In Vivo Topoisomerase II Cleavage Sites in the Ribosomal DNA of *Physarum polycephalum*†

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ABSTRACT: We have analyzed the topoisomerase II cleavage sites in the extrachromosomal ribosomal DNA of the lower eukaryote *Physarum polycephalum* using the topoisomerase II-specific inhibitor, 6,8-difluoro-7-(4-hydroxyphenyl)-1-cyclopropyl-4-quinolone-3-carboxylic acid. Most of the *in vivo* topoisomerase II cleavage sites were found either in the transcribed region of ribosomal DNA or in the palindromic region surrounded by the replication origins. Two classes of sites were identified: those which correlate with DNase I hypersensitive sites and corresponding to an open chromatin configuration (transcribed region) and internucleosomal cleavage sites (in the region of replication origins). Topoisomerase II drug-induced cleavage in the ribosomal DNA was considerably reduced upon *Physarum* differentiation to a dormant stage of life, the spherules. In contrast, the amount of drug-dependent cleavage was found to increase during the metaphase of mitosis, when rDNA transcription is shut off. These findings suggest a role for topoisomerase II in the ribosomal DNA minichromosomes segregation, in addition to its role in transcription. Finally, the similarity between *in vivo* sites and those observed following drug treatment of isolated nuclei indicates that no profound change occurs in rDNA chromatin conformation during nuclei isolation. By contrast, *in vitro* cleavage sites with purified topoisomerase II weakly correlate to *in vivo*, indicating a prominent role for chromatin structure in determining the interaction sites of topoisomerase II with DNA *in vivo*.

Through their transient breakage and rejoining of DNA strands, DNA topoisomerases are able to resolve the topological problems that arise during DNA-processing events such as transcription, replication, recombination, and chromosome segregation [for a review, see Wang (1987)]. Eukaryotic topoisomerases, both type I and II enzymes, can relax both positively and negatively supercoiled DNA, but only topoisomerase II is able to decatenate intact duplex DNA molecules. Since both classes of enzymes can substitute for one another except during decatenation, this has made genetic analysis of their individual functions difficult. By genetic studies in yeast, it has been shown that topoisomerase II is required at the time of mitosis to fully segregate replicated chromosomes (Holm et al., 1985). In addition to its catalytic activities, topoisomerase II has also been implicated as a structural component of eukaryotic chromosomes (Earnshaw et al., 1985). By localization to the base of chromatin loops (Berrios et al., 1985) and through its preferential interaction with SAR sequences (Adachi et al., 1989), topoisomerase II is thought to regulate the topology of individual chromatin loops. Little is known, however, about the physiological roles of topoisomerase II in the eukaryotic cell, in particular in replication and in transcription.

One way of elucidating physiological roles for topoisomerases is to look at drug-induced topoisomerase cleavage sites *in vivo*. Using such an approach, it has been shown that topoisomerase II is involved in the re-establishment of condensed chromatin during heat-shock recovery (Udvardy & Schedl, 1993), in *c-myc* gene transcription (Riou et al., 1993), and in the anchorage of histone genes to the nuclear scaffold (Käs & Laemmli, 1992). Use of topoisomerase II inhibitors has also allowed estimation of the size of chromatin loops, as revealed by the length of DNA fragments released from the nuclear scafflod following drug treatment of cells (Razin et al., 1993).

We have chosen a very simple and ideal organism, the lower eukaryote *Physarum polycephalum*, to study the topoisomerase II cleavage during the cell cycle and during differentiation. Indeed, *Physarum*'s plasmodium offers the unique advantage of a naturally synchronous division cycle, allowing fine studies of the cell cycle events. Moreover, when placed in starvation medium conditions, *Physarum* undergo differentiation to a dormant life stage, the spherules, in which many DNA metabolic processes are repressed (Aldrich & Daniel, 1982).

We focused our analysis on the extrachromosomal ribosomal DNA, which has been extensively characterized, in regards to both its DNA sequence and its chromatin structure (Amero et al., 1988; Lucchini et al., 1987; Pauli et al., 1988). The replication origins were also recently precisely localized in the minichromosomes carrying the ribosomal genes. The ribosomal DNA is not regulated as the bulk chromosomal genes in terms of its replication, which is unscheduled throughout both S and G2 phases. However, its replication and transcription are blocked during the metaphase of mitosis (Davies & Walker, 1978).

The ribosomal genes of *P. polycephalum* are located on a palindromic 60 kb (kilobase) minichromosome, each half

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containing a 12.7 kb transcription unit and two replication origins (Vogt & Braun, 1976). They are repeated at about 200 copies per nucleus. During *Physarum* differentiation, transcription of the ribosomal genes is progressively repressed.

Through use of CP-115,953,[1] a quinolone inhibiting eukaryotic topoisomerase II activity, we have mapped the *in vivo* topoisomerase II cleavage sites in the *Physarum* rDNA minichromosome. We show that, in the G2 phase, the topoisomerase II cleavage sites are located mainly in the transcription unit, whose chromatin is in an extended, open conformation. The other major topoisomerase II cleavage sites are located between the two rDNA replication origins, where DNase I studies suggest that chromatin is organized in a nucleosomal state. During mitosis, the cleavage sites were enhanced at all positions, with some new sites appearing, despite there being an arrest of rDNA transcription. This latter finding suggests an additional role of topoisomerase II at the time of mitosis in the segregation of the minichromosomes, whose mechanism is yet unknown.

During differentiation, we observed that general topoisomerase II cleavage activity progressively decreased with time. We thus propose two distinct roles for topoisomerase II in *Physarum* rDNA metabolism, one in the high level of transcription during the S and G2 phases and the other at mitosis to allow the rDNA minichromosomes to segregate.

MATERIALS AND METHODS

Culture of Physarum. Microplasmodia of *P. polycephalum* (strain M3CIV) were grown in shaken cultures at 24 °C in semidefined medium (Daniel & Baldwin, 1964). Synchronous macroplasmodia were obtained by coalescence of microplasmodia on filter paper as described (Daniel & Baldwin, 1964). Mitosis was followed in ethanol-fixed smears using phase contrast microscopy. After microplasmodia fusion, the second mitosis (mitosis II) and the third mitosis (mitosis III) were observed to take place roughly 16 and 26 h after nutrient medium was added, respectively.

For spherulation experiments, exponentially growing microplasmodia (48 h old cultures) were transferred to starvation medium (Aldrich & Daniel, 1982). Spherule formation was monitored microscopically.

