# The Specificity of Human Lymphocyte Nucleolar DNA Long-Range Fragmentation by Endogenous Topoisomerase II and Exogenous Bal 31 Nuclease Depends on Cell Proliferation Status†

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ABSTRACT: The specificity of nucleolar DNA organization into loops in normal and activated to proliferation human lymphocytes has been studied using two different procedures of DNA loop excision. In the activated lymphocytes the nucleolar genes were found to be organized into loops of the same size as the size of individual rDNA repeat. The loops could be excised from the genome by DNA cleavage at matrix attachment sites with either the endogenous topoisomerase II or an exogenous nuclease Bal 31. In normal lymphocytes none of these enzymes generated any specific pattern of nucleolar gene long-range fragmentation, indicating that proliferation arrest correlates with a certain reorganization at higher orders of DNA packaging.

Eukaryotic DNA is believed to be organized into topologically independent domains (loops) by periodic attachment to the high salt-insoluble nuclear matrix (Cook & Brazell, 1976; Berezney & Buccholtz, 1981; Hancock & Huges, 1982; for reviews see Hancock, 1982; Jackson et al., 1990). The specificity of this topological organization (with respect to DNA sequence organization) has been studied by different approaches with conflicting results (reviewed by Gasser et al., 1989; Georgiev et al., 1991; Getzenberg et al., 1991; Laemmli et al., 1992; Razin & Vassetzky, 1992). Recently, we have suggested the use of the topoisomerase II-mediated DNA cleavage for mapping the basements of DNA loops (Razin et al., 1991; Iarovaia et al., 1993; Razin et al., 1993). In experiments with a ribosomal gene cluster it has been demonstrated that treatment of living cells with VM-26 (dimethylepipodophyllotoxinthenylidene β-D-glucoside), an inhibitor known to block the religation half-reaction catalyzed by the DNA topoisomerase II (Chen et al., 1984), results in highly preferential DNA cleavage within a short region of the nontranscribed spacer. The same specificity of rDNA cleavage was observed in high salt-extracted nuclei (Razin et al., 1993), indicating that the target DNA sequence is permanently bound to the nuclear matrix of which the DNA topoisomerase II is an integral part (Berrious et al., 1985). Similar observations were made when topoisomerase II-mediated cleavage of DNA at matrix attachment sites was used for mapping the DNA loop organization in an amplified human c-*myc* gene locus (Razin et al., 1993), indicating that the validity of the proposed approach is not restricted to the nucleolar region.

Other experimental evidences suggest that single-stranded DNA-specific nucleases can also be used for excision of DNA loops (Recillas-Targa et al., 1994), possibly due to the presence in loop basements of DNA sequence elements capable of melting in mild conditions or to form a noncanonical DNA structure (Bode et al., 1992).

All the above observations were however made in experiments with cell cultures (i.e., with proliferating cells). The experiments described in this paper were carried out in order to find out whether the mode of genomic DNA interaction with the nuclear matrix may be different in resting cells. With this aim we have compared the patterns of rDNA cleavage by endogenous topoisomerase II and by exogenous Bal 31 nuclease in normal human lymphocytes and lymphocytes induced to proliferation by PHA treatment. The results strongly support a conclusion that the mode of DNA interaction with the nuclear matrix and hence possibly the spatial organization of the genome are not the same in proliferating and resting lymphocytes.

## MATERIALS AND METHODS

*Purification of Lymphocytes and Stimulation of Lymphocytes to Proliferation by Treatment with Phytohemagglutinin (PHA).* Normal lymphocytes were isolated from heparinized peripheral human blood by sedimentation in a Ficoll−verografin gradient as has been described (Timonen & Soskela, 1980). Induction of lymphocyte proliferation by PHA treatment was carried out as described in the Difco manual for usage of phytohemagglutinin. In brief, lymphocytes were concentrated by centrifugation, washed twice with PBS buffer, and resuspended ($1 \times 10^6$ cells/mL) in RPMI medium (Gibco) supplemented with 6% of foetal calf serum. PHA was added to a final concentration of 100 μg/mL. The cell suspension was than placed in plastic Petri dishes and was incubated for 72 h at 37 °C in a $CO_2$ incubator.

