribosomal RNA and caused ribosomal DNA fragmentation, thus suggesting an involvement of topo II in the transcription of ribosomal genes, as confirmed at morphological level by the segregation of nucleolar components (12). In view of these results we have mapped topo II cleavage activity *in vivo* on transcribed sequences and non-transcribed spacer of ribosomal DNA and we have investigated which form of enzyme is localized on ribosomal DNA of TG cells treated with VM26, the specific inhibitor of topo II activities that increases potential cleavage sites of DNA by stabilizing the DNA-enzyme complex (13).

MATERIALS AND METHODS

Human TG cells were grown in RPMI-1640 medium, supplemented with non-essential amino acids, 100 U/ml penicillin, 100 ml/ml streptomycin and 10% fetal calf serum. VM26 was added to the culture medium at 2.5 μM final concentration for 1 or 3 hours before harvesting.

To analyze the VM26-induced formation of stable ribosomal DNA-protein complexes, we have isolated nucleoli as below described. TG cells were homogenized in 0.25 M sucrose containing 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$ and 0.5% Triton X-100, then nuclei were sedimented by centrifugation at 1500×g for 10 minutes. After two washes with the same homogenization buffer without detergent, the nuclei were resuspended in 0.25 M sucrose, 10 mM Tris-HCl (pH 7.5) and 5 mM $MgCl_2$ and sonicated for 10 min (15 sec of sonication followed by 15 sec of pause) with 70 watts of power. The nuclei disrupted by sonication were layered on 0.88 M sucrose, 10 mM Tris-HCl (pH 7.5) and 5 mM $MgCl_2$ and nucleoli were pelletted at 400×g for 20 min. Purification of nucleolar DNA-protein complexes was carried out following the methods described by Udvardy and Schedl (14); DNA from complexes did not hybridize with pF0422 probe, complementary to H2A, H2B and H3 histone DNA (15), indicating the absence of extranucleolar material (data not reported).

To analyze the nucleolar rDNA in the pellets a part of DNA-protein complexes was phenol extracted and ethanol precipitated. 5 μg of DNA digested sequentially by RNase A (100 μg/ml) and *Eco* RI restriction enzyme were electrophoresed and transferred to a nylon membrane. After hybridization with ^{32}P-labeled probes, filters were washed in stringent conditions with 0.1×SSC (1×SSC: 0.15 M NaCl, 17.5 mM sodium citrate) and hybrids were revealed by autoradiography.

After reduction and denaturation with SDS, proteins complexed to nucleolar DNA were separated on a 10% acrylamide gel and electroblotted to nitrocellulose. The blot was reacted with a rabbit serum that recognizes both the α and the β forms of human topo II (MAC) (16).

RESULTS

In Fig. 1, a schematic map is reported of the probes used to analyze the entire transcribed sequences and almost all of IGS spacer of human rDNA, because in a previous study a prominent site of cleavage has been localized in the 3' region of the transcribed sequence in rDNA of TG cells after VM26 incubation (12).