BrdU Incorporation. A synchronous macroplasmodium was divided into two equal parts and each part placed on 20 mL of nutrient medium containing 100 μg/mL of bromodeoxyuridine (BrdU), 100 μg/mL uridine, 5 μg/mL fluorodeoxyuridine (Pierron et al., 1984), and 0 or 100 μM quinolone CP-115,953 (kindly provided by Dr. McGuirk, Pfizer Inc.). After 6 h at 26 °C, each sample was harvested using a Warring Blender in 200 mL of buffer A (10 mM $CaCl_2$, 10 mM Tris-HCl, pH 8.0, 0.1% Nonidet P40, and 0.25 M sucrose), filtered, spun down at 4 °C for 5 min at 800*g*, washed in 20 mL of the same buffer, and pelleted again. Nuclei were then lysed by resuspending in 2 mL of lysis buffer (10 mM Tris-HCl, pH 7.5, 20 mM NaCl, and 1% SDS), followed by addition of 100 mM ethylenediaminetetraacetic acid (EDTA) and 500 μg/mL proteinase K. After incubation at 50 °C for 16 h, samples were extracted twice with phenol/chloroform and once with chloroform and ethanol precipitated. After resuspension in 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA, 150 μg of each genomic DNA sample was restricted overnight with *Eco*RI (Biolabs). The solution was adjusted to 9 mL with 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA, and 11 g of cesium chloride was added to give a final density of 1.69. After ultracentrifugation at 36 000 rpm for 72 h in a 70Ti rotor (Beckman), the position and density of "light/light" and "heavy/light" DNA bands were noted.

In Vivo Topoisomerase II Cleavage. A synchronous macroplasmodium in either mitosis II (M II) or G2 phase was divided into equal portions which were placed on 2 mL of nutrient medium containing either 0, 25, 50, or 100 μM CP-115,953. After various incubation times at 26 °C (see text), the plasmodium portion was homogenized using a Warring Blender at 4 °C in 200 mL of buffer A. The nuclei obtained were filtered, pelleted by centrifugation for 5 min at 800*g* at 4 °C, washed in the same buffer, pelleted again, and lysed by resuspending in 2 mL of lysis buffer. EDTA (100 mM) and proteinase K (500 μg/mL) were then added, and the lysate was incubated at 50 °C for 16 h. Genomic DNA was subsequently purified using phenol/chloroform extraction, RNase treatment (100 μg/mL for 30 min at 37 °C), re-extraction once with phenol/chloroform and once with chloroform, and finally ethanol precipitation. Purified genomic DNA was dissolved in 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA.

Topoisomerase II and DNase I Cleavage Sites in Isolated Nuclei. Nuclei were prepared by homogenization of a synchronous macroplasmodium in 200 mL of buffer A using a Warring Blender. Nuclei were filtered, pelleted at 4 °C for 5 min at 800*g*, washed in 20 mL of the same buffer, and pelleted again. For isolation of spherules, as spherules become very difficult to break after 48 h in starvation medium, a Dounce homogenizer (Potter) was used instead of the Warring Blender; pelleted spherules were resuspended in 20 mL of buffer A and broken after 20 strokes at 4 °C. Nuclei isolation was checked under a phase-contrast microscope. About 80% of the spherules were broken.

For determination of topoisomerase II cleavage sites, isolated nuclei were resuspended in 10 mL of buffer B [100 mM NaCl, 5 mM KH_2PO_4, 5 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.5 mM phenylmethanesulfonyl fluoride, 1 mM DTT, and pH 6.2] and, following pelleting, were resuspended in buffer B plus 0.2 mM ATPγS. After 5 min on ice, nuclei suspension was divided into 1 mL aliquots and CP-115,953 was added at the final concentration indicated in the text. After 30 min of incubation at 26 °C, the nuclei were pelleted at 4 °C and lysed, and the DNA was purified as described above.

For salt extraction of nuclei prior to incubation with CP-115,953, isolated nuclei were resuspended in 10 mL of buffer B and pelleted by centrifugation, and the resultant pellets were resuspended in buffer B and the isolated nuclei dialyzed stepwise against buffer B containing in succession either 0.3, O.6, 1.2, or 2 M NaCl at 4 °C to the maximal salt concentration indicated. Dialysis in each buffer was at 4 °C for 1 h. The solutions were then dialyzed back to buffer B in inverse, stepwise order. Nuclei were inspected microscopically, and the yield of intact structures was determined. Nuclei were then spun down at 800*g* for 5 min and

[1] Abbreviations: ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); BrdU, bromodeoxyuridine; CP-115,953, 6,8-difluoro-7-(4-hydroxyphenyl)-1-cyclopropyl-4-quinolone-3-carboxylic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate.

resuspended in buffer B containing 0.2 mM ATPγS. After 5 min on ice, CP-115,953 was added, nuclei were treated, and the DNA was purified as described above.

For the identification of DNase I sensitive sites, isolated nuclei were washed in 5 mL of DNase I buffer (15 mM Tris-HCl, pH 7.4, 60 mM KCl, 15 mM NaCl, 0.5 mM DTT, 0.05 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.25 M sucrose). After being pelleted for 5 min at 800*g* and 4 °C, nuclei were resuspended in 1.2 mL of DNase I buffer, divided into 0.2 mL aliquots, and kept at 4 °C. DNase I (Boehringer) was diluted in 25 mM Tris-HCl (pH 7.6) and 30% glycerol before use and added to nuclei for 5 min at 26 °C. The DNase I reactions were stopped by the addition of 40 μL of 0.1 M EDTA and 2.5% SDS. Finally, proteinase K was added to 500 μg/mL and incubation continued for 16 h at 50 °C. Genomic DNA was then purified as described above.

In Vitro Topoisomerase II Cleavage Reaction. Eukaryotic topoisomerase II (from calf thymus) was provided by J. F. Riou (Rhône Poulenc - Rorer, Vitry-sur-Seine). One unit of topoisomerase II activity was defined as the amount of enzyme necessary to decatenate 200 ng of kDNA at 37 °C for 30 min in TopoII reaction buffer (50 mM Tris-HCl, pH 7.9, 100 mM KCl, 15 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 15 μg/mL BSA, and 1 mM ATP).

Physarum. rDNA was purified in a potassium iodure gradient as previously described (Ferris & Vogt, 1982). Total rDNA (400 ng) was incubated with purified topoisomerase II and CP-115,953 in a 50 μL reaction mixture containing TopoII reaction buffer for 10 min at 37 °C. The reaction was stopped by the addition of SDS to 0.25% and proteinase K to 250 μg/mL, and the incubation continued for 30 min at 50 °C. The samples were then phenol/chloroform and chloroform extracted and after ethanol precipitation were resuspended in 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA.

Hybridization Probes. DNA probes were obtained from two pBR322-derived plasmids provided by Richard Braun (Bern, Switzerland). They are represented in Figure 2; pPHR117 contains a 0.6 kb *Mbo*I insert which is repeated twice per half-molecule of rDNA and corresponds to the region of the replication origins. These two regions are located respectively 2.7 and 7.8 kb upstream from the transcription start. pPHR102 has a 5 kb *Hin*dIII insert from which we derived probe 2, a 0.4 kb *Hin*dIII-*Pvu*II fragment located in the 5′ part of the 19S gene, probe 3, a *Bgl*II-*Hin*dIII 0.3 kb fragment in the first exon of the 26S gene, and probe 4, a *Pvu*II-*Hin*dIII 0.6 kb fragment overlapping probe 3.