*Preparation of Agarose Blocks with Living Cells.* Normal or activated lymphocytes were collected by centrifugation and washed once with RPMI 1640 medium. Approximately

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$(1-4) \times 10^6$ cells were resuspended in 50 $\mu$L of PBS buffer (preheated to 37 °C) and mixed with an equal volume of molten 1.5% low-melting agarose prepared in PBS buffer. The suspension was placed into a plug molt former and left at room temperature for 5 min.

*Treatment of Living Cells with Dimethylepipodophyllotoxinthenylidene β-D-Glucoside (VM-26).* The agarose blocks with immobilized cells were incubated for 30 min at 37 °C in RPMI media supplemented with VM-26 (from 0 to 60 $\mu$g/mL, as specified in the figure legends). VM-26 was provided by Bristol-Myers Pharmaceutical Co. After incubation, the agarose blocks were placed into the "stop buffer": 1% SDS, 0.4 M Na-EDTA (pH 8.0), 0.5 mg/mL proteinase K (Merck). The digestion was carried out for 36 h at 55 °C with constant gentle rotation. Then the blocks were washed with 0.2 M Na-EDTA and either were directly loaded on agarose gels for the pulsed field gel electrophoresis (PFGE) or were stored in the same solution at 4 °C.

*Extraction of Permeabilized Cells with the 2 M NaCl Solution and Treatment with VM-26.* To permeabilize cells and extract them with the 2 M NaCl solution, the blocks with embedded cells were incubated in a buffer containing 0.2% Nonidet P40 (NP40), 2 M NaCl, 2 mM Na-EDTA, 1 mM phenylmethanesulfonyl fluoride (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 (50 $\mu$g/mL) 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 Permeabilized Cells with Nuclease Bal 31.* The agarose blocks with embedded cells were placed into the buffer solution containing 0.5% NP40, 12 mM $CaCl_2$, 12 mM $MgCl_2$, 1 mM Na-EDTA (pH 8.0), 200 mM NaCl, 20 mM Tris-HCl (pH 8.0), and 75 units/mL Bal 31. The samples were then incubated at 22 °C for different time intervals (up to 3 h) as specified in the legend to Figure 4. In control experiments the blocks were incubated in the same conditions without enzyme. The reaction was terminated by replacement of the incubation mixture with the stop buffer, and digestion of proteins was carried out as described above.

*Pulsed Field Gel Electrophoresis (PFGE), Southern Transfer, and Hybridization.* Agarose blocks were loaded into wells of 1% agarose gels cast in 0.5× TBE buffer, and PFGE was carried out in a Bio-Rad CHEF DR-III system in 0.5× TBE buffer at 14 °C for 18−22 h at voltage gradient 6 V/cm with the switch time ramped linearly from 10 to 90 s. Concatemers of $\lambda$ DNA were used as marker. Gels were stained with ethidium bromide and photographed in UV light. For Southern transfer gels were incubated in 0.25 M HCl for 15 min at room temperature, washed twice in $H_2O$ for 10 min, and incubated in 0.4 NaOH for 30 min. Alkaline transfer of DNA to Hybond $N^+$ membranes (Amersham) was carried out as described in the manufacturer manual. Prehybridization (5−10 h) and hybridization (12−16 h) were carried out at 65 °C in the following solution: 5× SSPE, 5× Denhardt's solution, 0.5% (w/v) SDS (Bio-Rad), and 200 $\mu$g/mL denatured salmon sperm DNA (Sigma). After hybridization the filters were washed two times (30 min each) at 65 °C in 2× SSPE−0.5% SDS solution and one time (30 min) at 65 °C in 0.1× SSPE−0.5% SDS solution. The filters were then exposed to Fuji RX film at −75 °C with intensifying screen (DuPont).