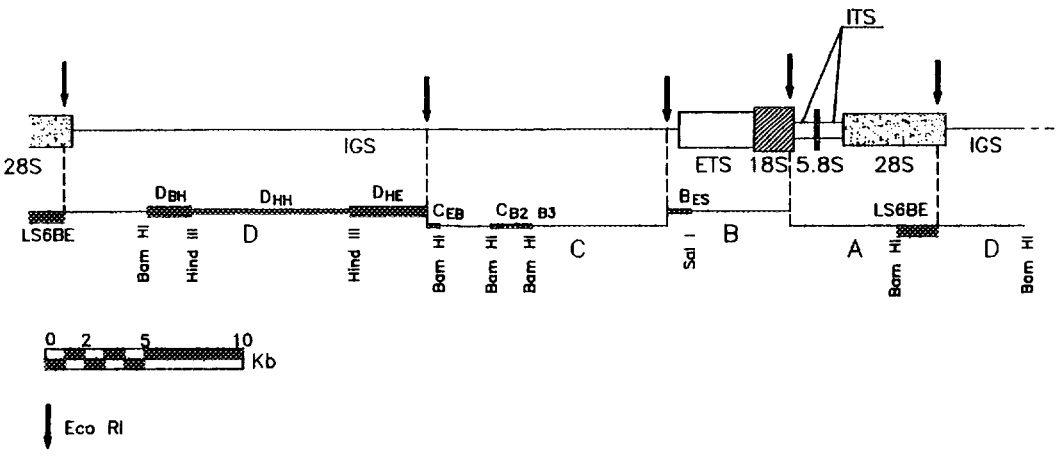

Fig.1. Restriction map of human DNA ribosomal genes. IGS, intergenic spacer; ETS, external transcribed spacer; 18S, coding region for the 18S RNA; ITS, internal transcribed spacer; 5.8S, coding region for the 5.8S RNA; 28S, coding region for the 28S RNA. *Eco* RI restriction sites define rDNA in four zones: A, B, C and D as described by La Volpe et al.(17). The subcloned rDNA sequences used as radioactive probes are also shown. Three ^{32}P-labeled *Eco* RI fragments of the rDNA repeating unit (A, B, and C) and one or more of subcloned sequences are utilized to investigate topo II cleavage sites on ribosomal genes (17, 18): LS6BE, a 1.70 kb *Bam* HI-*Eco* RI fragment covering the 3' portion of 28S gene, provided by G. Ranzani of Pavia University (Italy) (19); B_{ES}, a 1.20 kb *Eco* RI-*Sal* I fragment covering the 5' portion of B zone described by Labella and Schlessinger (20), C_{EB} and C_{B2B3}, 0.53 kb *Eco* RI-*Bam* HI and 2.00 kb *Bam* HI-*Bam* HI,respectively, derived from C zone; D_{BH}, D_{HH} and D_{HE}, 2.15 kb *Bam* HI-*Hind* III, 8.00 kb *Hind* III-*Hind* III and 2.00 kb *Hind* III-*Eco* RI fragments in order, obtained from D zone (21).

Transcribed region: In Fig.2a, the cleavage sites on rDNA are mapped with the A probe revealing in all samples both the 7.20 kb *Eco* RI fragment, sequence complementary to itself, and a pattern of minor bands derived from the *in vivo* fragmentation of A region. In the VM26 treated samples, the bands of lower molecular weights are more intense, in a time dependent way. The hybridization of the same samples with LS6BE, a subcloned fragment of A, shows much more clearly the cleavage effect, revealing numerous discrete bands of different length with an intensity increased in relation to VM26-treatment time, e.g. the 1.10 band in the 3 hrs VM26 treated sample. The B probe, complementary to remaining transcribed region of rDNA unit, recognizes the 5.80 kb fragment *Eco* RI and shows a diffuse pattern of fragments that is much more evident in the DNA samples from VM26 treated TG cells (Fig.2b). By the use of the subcloned probe B_{ES} a series of sharply defined bands can be detected (Fig.2b). These results are indicative that the breaks present in the rDNA are due to preferential cleavage sites for topo II activity in the transcribed portion of ribosomal genes.

IGS region: Very different results are been obtained mapping the non-transcribed zones by C and D probes (Fig.3). With C and the two subcloned probes used (Fig.3a), no fragmentation or difference can be detected in control and VM26 treated cells. In the autoradiography with the entire C probe smears