Gel Electrophoresis and Southern Hybridization. The DNA was cleaved with restriction enzymes (New England Biolabs) and electrophoresed through 0.8−1% 20 cm agarose gels at 1.2 V/cm overnight in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, and pH 8.0). After HCl depurination, the DNA was transferred to Hybond N^+ (Amersham) in 20x SSC solution (1x SSC is 0.15 M NaCl and 0.015 M sodium citrate), using the procedure described in Maniatis et al. (1982). ^{32}P-labeled probes were made by the random-priming method as recommended by the manufacturer (Boehringer). Prehybridization and hybridization were carried out at 42 °C in the following solution: 50% formamide, 5x SSC, 5x Denhardt's reagent, 1% SDS, 25 mM NaH_2PO_4, pH 6.8, and 100 μg/mL denatured salmon sperm DNA. Membranes were rinsed at a final stringency of 0.2x SSC and 0.1% SDS at 65 °C and later exposed for 4−48 h with intensifying screens at −80 °C. For removal of the probe, filters were washed twice with 0.5% SDS at 80 °C for 30 min each time.

A

	control	100μM CP-115,953
microplasmodia growth	+	-
plasmodium formation after 48H drug treatment	+	-
plasmodium growth	+	-
mitosis in plasmodium nuclei	+	-

B

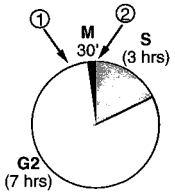


FIGURE 1: Effects of topoisomerase II inhibition on the *Physarum* cell cycle. (A) Summary of cellular effects of CP-115,953 treatment. See text for details. (B) Represented in this diagram is the cell cycle of *Physarum* plasmodium. The total time of duration of each cell cycle stage is indicated. Arrows 1 and 2 refer to the beginning of drug treatments in BrdU experiments: 1, 9 h after the first mitosis; 2, 5 min after mitosis II metaphase.

For quantitation and positioning of the topoisomerase II cleavage sites, we used a Molecular Dynamics 400A Phosphorimager and Image Quant software.

RESULTS

The Quinolone Antitumor Drug CP-115,953 Prevents Mitosis in Physarum Plasmodia. The quinolone topoisomerase II inhibitor, CP-115,953, was tested for its ability to inhibit the growth of *Physarum*. The results are summarized in Figure 1A. CP-115,953 (100 μM) totally inhibited nuclear division of microplasmodia, as estimated by nuclei counting using a haemocytometer (data not shown). After 48 h of drug treatment, the microplasmodia were tested for their ability to fuse into a synchronous macroplasmodium; this did not occur, even after transfer to a drug-free medium. We also tested for plasmodium growth in the presence of the inhibitor. After the fusion of exponentially growing microplasmodia, 100 μM CP-115,953 was added with the nutrient medium. This totally inhibited plasmodium growth, compared with the untreated control, as deduced by measuring the plasmodium area (not shown). Also, from the observation of nuclei under a phase-contrast microscope, we could clearly see that no mitosis occurred in the treated plasmodium; the nucleolus remained present and no chromatin condensation was observed, while untreated plasmodium underwent mitosis II and III 16 and 26 h, respectively, after nutrient medium was added.

To determine more precisely where the cell cycle arrest takes place in the synchronous cycle of *Physarum*, following CP-115,953 addition, BrdU incorporation into total DNA was monitored after drug addition. The division cycle of *Physarum* is described in Figure 1B; BrdU and CP-115,953 were added to a synchronous plasmodium either in late G2 (arrow 1) or at the beginning of S phase (arrow 2). After 6 h of drug treatment, genomic DNA was prepared and unreplicated (light/light) DNA was separated from newly replicated (heavy/light) DNA on a CsCl gradient (see

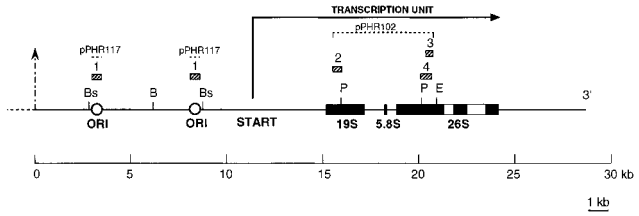


FIGURE 2: Map of *Physarum* rDNA and localization of the probes. Only one-half of the palindromic rDNA molecule is shown. The vertical broken arrow indicates the symmetry axis of the palindrome. The horizontal arrow represents the 12.7 kb primary transcript. Black boxes represent the ribosomal genes coding for 19S, 5.8S, and 26S rRNAs, respectively. White boxes represent the two 26S gene introns. The position of replication origins (circles) is also indicated (Benard et al., 1995; Vogt & Braun, 1977). Probes 1−4 (hatched boxes) are described in Materiels and Methods. Probe 1 was used for indirect end labeling of *Bst*EII (Bs)-digested DNA, probes 2 and 4 were used with *Pvu*II (P)-restricted DNA, and probe 3 was used with either *Eco*RI (E) or *Eco*RI plus *Bam*HI (B) digestion. The positions of the plasmids pPHR117 and pPHR102 inserts are indicated by the dotted lines. The kb numeration starts from the center of the palindrome and is according to Ferris (1985).

Materials and Methods). The data obtained indicate that, when the quinolone was added immediately after mitosis, in early S phase (arrow 2 in Figure 1B), BrdU incorporation occurred normally since a heavy/light band indicating semiconservative replication was observed in the density gradient and was comparable to that of the untreated control (not shown). When the topoisomerase II inhibitor was applied just before mitosis, in late G2 (arrow 1 in Figure 1 B), BrdU incorporation was blocked and no heavy/light band was detected. These experiments suggest that the quinolone blocks the plasmodium cycle at the G2/M transition by inhibiting mitosis.