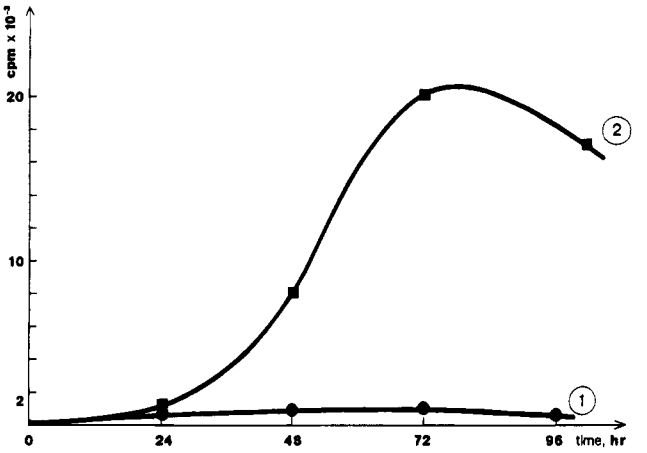


FIGURE 1: Incorporation of [$^3$H]thymidine by lymphocytes treated and nontreated with PHA. Purified human lymphocytes were incubated for 96 h in a $CO_2$ incubator at 37 °C in Petri dishes with culture medium supplemented (curve 2, rectangles) or not supplemented (curve 1, circles) with PHA. Every 24 h, equal aliquots were taken from each dish and incorporation of [$^3$H]thymidine into the acid-insoluble fraction was calculated. Each experimental point on the curves represents the average of three independent measurements.

## RESULTS

*Activation of Lymphocytes.* Induction of lymphocytes to proliferation was monitored by registration of incorporation of [$^3$H]thymidine into the acid-insoluble fraction. The data represented in Figure 1 show that normal lymphocytes do not incorporate [$^3$H]thymidine. When lymphocytes were stimulated to proliferation by PHA, the incorporation of [$^3$H]-thymidine became detectable about 24 h after the stimulation. In the following 48 h of cultivation the amount of incorporated [$^3$H]thymidine increased almost linearly (Figure 1), indicating that stimulated cells indeed started to replicate their DNA. Characteristic changes in the morphology of stimulated lymphocytes were also observed. The cells and also the nuclei became larger with pronounced internal compartmentalization. Several nucleoli could be observed in each nuclei. Mitotic cells could also be easily found (not shown).

*Analysis of the Size Distribution of DNA Fragments Released from the Genome of Normal and Activated Lymphocytes by Topoisomerase II-Mediated Cleavage in Living Cells.* In all experiments the activated lymphocytes were collected 72 h after treatment with PHA. The agarose blocks with embedded cells (either normal or activated lymphocytes) were incubated in a culture medium supplemented with different amounts of VM-26, as specified in the legend to Figure 2. After incubation, the cells were lysed and proteins were digested by extensive treatment with proteinase K (see the Materials and Methods section for the details). The size distribution of released DNA fragments, after separation by PFGE, is shown in the Figure 2 A,C. In both cases virtually no fragmentation in the absence of the drug was observed (lane 1 in Figure 2A and lane 1 in Figure 2C). The consequences of treatment of embedded cells with VM-26 were different when normal and activated lymphocytes were subjected to this treatment. In activated lymphocytes an