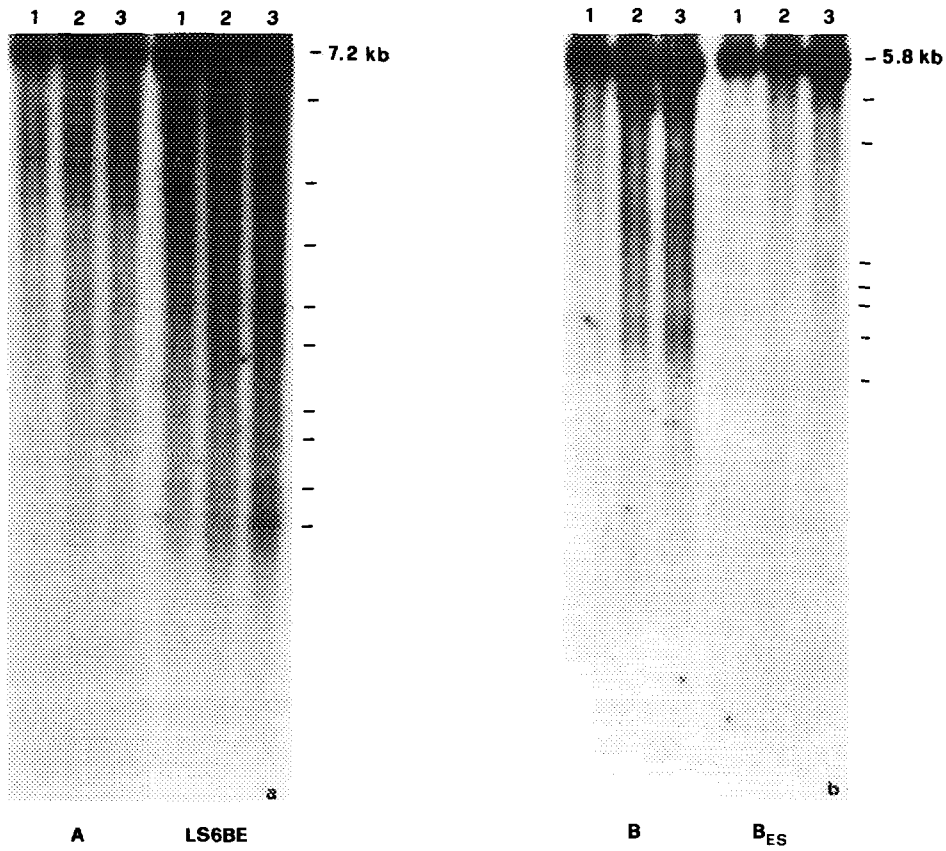

Fig.2. Cleavage sites induced *in vivo* by VM26 within ribosomal genes of TG cells. (a) Hybridization pattern obtained with A probe and its subcloned fragment LS6BE (see Fig. 1). (b) Hybridization pattern obtained with B probe and its subcloned fragment B_{ES}.
1: control cells; 2: VM26 treated TG cells (2.5 μM for 60 min); 3: VM26 treated cells (2.5 μM for 180 min). On the right the length of the *Eco* RI genomic fragments is reported and the main fragmentation bands are pointed out.

are present in all DNA samples, probably due to the combination of repeated sequences (22, 23) and length probe effect.

When mapped with probes D_{BH}, D_{HH} and D_{HE},, a considerable diffuse smear is present in DNA from drug-treated and untreated cells, again due to repeated sequences (Fig.3b) (24, 25). We can not exclude that very faint bands of fragmentation are covered by the smears.

If DNA cleavage sites are caused by topo II activity, the enzyme should copurify complexed to nucleolar rDNA. Proteins from these complexes are analyzed by Western immunoblotting with the MAC antiserum directed against human topo IIα and ß forms (16) (Fig.4). The antiserum reacts in all the samples with an high molecular weight protein having an electrophoretic mobility consistent with a 180 kDa molecular mass and a more evident band is present in samples from VM26 treated cells. A silver stained gel has shown that precipitable complexes have a similar pattern of proteins in the range of

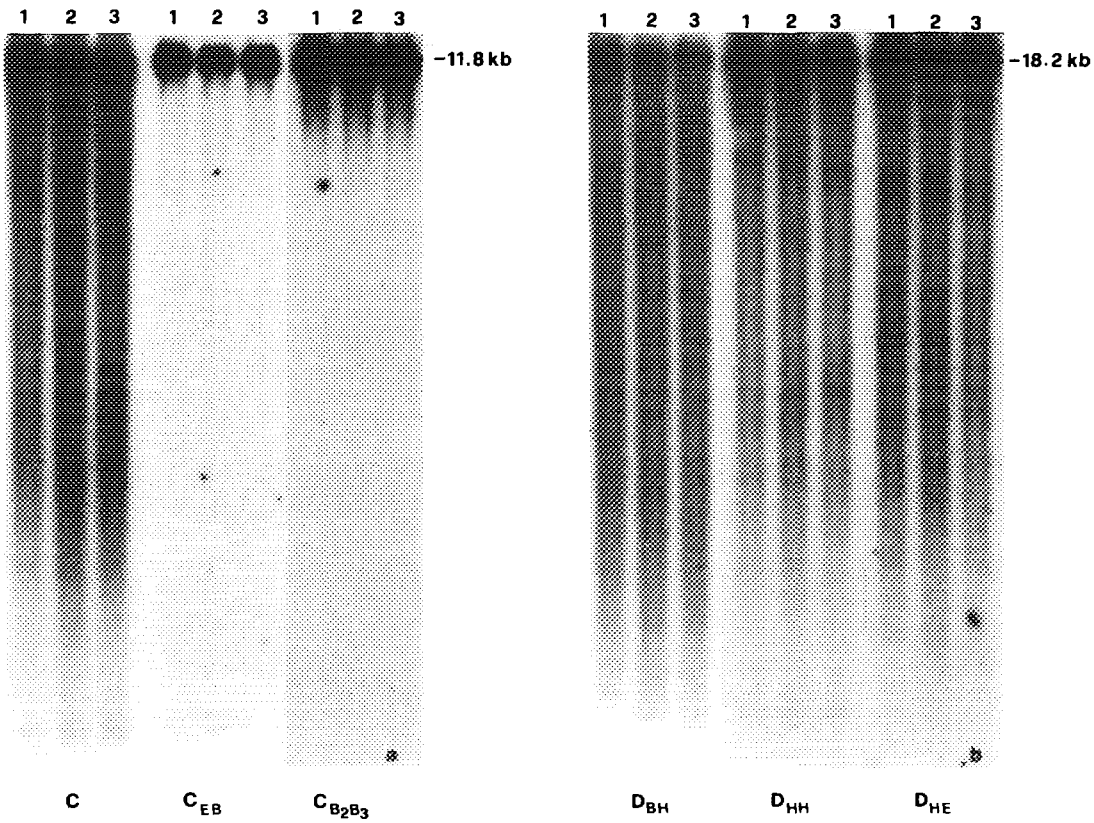

Fig.3. Probing of IGS region of ribosomal genes in TG cells treated with VM26. (a) Nucleolar DNA hybridized with C, and C_{EB}, C_{B2B3} subclones. (b) Nucleolar DNA hybridized with D_{BH}, D_{HH}, D_{HE} subcloned fragments of D (see Fig. 1 for details).
1: control cells; 2: TG cellsi ncubated with VM26 for 60 min.; 3: TG cells incubated with VM26 for 180 min. On the right the length of the *Eco* RI genomic fragments is reported.