In Vivo Topoisomerase II Cleavage Sites Are Preferentially Induced in the Transcribed Region of Ribosomal DNA. The next step was to examine whether *in vivo* treatment by the quinolone induced topoisomerase II cleavage in the DNA of *Physarum*, as it has been observed in higher eukaryotic cells. For this purpose, synchronous plasmodia either in M or in G2 phase were treated for 30 min with 100 μM quinolone. The DNA was prepared as described in Materials and Methods, cleaved by appropriate restriction enzymes, and electrophoresed. Drug-induced cleavage was analyzed by indirect end labeling with various probes of single or low-copy number genes of chromosomal DNA (i.e. profillin, actin, and histones). Unexpectedly, no topoisomerase II cleavage sites were detected in these genes (not shown), despite the fact they are actively transcribed (Binette et al., 1990; Gonzalez-y-Merchand & Cox, 1988; Wilhelm & Wilhelm, 1989). In contrast, when DNA from treated cells was hybridized with probes homologous to the ribosomal DNA region (see organization in Figure 2), numerous topoisomerase II cleavage sites were detected (Figure 3). Analysis of the same DNA samples with a variety of different probes allowed us to map the *in vivo* sites within the whole rDNA domain. The cleavage sites were positioned on the rDNA map (Figure 5, *in vivo*). We could distinguish two groups of sites. The first covers the whole transcribed region (starting from the site marked y to the 3′ end of the molecule). The second group of sites is located in the nontranscribed spacer (NTS), in the palindromic area limited by the two replication origins; it is composed of a cluster of weak sites, which are not resolved in the electrophoresis

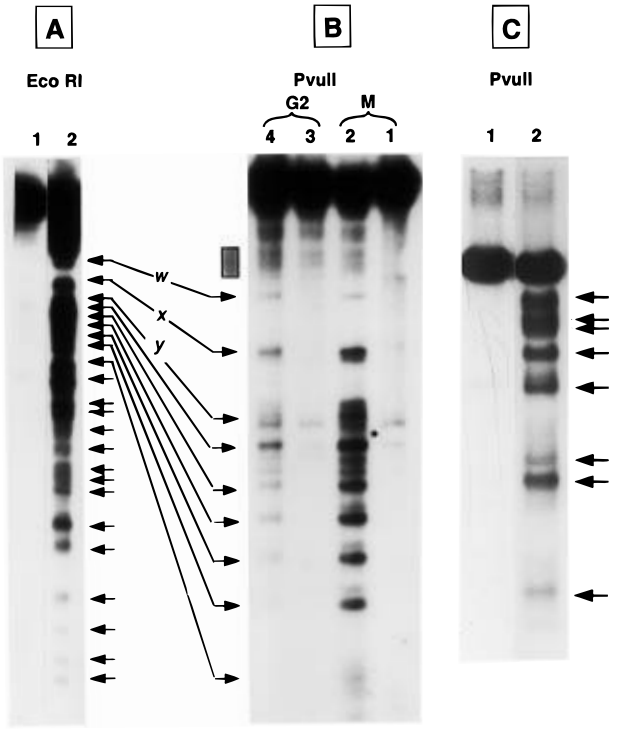


FIGURE 3: *In vivo* topoisomerase II cleavage sites. DNA from a plasmodium treated with 100 μM CP-115,953 for 30 min was restricted and analyzed by indirect end labeling (see Figure 2 for localization of enzymes and probes). (A) The DNA from a plasmodium treated in G2 was digested by *Eco*RI and hybridized with probe 3, generating a 43 kb fragment: lane 1, no drug; lane 2, CP-115,953-treated. (B) DNA digested with *Pvu*II and hybridized with probe 2, generating a 31 kb fragment: lanes 1 and 2, plasmodium treated in M; lanes 3 and 4, plasmodium treated in G2. Lanes 1 and 3 are controls without drug. (C) DNA digested with *Pvu*II and hybridized with probe 4, generating a 8 kb fragment. Lane 1 is a control without drug. Cleavage sites are indicated by arrowheads. Sites w, x, and y are described in the text.

conditions employed (represented by a gray rectangle in Figures 3B and 5). The domain between these two groups of sites contains only one prominent topoisomerase II cleavage site, marked x and located about 0.7 kb upstream of the transcription start. On the 5′ side of the transcribed rDNA region, starting from site y, cleavage sites are regularly distributed, being spaced every 450 bp (see Figures 3A,B and 5). The regularity of this distribution suggests that in this region cleavage of the DNA is occurring every two nucleosomes, with nucleosomes phased with respect to the DNA sequence. This regular spacing disappears in the 19S and 26S sequences.

Unexpectedly, topoisomerase II-induced cleavage activity appeared stronger in mitosis than in G2 phase (Figure 3, panel B, compare lanes 2 and 4), although the rDNA is not transcribed during mitosis (Davies & Walker, 1978; Hall & Turnock, 1976; Seebeck & Braun, 1980).

Incubation of Isolated Nuclei with CP-115,953 Induces Extensive Topoisomerase II Cleavage in Ribosomal DNA, with a Pattern Similar to That Obtained in Vivo. As shown in Figure 4, incubation of plasmodium nuclei with increasing concentrations of the topoisomerase II inhibitor, CP-115,-953, produced extensive cleavage within the rDNA region. For instance, up to 100% of the input *Eco*RI DNA fragment is cleaved using only 25 μM quinolone (panel A, lane 3). With increasing concentrations of drug (i.e. 50 and 100 μM), the amount of large DNA fragments was reduced to the

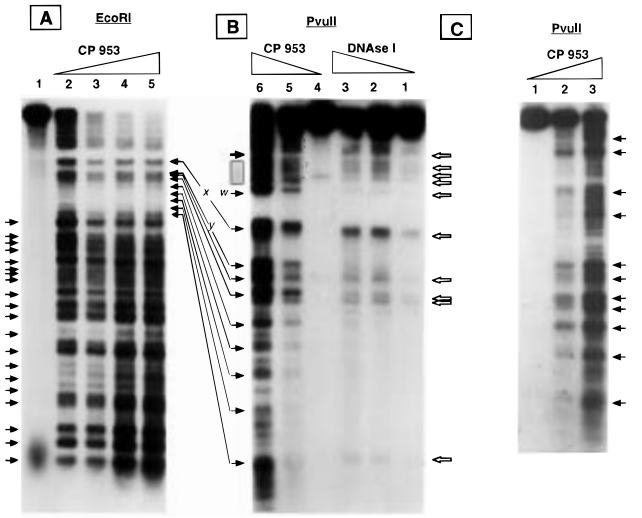


FIGURE 4: Topoisomerase II and DNase I cleavage sites in isolated nuclei. Isolated nuclei from a G2 plasmodium were incubated either with CP-115,953 to generate topoisomerase II cleavage sites (panels A−C) or with DNase I (panel B, lanes 1−3). (A) CP-115,953 treatments were for 30 min with the following: lane 1, no drug; lane 2, 10 μM drug; lane 3, 25 μM drug; lane 4, 50 μM drug; lane 5, 100 μM drug. DNA was digested with *Eco*RI, giving a 43 kb fragment. In panels B and C, the DNA was digested with *Pvu*II and hybridized with probes 2 and 4, respectively. (B) CP-115,953 treatments were for 30 min with the following: lane 4, no drug; lane 5, 10 μM; lane 6, 50 μM drug. DNase I treatments were as follows: lane 1, no enzyme; lane 2, 0.1 u/mL enzyme; lane 3, 1 u/mL enzyme. (C) Lane 1, no drug; lane 2, 10 μM CP-115,953; lane 3, 50 μM CP 115,953. In panels A−C, black arrowheads indicate the topoisomerase II cleavage sites and open arrows the DNase I sensitive cleavage sites. x, y, and y′ refer to topoisomerase II sites described in the text. The gray rectangle in B indicates a cluster of topoisomerase II sites that are not resolved under the electrophoresis conditions used (see Materials and Methods).

benefit of smaller ones (lanes 4 and 5, panel A). In order to estimate the relative intensities of the topoisomerase II cleavage sites and to map them in the rDNA, we used a concentration of drug that resulted in only partial cleavage of the 43 kb parental DNA band (lane 2, 10 μM). The data of Figure 4 were quantified by using a Phosphorimager (see Materials and Methods) and allowed to establish the topoisomerase II cleavage site map shown in Figure 5. In parallel with these experiments, we also performed DNase I digestions and mapped DNase I hypersensitive sites in the whole rDNA molecule using the same probes and digestions as for topoisomerase II cleavage (Figures 4B and 5).