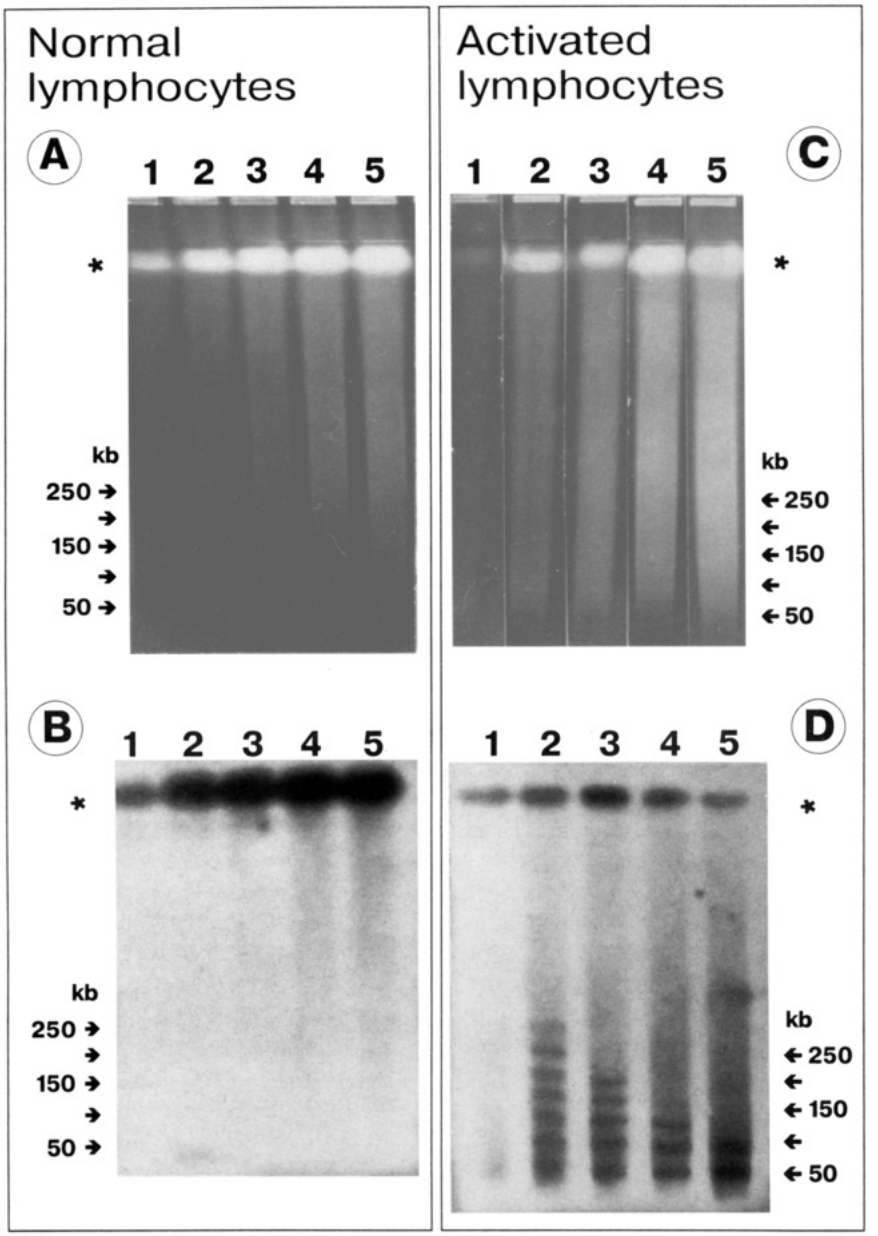


FIGURE 2: Analysis of patterns of topoisomerase II-mediated cleavage of rDNA *in vivo* in normal (A, B) and activated to proliferation (C, D) human lymphocytes. The cells embedded in agarose blocks were incubated for 30 min at 37 °C in a culture medium supplemented with 0, 10, 20, 40, or 60 $\mu$g/mL VM-26 (lanes 1−5, respectively, in all cases). Panels A and C show the released fragments separated by PFGE (ethidium bromide staining) The compressed band of ~1 Mb is indicated by an asterisk. Positions of molecular weight markers (phage $\lambda$ DNA concatemers) are indicated by arrows. Panels B and D demonstrate the results of hybridization of the 28S rDNA probe with the material transferred from gels shown correspondingly in panels A and C. Note the presence of a regular pattern of bands in panel D (activated lymphocytes) and absence of any bands (with the exception of the compression band) in panel B (normal lymphocytes).

accumulation of topoisomerase II-mediated scissions resulting in prominent DNA fragmentation was observed (Figure 2C, lanes 2−5). The size distribution of released fragments depended on the concentration of VM-26 in the incubation mixture. When cells were incubated in the media containing 40 or 60 $\mu$g/mL of VM-26, a prominent portion of the material was recovered in the 50−500 kb size area. The other part of DNA resided in a compressed band of ~1 Mb (indicated by the asterisk). Only slight fragmentation of DNA was observed when normal lymphocytes were subjected to treatment with the same concentrations of VM-26 (Figure 2A, lanes 2−5). This relative inefficiency of the fragmentation cannot be explained by the inability of VM-26 to penetrate into normal lymphocytes, as essentially the same result was obtained when normal lymphocytes permeabilized by NP-40 were incubated in topoisomerase II cleavage buffer supplemented with VM-26 (not shown).