90 and 200 kDa molecular weight (data not shown). The detectable 180 kDa band is the proof for the presence of topo IIß molecules on nucleolar DNA-protein complexes isolated from nucleoli.

DISCUSSION

The fragmentation map of rDNA purified from nucleolar DNA-protein complexes shows bands of different lengths, indicative for the presence of topo II cleavage sites. Although evident on rDNA genes of control TG cells, the same pattern of fragmentation is increased in TG cells exposed to VM26, due to the inhibitory trapping mechanism of the topo II by the drug.

Mapping of topo II cleavage sites has revealed cleavage points in the transcribed part of rDNA genes and in particular, using VM26, a significant increase on 3' end of the gene, while in the IGS spacer it is not possible to detect any cleavage effect. At present we can not explain the meaning of these topo II preferential cleavage sites in ribosomal DNA, but similar effect has been found on the 3' end of *hsp* 70 gene upon induction of heat shock transcription (8).

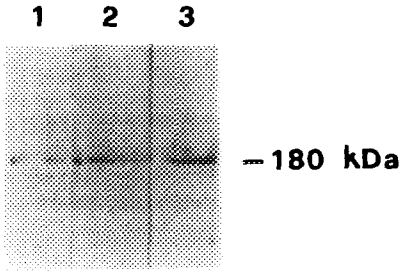

Fig.4. Copurification of topo IIß with nucleolar DNA isolated from control and VM26-treated TG cells. Proteins from nucleolar DNA-proteins complexes have been electrophoresed, blotted to nitrocellulose filter, and probed with a rabbit serum (MAC antiserum) that recognizes both human topo II forms (16). The molecular weight of 180 kDa, corresponding to the topo IIβ monomer, is indicated. Lane: 1, nucleolar proteins from TG control cells; 2 and 3, proteins from TG cells incubated with 2.5 μM VM26 for 60 and 180 min, respectively.

The preferential cleavage of transcribed region by topo II may be functionally related to ribosomal DNA activities. Previously we have found a relationship between topo II activity and ribosomal DNA transcription, in fact a reduced rRNA synthesis and a segregation of nucleolar components in VM26 treated TG cells have been reported (12). It has been proposed a twin-supercoiled-domain model of DNA transcription (6): on the base of this model the nucleolar topo IIß activity might be necessary to relax supercoiled loops generated by the RNA polymerase I complex movement, respectively at the ETS region and at the 28S ending region of the actively transcribed rDNA gene These results may lead to the speculation that rDNA specific sites are critical regions for the transcriptional process.

The topo II enzymes bind, break and rejoin DNA at specific sequences, but the positions of the break depend on the presence and on the type of inhibitor (26). Furthermore the topo II cleavage pattern may be modified by DNA-methylation (27) and by secondary structure of DNA (28, 29) and it is restricted by the nucleoprotein organization (14). The *in vivo* cleaved sites, shown by the rDNA genes mapping, may be the results of specific consensus sequences and of their relative accessibility to topo IIβ. After purification on a sucrose gradient, only a small part of ribosomal genes shows topo II induced cleaved sites, thus suggesting that the interaction of topo II with specific sequences is dictated by the chromatin structure. In the nucleolus, topo IIß might be associated to ribosomal chromatin with an accessible configuration, for example ribosomal genes in a highly dispersed state with a non-nucleosomal structure (30) or/and with actively transcribed genes.

After Western blot of proteins from nucleolar complexes (Fig.4), an antiserum against both human topo II forms, reveals the presence of topo II in such complexes: in particular the 180 kDa protein, monomer of topo IIß form, is found covalently linked to nucleolar DNA, in agreement with previous demonstrations of a nucleolar localization of topo II (11). When compared to control, a more evident 180 kDa band is detected by immunostaining of proteins from VM26 treated cells, confirming that, also *in vivo*, topo II inhibitor VM26 increases and stabilizes the enzyme linkage to substrate DNA.

ACKNOWLEDGMENTS

We are grateful to Prof. W.T. Beck (St. jude Children's Research Hospital of Memphis, Tennessee, U.S.A.) for providing the antitopoisomerase II serum. Further we thank V. Cristofori for support in preparing photographs. This work was supplied by MPI (60 %) and by AIRC (Italian Association for Cancer Research).

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