It is clear from the maps of Figure 5 that the pattern of drug-induced topoisomerase II cleavage sites revealed in isolated nuclei was similar to that obtained *in vivo*. Some minor differences were observed, however, which correspond to sites present in the isolated nuclei but absent *in vivo*. Thus, the set of *in vivo* cleavage sites appears as a subset of those obtained in isolated nuclei. This is especially apparent for sites in the nontranscribed spacer region in between replication origins, where the unresolved sites (gray rectangle) are more intense than *in vivo* (compare lane 6 in Figure 4B and lane 4 in Figure 3B). As seen *in vivo*, the transcribed region just upstream from the 19S coding sequence maintains its regularly spaced topoisomerase II sites. The spacer region between the replication origins region and the transcribed region (from site w to site y) is also free of DNase I sensitive sites, except the prominent topoisomerase II site x (Figures 4B and 5).

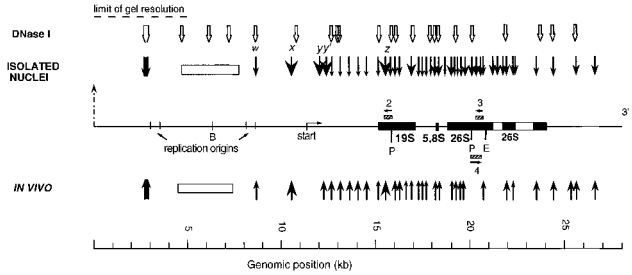


FIGURE 5: Comparison of the topoisomerase II cleavage sites induced *in vivo* with the sites in isolated nuclei and with DNase I sensitive sites. The maps shown here are based on cleavage data generated in the experiments described in the legends to Figures 3 and 4. *In vivo* sites (arrows pointing up), sites in isolated nuclei (arrows pointing down), and DNase I sensitive sites (open arrows) have been reported on the rDNA map. The rDNA transcription initiation site (start) is also indicated. Only half of the rDNA molecule is shown, since the electrophoresis did not resolve DNA fragments above 15 kb length (the limit of gel resolution is indicated by a dashed line). The gray rectangle corresponds to the cluster of topoisomerase II cleavage sites which are not resolved using probe 3 (see Figures 3B and 4B). The numeration of genomic positions is the same as in Figure 2.

DNase I digestions performed in parallel indicate that the DNase I hypersensitive sites in all rDNA regions reasonably correlate to topoisomerase II sites induced by the quinolone (Figure 5). One notable exception is the region with topoisomerase II sites spaced every 450 bp before the coding sequence of rDNA. This region is free of DNase I hypersensitive sites.

Topoisomerase II Cleavage Sites in the Central Region of the Nontranscribed Spacer (NTS). The relatively long distance of probe 2 from the nontranscribed spacer (starting from *Pvu*II, see Figure 2) did not allow for resolution of the cluster of topoisomerase II cleavage sites in this region of the rDNA, revealed *in vivo* and in the isolated nuclei (see gray rectangles in Figures 3 and 4). The complex organization of this palindromic region is depicted in Figure 6B; it is limited by two unique regions corresponding to the replication origins between which there are two series of four inverted repeats 308 bp long each and two series of two inverted repeats of 222 bp around the center of the palindrome. After digestion of genomic DNA with *Bst*EII, probe 1 hybridizes to the 6 kb palindromic fragment at each of its extremities.

As shown in Figure 6A (lanes 1−3), extensive topoisomerase II cleavage (up to 60% of the fragment with 50 μM CP-115,953) occurs in isolated nuclei within this NTS region. The cleavage sites detected exhibit periodic spacing, especially within the 308 bp repeat region where they are spaced by about 80−100 bp. Among the numerous sites observed in isolated nuclei, some sites detected were more prominent than others. These sites are represented by longer arrows in Figure 6 B. These sites are spaced with an average of 250 bp.

A lower (3−5%) but significant extent of cleavage also appears in the palindromic 6 kb region after *in vivo* quinolone treatment (Figure 6A, lanes 4 and 5). These sites are similar to the prominent sites observed in isolated nuclei (Figure 6B) and correspond to the DNase I hypersensitive sites in this region (Figure 5B, white arrows). Comparison of the cleavage patterns of lanes 4 and 5 in Figure 6A demonstrates that the relative intensities of *in vivo* cleavage sites were different in mitosis (M) and G2 phase, as observed in the transcribed region (Figure 3B). Several sites are detected