*The Specific Pattern of Nucleolar DNA Cleavage by Endogenous Topoisomerase II Can Be Observed Only after Activation of Lymphocytes to Proliferation.* After separation by PFGE, the released fragments of the genomic DNA of either normal or activated lymphocytes were transferred to a nylon filter and hybridized with a cloned DNA fragment representing a part of 28S rRNA transcription unit. Results of the hybridization are shown in Figure 2B,D. In slots loaded with DNA from activated lymphocytes (Figure 2D, lanes 2−5) a clear pattern of bands with the sizes divisible by the size of rDNA repeat in human cells (~45 kb) was seen. An essentially similar pattern was observed previously in experiments with cultured cells (Razin et al., 1993;

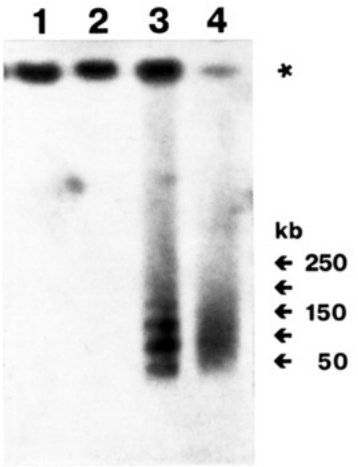


FIGURE 3: Analysis of patterns of topoisomerase II-mediated cleavage of rDNA in high salt-extracted nuclei of activated (lanes 1 and 3) and normal (lanes 2 and 4) human lymphocytes. Permeabilized cells were extracted with 2 M NaCl solution and then were incubated in topoisomerase II cleavage buffer without VM-26 (lanes 1 and 2) or supplemented with 50 $\mu$g/mL of VM-26 (lanes 3 and 4). After separation by PFGE, the released DNA fragments were hybridized to the 28S rDNA probe. Arrows at the right side of line 4 show positions of molecular weight markers (phage $\lambda$ DNA concatemers).

Iarovaia et al., 1993). The next set of experiments (Figure 3, lane 3) demonstrated that the specificity of rDNA long-range fragmentation by endogenous topoisomerase II in activated lymphocytes was not affected by the preextraction of permeabilized cells with 2 M NaCl solution. Hence, this specific fragmentation of nucleolar genes was generated by the high salt-insoluble topoisomerase II interacting with basements of DNA loops at sites of DNA attachment to the nuclear matrix, again in full accordance with the previous observations made in experiments with cultured cells (Razin et al., 1993; Iarovaia et al., 1993). In normal lymphocytes no specific fragmentation of nucleolar genes by the endogenous topoisomerase II was usually observed either in living cells or in high salt-extracted nuclei (Figure 2B, lanes 2−5, and Figure 3, lane 4). Only in some experiments a faint pattern of characteristic bands was seen after overexposure (not shown). This pattern was possibly due to the presence of a small population of activated cells in normal lymphocyte samples.

*Analysis of the Specificity of Long-Range DNA Fragmentation by Exogenous Nuclease Bal 31 in Permeabilized Normal and Activated Lymphocytes.* The inability to excise DNA loops from the genome of normal lymphocytes by the topoisomerase II-mediated DNA cleavage could be explained either by a low level of topoisomerase II in these nonproliferating cells or by some major rearrangement of the mode of DNA packaging occurring in connection with proliferation arrest. In order to choose between these two possibilities, the specificity of nucleolar DNA cleavage by an exogenous Bal 31 nuclease was studied in normal and activated lymphocytes. The rationale for utilization of Bal 31 nuclease in this experiment will be addressed in the Discussion.

Agarose blocks with embedded cells (either normal or activated lymphocytes) were incubated at 22 °C for different time intervals in the reaction buffer (see Materials and Methods section), supplemented with 75 units/mL Bal 31. After termination of the reaction and digestion of protein moiety, the released DNA fragments were separated by PFGE. As follows from the data represented in Figure 4, treatment of permeabilized cells with Bal 31 caused prominent fragmentation of DNA in both activated and normal lymphocytes, although a longer incubation time was necessary in order to achieve the degree of fragmentation of normal lymphocyte DNA similar to that observed in activated lymphocytes. Importantly, in both cases the fragmentation of DNA with endogenous nucleases was neglectable in experimental conditions used for treatment with Bal 31 (Figure 4A, lanes 1 and 2, and Figure 4C, lanes 1 and 2). Hybridization of separated DNA fragments (after Southern transfer) with the 28S rRNA coding DNA sequence revealed the specific pattern of rDNA bands in the case of activated lymphocytes (Figure 4D) and no specificity in the case of normal lymphocytes (Figure 4B).