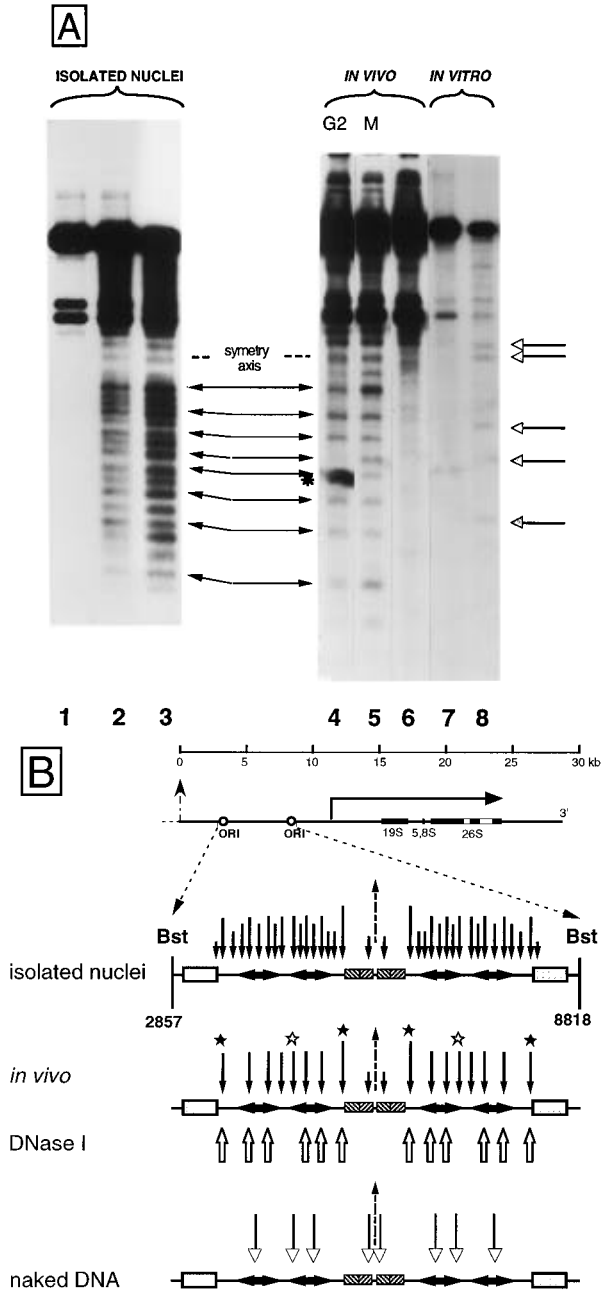


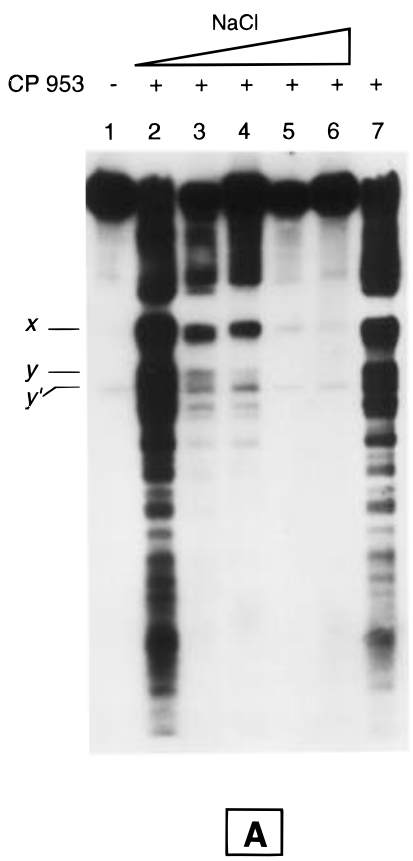
FIGURE 6: Topoisomerase II cleavage in the replication origins region. (A) DNA was digested with *Bst*EII and hybridized with probe 1 to give a 6 kb fragment (see Figure 2). In lanes 1−3, isolated nuclei from a G2 plasmodium were incubated with 10 μM (lane 2) or 50 μM (lane 3) CP-115,953 as described in Materials and Methods. Lane 1 is a control wihout drug. In lanes 4−6, a plasmodium, either in G2 (lane 4) or in M phase (lanes 5 and 6) was treated with 0 μM (lane 6) or 100 μM (lanes 4 and 5) CP-115,953 for 30 min. In lanes 7 and 8, purified rDNA was incubated with 100 units of calf thymus topoisomerase II and either 0 μM (lane 7) or 100 μM CP-115,953 (lane 8). Black arrows indicate the topoisomerase II sites seen both *in vivo* and in isolated nuclei. White arrows indicate the *in vitro* topoisomerase II sites. The asterisk indicates a hybridization artifact. (B) Comparison of the topoisomerase II and DNase I cleavage sites in the 6 kb *Bst*EII region. The horizontal arrows indicate the 308 pb inverted repeated sequences, and the hatched boxes indicate the 220 pb inverted repeated sequences. The dotted vertical arrow is the axis of symmetry found in this region. The gray box is the region homolgous to probe 1 and corresponds to the replication origins. Black stars indicate *in vivo* sites more intense in M than in G2 phase and white stars the *in vivo* sites present during M phase only.

only in mitosis (Figure 6B, indicated by a white star), while others are more intense (black star).

Finally, to estimate the overall influence of chromatin structure on the position of topoisomerase II cleavage sites in this region, purified rDNA was incubated *in vitro* with eukaryotic topoisomerase II in the presence of the inhibitor. As shown in Figure 6, the pattern of sites obtained *in vitro* is different from that observed *in vivo* or in isolated nuclei, and it was also different from the pattern of DNase I sensitive sites.

Topoisomerase II Cleavage in Ribosomal DNA Is Suppressed by High Salt Treatment of Nuclei. To examine whether any of the quinolone-induced cleavage sites observed in *Physarum* rDNA are due to topoisomerase II molecules tightly bound to nucleolar structures, high salt extraction of nuclei was performed prior to quinolone treatment. As shown in Figure 7A, rDNA cleavage disappeared after high salt (over 1.2 M) treatment of the nuclei (lanes 5 and 6). To check that this drop in cleavage level was not due to an alteration in topoisomerase II activity during dialysis of the samples (see Materials and Methods), we dialyzed a nuclei sample without salt extraction during the maximal dialysis time of the experiment (8 h, lane 7 in panel A). A total of 84.5% of the DNA was cleaved in these conditions, compared to 88.5% cleaved at the beginning of the experiment (lane 2). This indicates that the topoisomerase II activity remaining in the nuclei was not affected by prolonged times of dialysis. Although the most prominent sites (e.g. site x, upstream from the transcription start) remain visible after 0.6 M salt extraction, quantitative analysis shows that they decrease in parallel with the other sites (Figure 7B, 1−3). Thus, this experiment does not reveal anchorage sites to the nucleolar matrix where topoisomerase II is supposed to be tightly bound to ribosomal DNA. We observed the same disappearence of topoisomerase II cleavage sites in high salt-treated nuclei, in the other regions of rDNA (following *Eco*RI digestion and hybidizing with probe 3 and following *Pvu*II digestion and hybridizing with probe 4) (data not shown).

The Extent of Topoisomerase II Cleavage in rDNA Is Reduced upon Differentiation of Microplasmodia into Spherula. In several systems, the intensity of drug-induced topoisomerase cleavage in a DNA region was related to the transcriptional activity of this region (Reitman & Felsenfeld, 1990; Riou et al., 1989). We therefore tested if reduced transcription in *Physarum* rDNA affected the extent of cleavage induced by the quinolone. To do this, we took advantage of the possibility of differentiation of *Physarum*; when placed in adverse conditions (salt-only containing medium), *Physarum* microplasmodia undergo differentiation into spherula, and the expression of numerous genes is repressed (Sauer et al., 1969). Among them, the ribosomal genes are the most severely repressed, and it has been shown that it is due to an inhibition of RNA polymerase I during spherulation (Hildebrandt & Sauer, 1977). As spherules are surrounded by a wall, becoming completely resistant to drug penetration, free nuclei were isolated from spherules and incubated with the quinolone CP-115,953. Figure 8 shows that the extent of durg-induced cleavage in rDNA is considerably reduced as spherulation takes place, possibly in connection with the reduced expression of ribosomal genes and/or possible changes in chromatin accessibility or topoisomerase content.



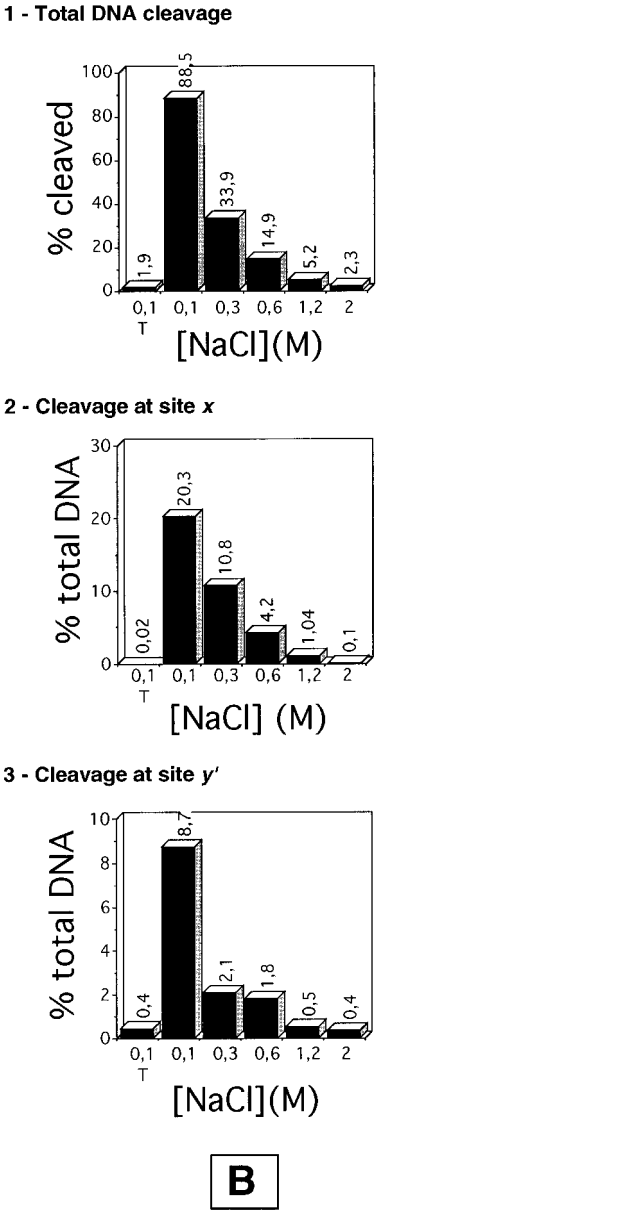


FIGURE 7: Topoisomerase II cleavage in isolated nuclei after salt extraction. (A) Nuclei from a G2 plasmodium were treated with increasing concentrations of NaCl prior to incubation with CP-115,953 (see Materials and Methods). DNA was digested with *Pvu*II and analyzed with probe 3 as in Figure 4B. Lane 1 is a control without drug or NaCl treatment. In lanes 2−7, nuclei were incubated with 50 μM CP-115,953 for 30 min. In lane 2, no NaCl treatment. In lane 3, there was 0.3 M NaCl treatment. In lane 4, there was 0.6 M NaCl treatment. In lane 5, there was 1.2 M NaCl treatment. In lane 6, there was 2 M NaCl treatment. Lane 7 is a control without NaCl treatment but which was kept in buffer B (see Materials and Methods) at 4 °C for 8 h prior to 50 μM CP-115,953 treatment. (B) Quantification of topoisomerase II cleavage after NaCl extraction. The blot presented in A was analyzed using a Phosphorimager (see Materials and Methods). In 1 is shown the level of DNA cleavage after extraction of nuclei with different NaCl concentrations. In 2 and 3 are shown the yield of sites x and y′, respectively.

DISCUSSION

The experiments described in this paper constitute the first attempt to map topoisomerase II sites *in vivo* in the slime mold *P. polycephalum*. We focused our work on ribosomal DNA whose chromatin has been the subject of several studies in the past years (Amero et al., 1988; Lucchini et al., 1987; Pauli et al., 1988). The present work addresses several questions concerning the accessibility of rDNA chromatin in diverse situations: active transcription, cell cycle stage, differentiation state, and the role of topoisomerase II in these processes.

Only the quinolone CP-115,953 was active among several topoisomerase II inhibitors we tested, for their ability either to inhibit *Physarum* growth or to induce *in vivo* topoisomerase II cleavage in the rDNA. They were probably unable to penetrate *Physarum* plasmodia, which has been shown previously to be resistant to drug penetrationon (Mittermayer et al., 1966). In this work, we found that CP-115,953, whose primary target *in vivo* is the topoisomerase II (Elsea et al., 1992), blocks the synchronous cell cycle of *Physarum* plasmodia at the G2/M transition, thus preventing entry into mitosis. *Physarum* rDNA is cleaved after *in vivo* incubation with the quinolone. However, in contrast to results obtained in mammalian cells, no detectable cleavage was observed in the *Physarum* chromosomal DNA, by using several probes of single copy genes (not shown). This situation was also described for the yeast *Saccharomyces cerevisiae* (C. Esnault, personal communication). This

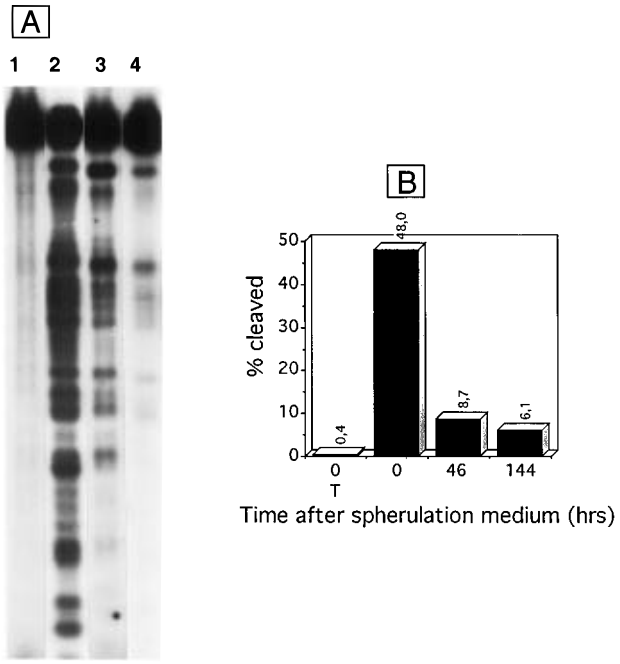


FIGURE 8: Topoisomerase II cleavage sites in isolated nuclei during spherulation. Microplasmodia in exponentially growing phase were transferred in starvation medium in order to induce spherulation. (A) Isolated nuclei were incubated with 10 μM CP-115,953 for 30 min. Purified DNA was restricted with *Eco*RI plus *Bam*HI and probed with probe 2, giving a 16 kb fragment (see Figure 2): lane 1, control without drug treatment, before starvation medium; lane 2, treatment with 10 μM CP-115,953 before starvation medium; lanes 3 and 4, 10 μM CP-115,953, 46 and 144 h in starvation medium, respectively. (B) Quantitative analysis of the topoisomerase II cleavage during spherulation.

preferential rDNA topoisomerase II cleavage might be due either to its constitutive high level of transcription or to its extrachromosomal nature, rendering its chromatin more accessible than chromosomal regions.