## DISCUSSION

It has been proposed previously that the organization of the eukaryotic genome into loops may be related somehow to its functional organization. Some experimental evidence suggests that loops may constitute the units of replication and/or transcription (for review see Razin, 1987; Gasser et al., 1989; Georgiev et al., 1991; Laemmli et al., 1992; Razin & Vassetzky, 1992). In order to obtain more information about the possible relationship between the functioning of the eukaryotic genome and the mode of its packaging within nuclei, it seemed reasonable to check whether changes in the genome functional activity correlate with changes at higher orders of DNA packaging. With this aim we have compared the mode of nucleolar DNA organization into loops in normal (nonproliferating) and activated by PHA (proliferating) human lymphocytes. The inability to excise the rDNA loops by topoisomerase II-mediated DNA cleavage in normal lymphocytes suggested that ribosomal genes could be packed differently in proliferating and resting cells. It was, however, necessary to take into account the possible technical limitations of the approach used in these experiments. A simple decrease in the amount of active topoisomerase II in resting cells which may not necessarily correlate with a major rearrangement of the mode of DNA packaging would also explain the observed differences in the patterns of long-range cleavage of nucleolar genes by this enzyme. Hence, we have also studied the patterns of nucleolar genes cleavage by an exogenous Bal 31 nuclease. It seems that DNA sequence elements involved in organization of matrix attachment sites are either permanently melted or have a noncanonical secondary structure (Bode et al., 1992). For this reason they can be expected to constitute selective targets for cleavage with single-stranded DNA-specific nucleases. Indeed, we have shown that large DNA fragments with the expected properties of released loops can be excised from the genome by treatment of permeabilized cells with S1 nuclease (Recillas-Targa et al., 1994). Unfortunately, this nuclease is active only at nonphysiological pH, which may complicate interpretation of the results. Hence, in the present studies we have used for the same purpose Bal 31 nuclease which is active at physiological pH. This enzyme is characterized by a complex spectrum of activities. However, when the ends of DNA chains are not accessible, it cleaves DNA preferentially at melted regions and regions with a noncanonical secondary structure. It has been shown previously that brief treatment of isolated metaphase chromosomes with Bal 31 nuclease results in excision of loop-