A fundamental question was first whether isolation of *Physarum* nuclei perturbs chromatin structure so that the pattern of topoisomerase II cleavage is different in isolated nuclei from *in vivo*. Käs proposed that a redistribution of histones during nuclei isolation may produce a different cleavage pattern (Käs et al., 1993). In *Physarum* rDNA, this pattern is not markedly changed; the map obtained in isolated nuclei is similar to that obtained *in vivo* except for relatively minor differences. These differences reside only in the higher intensity of cleavage in isolated nuclei and the appearence of a number of extra sites. In *Physarum*, it is thus valuable to study topoisomerase II cleavage in isolated nuclei, where a better response to the inhibitor seems to occur.

Mapping of the topoisomerase II sites either *in vivo* or in isolated nuclei allows us to define two regions of topoisomerase II cleavage in *Physarum* rDNA: the transcribed region, where the essence of the cleavage occurs, and the nontranscribed spacer (NTS) region, more weakly cleaved *in vivo*. The regions of rDNA free of topoisomerase II cleavage are also free of DNase I, suggesting that they are not in an accessible chromatin conformation. Our DNase I studies are consistent with previous results suggesting that the transcribed region of *Physarum* rDNA is in an extended, more accessible conformation, compared to the nontranscribed spacer (Amero et al., 1988; Lucchini et al., 1987; Pauli et al., 1988). However, it is noteworthy that a regular pattern of topoisomerase II cleavage sites (every 450 bp) occurs in the 5′ part of the transcription unit, which is not sensitive to DNase I, suggesting that a nucleosomal conformation of chromatin is still present. This pattern of *in vivo* topoisomerase II cleavage every two nucleosomes has been described in other systems (Käs & Laemmli, 1992).

A large region between the transcription unit and the NTS is free from topoisomerase II cleavage, except for one prominent site in the middle of this region. This "protection" from topoisomerase II cleavage and DNase I hypersensitivity might be due to a transcriptionnal initiation complex ever-present to allow for continual transcription throughout the *Physarum* cell cycle or correspond to the anchoring protein complex to a nucleolar matrix, described by Künzler et al. (1984) in this region.

Detailed examination of the 6 kb palindromic region of the NTS limited by the two replication origins allowded us to compare four different maps of this region: (i) an *in vivo* map, (ii) a map generated from isolated nuclei, (iii) a map derived from *in vitro* cleavage studies, and (iv) a DNase I hypersensitivity map. The similarity between some of these maps is evident; the *in vivo*-generated sites correspond to the prominent sites described in isolated nuclei. DNase I-cut sites also represent a subset of *in vivo* sites and suggest that the nucleosomes are more spaced (with an average of 250 pb) than the classical nucleosome phasing (every 200 pb). Sequencing of some topoisomerase cleavage points will determine whether *in vivo* and in isolated nuclei sites are precisely the same. In contrast to the other maps, that obtained with naked DNA is clearly different. This supports the idea that the choice of topoisomerase II sites from a vast repertoire of sequences is largely dependent on chromatin structure. It should be noted that the multiplicity of cleavage sites found in this palindromic region is probably in connection with the numerous inverted repeats present in the region.

A fundamental question is about a possible correlation between topoisomerase cleavage and transcriptional activity; several studies stress this correlation in various systems: heat-shock genes (Udvardy & Schedl, 1993), globin cluster (Reitman & Felsenfeld, 1990), and the *c-myc* gene (Riou et al., 1989). Such a correlation also seems to exist for *Physarum* rDNA, where a high level of transcriptional activity is accompanied by a high level of drug-induced topoisomerase II cleavage activity. Moreover, during differentiation of microplasmodia to spherula (a dormant stage of life), the rDNA transcription is shut off. We show here that at the same time topoisomerase II sites almost completely disappear, presumably because topoisomerase II is no longer required for the rDNA genes' transcription and/or because of a change in chromatin structure.

However, in apparent contradiction to the above cited results are the variations of topoisomerase II cleavage intensity we find during the synchronous division cycle of the plasmodium; rDNA is transcribed all along the cycle, except during the short interval of mitosis when the nucleolus vanishes and the rDNA molecules are dispersed in the whole nucleus (Pierron & Puvion-Dutilleul, 1993). The arrest in rDNA transcription at the time of metaphase has been clearly demonstrated in *in vitro* run-on transcription experiments (Davies & Walker, 1978). The perfect synchrony of *Physarum* nuclei allowed us to be sure that the quinolone treatment began exactly at the time of metaphase. We found

that the intensity of *in vivo* topoisomerase II cleavage in the rDNA increased during mitosis compared to that during G2 and S phases, both in the transcribed and nontranscribed regions. In addition, in the NTS region, there are some mitosis-specific topoisomerase II cleavage sites. It might signify that topoisomerase II is required not only for the rDNA transcription but also for the molecules' segregation at the time of mitosis, using the same preferential cleavage sites. It has been recently shown, in an electron microscope study, that rDNA minichromosomes do not condense during mitosis (Puvion-Dutilleul & Pierron, 1992). The mechanism of their segregation and their migration at the periphery of the dividing nucleus is yet unknown. It is possible that, after their replication, the minichromosomes remain partially catenated during the G2 phase, and this would allow at the time of mitosis for distribution of an equal number of rDNA molecules in each daughter nucleus. This "final" segregation would be mediated by the action of topoisomerase II. This was demonstrated by genetic studies in the yeast *Saccharomyces cerevisiae* (Holm et al., 1989). Another hypothesis could have been that, during the short time of mitosis, rDNA chromatin remains in an open configuration even more accessible to topoisomerase II, when dispersed in the nucleoplasm. But this would not explain why we have such a high level of topoisomerase II rDNA cleavage during the other phases of the cell cycle.

Finally, several authors have stressed the fact that some topoisomerase II sites may serve as anchorage of the chromatin loops to the nuclear scaffold (SAR sites) (Gromova et al., 1995; Iarovaia et al., 1995; Razin et al., 1993). These sites are resistant to a high salt treatment that removes the less tightly bound topoisomerase II molecules. In each of the 35 kb rDNA repeats of CHO cells, there is a unique topoisomerase II site, located about 0.5 kb upstream from the transcription start, highly resistant to salt (Razin et al., 1993). The authors concluded that it was involved in the scaffold attachment of the rDNA units. The situation seems different in *Physarum*, where the strong site 0.7 kb upstream from the start is not refractory to salt treatment and all topoisomerase II cleavage disappears from the rDNA molecule. It is possible that, contrary to CHO cells, *Physarum* rDNA, which is in an episomal form, is not organized as anchored loops or that proteins other than topoisomerase II anchor the *Physarum* rDNA to a nucleolar matrix.

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