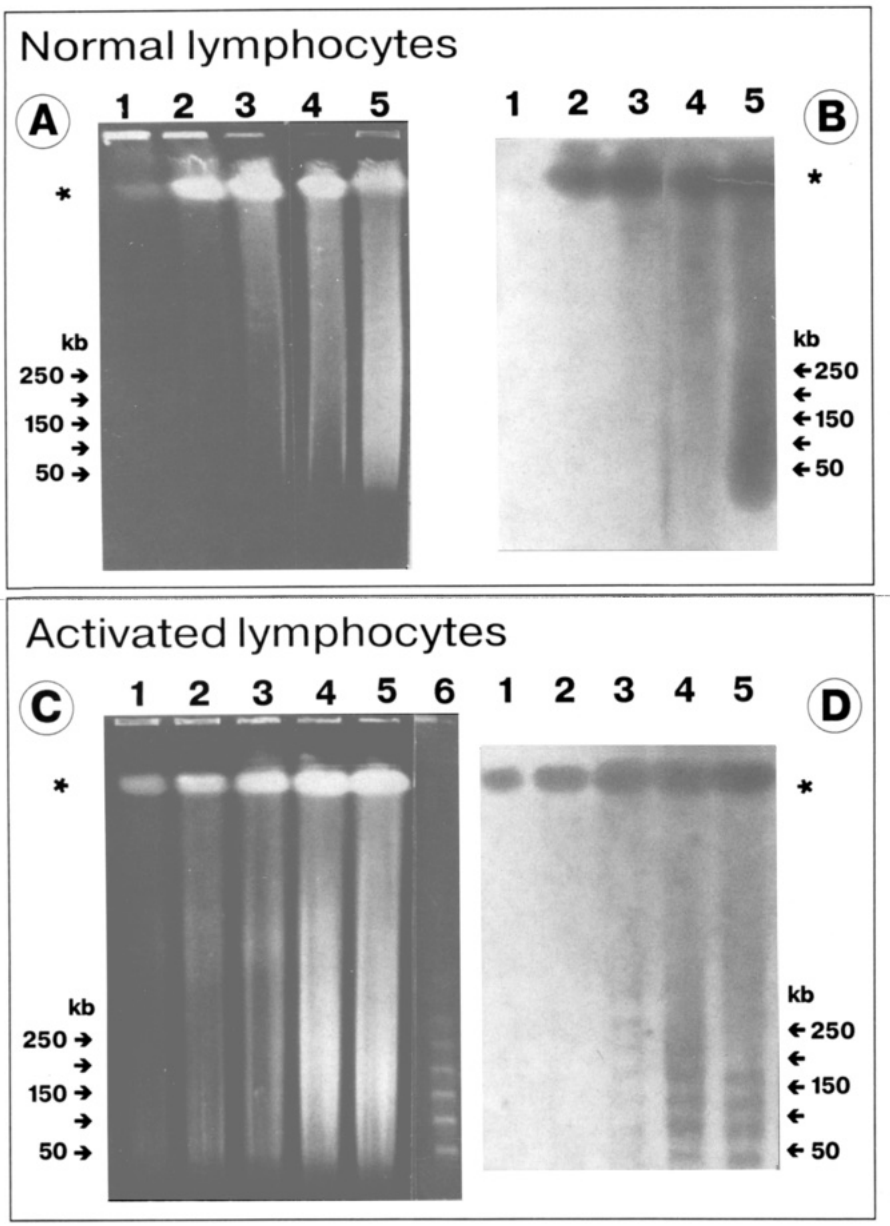


FIGURE 4: Analysis of patterns of rDNA cleavage by Bal 31 nuclease in permeabilized normal (A, B) and activated to proliferation (C, D) human lymphocytes. Panels A and C show the distribution of released DNA fragments after separation by PFGE. Results of hybridization of the 28S rDNA probe with corresponding Southern filters are shown in panels B and D. In all panels lanes 1−5 represent the material from permeabilized cells not incubated in Bal 31 reaction buffer (lane 1), permeabilized cells incubated in Bal 31 reaction buffer for 3 h without enzyme (lane 2), or permeabilized cells incubated in Bal 31 reaction buffer with 75 units of Bal 31 for 1, 2, and 3 h (lanes 3−5 correspondingly). Lane 6 in panel C shows distribution of the molecular weight markers (phage $\lambda$ DNA concatemers).

sized DNA fragments (Clark et al., 1987). Identity of patterns of activated lymphocyte nucleolar DNA long-range fragmentation by the nuclear matrix topoisomerase II and by the exogenous Bal 31 nuclease directly demonstrates that the latter cleaves DNA at matrix attachment sites. Hence, a treatment of permeabilized cells with Bal 31 nuclease can be used for mapping the DNA loop organization. Consequently, the drastic difference in the specificity of the nucleolar DNA fragmentation by Bal 31 nuclease in normal and activated lymphocytes is likely to indicate that the mode of genomic DNA interaction with the nuclear matrix is different in proliferating and resting cells. This does not necessarily mean that the DNA loop anchorage sites are destructed in quiescent cells. Indeed, our previous data support a conclusion that DNA is still organized into loops in completely inactive nuclei of chicken erythrocyte and sperm cells (Farache et al., 1990; Kalandadze et al., 1990). Hence, it is more likely that the pattern of DNA−protein interactions at the DNA loop anchorage sites is changed upon the proliferation arrest. These changes may occur in connection with inactivation of replication origins which are believed to be permanently attached to the nuclear matrix (Dijkwell et al., 1986; Razin et al., 1986).

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