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Topological Complexes between DNA and Topoisomerase II and Effects of Polyamines

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ABSTRACT: The polyamines spermine and spermidine were found to enhance the formation of a stable noncovalent complex between mammalian topoisomerase II and DNA. This complex is not associated with DNA strand breaks and forms to a greater extent with supercoiled than with relaxed circular or with linear DNA. Polyamine-induced complex formation is associated with a stimulation of the enzymatic relaxation of DNA supercoils. In these respects, the polyamine-enhanced complex differs from the covalent cleavable complexes stabilized by DNA intercalators such as amsacrine (*m*-AMSA) or epipodophylotoxins such as teniposide (VM-26). In the polyamine-enhanced complex, the topoisomerase II may be a donutlike structure topologically bound to the DNA and able to migrate and dissociate from the ends of linear DNA molecules. At relatively high concentrations, spermine (1 mM) enhances topoisomerase II induced cleavage at certain sites on the SV40 genome that could have regulatory significance.

Because the polyamines spermine and spermidine and topoisomerase II are both normal constituents of chromatin and may have important roles in the regulation of genetic transcription and replication, it appeared useful to determine their interaction in a purified enzyme system.

Polyamines are found at millimolar concentrations in mammalian cells and are present in the nucleus in quantities sufficient to neutralize 15–30% of the DNA negative charge (McCormick, 1978). Crystallographic, NMR, and molecular modeling studies suggest that polyamines bind to the DNA grooves with their charged amines in close proximity to the phosphates of the DNA backbones (Marton & Morris, 1987). Polyamine binding to DNA facilitates DNA condensation and aggregation (Hoopes & McClure, 1981; Baeza et al., 1987) and increases the stability of nucleosome core particles (Morgan et al., 1987).

Two types of DNA topoisomerases have been isolated from mammalian cells. Type I topoisomerase opens and seals reversibly DNA single-strand breaks, while type II topoisomerase opens and seals reversibly DNA double-strand breaks in the presence of ATP (Wang, 1985, 1987). Both topoisomerases can relax DNA supercoiling, and the regulation of DNA supercoiling may affect gene expression and DNA replication (Menzel & Gellert, 1987). DNA decatenation at the end of chromosome replication can be carried out only by topoisomerase II (Holm et al., 1985; Uemura et al., 1987). Topoisomerase II is inhibited by certain DNA intercalators, such as amsacrine (Minford et al., 1986; Nelson et al., 1985; Pommier et al., 1985), and by demethylepipodophyllotoxins, such as teniposide (Long et al., 1984; Chen et al., 1985). The drugs inhibit the enzyme primarily by stabilizing an enzyme–DNA complex in which one or both DNA strands are cleaved and the 5′ terminus of each strand break is covalently linked to a tyrosine residue on the enzyme (Kohn et al., 1987).

Spermidine has been shown to stimulate the activity of mammalian topoisomerase I and to inhibit the activity of the bacterial enzyme (Srivenugopal & Morris, 1985; Srivenugopal et al., 1987). Spermidine has been reported to stimulate the activity of bacterial DNA gyrase (Srivenugopal et al., 1987), a type of topoisomerase II quite different from that found in eukaryotic cells. Polyamines shift the reversible DNA ca-

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tenation–decatenation reactions of mammalian topoisomerase II toward catenation (Marini et al., 1980). Aside from this, there appear to be little or no data on the effects of polyamines on topoisomerase II.

The present study shows that polyamines enhance the formation of stable noncovalent complexes between topoisomerase II and DNA. A hypothesis is proposed concerning the nature and physiological significance of these complexes.

Materials and Methods

Materials. SV40 and λ *Hind*III DNAs, *Ban*I, *Eco*RI, *Hpa*II, and *Kpn*I restriction endonucleases, T4 polynucleotide kinase, and agarose were purchased from Bethesda Research Laboratories (Gaithersburg, MD). ^{3}H-Labeled SV40 DNA was purchased from Lofstrand Laboratory (Rockville, MD). Phosphatase was purchased from New England Biolabs (Beverly, MA) and [γ-^{32}P]ATP from New England Nuclear Research Products (Boston, MA). XAR-5 films (Eastman Kodak Co., Rochester, NY) were used for autoradiography.

Spermine and spermidine were obtained from Sigma (St. Louis, MO). Stock solutions were made in distilled water at 0.1 M and kept frozen at −20 °C. 4′-(9-Acridinylamino)-methanesulfon-*m*-anisidide (*m*-AMSA, amsacrine) (NSC 249992) and teniposide (VM-26) (NSC 122819) were obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, NCI, and from the Bristol Myers Co., respectively. Amsacrine and teniposide stock solutions were made in dimethyl sulfoxide at 10 mM. Amsacrine stock solutions were kept frozen at −20 °C, and teniposide stock solutions were made fresh immediately before use.

Linear and circular SV40 DNAs were prepared by complete *Eco*RI and topoisomerase I digestion of native SV40 DNA, respectively. Enzyme reactions were stopped by $NaDodSO_4$ and proteinase K digestion (1% and 0.5 mg/mL, respectively). DNA was purified by ethanol precipitation after phenol–chloroform extraction and resuspended in TE buffer.

Topoisomerases II and I were purified from mouse leukemia (L1210) cell nuclei, as described previously (Minford et al., 1986). Enzyme purities were checked by SDS–PAGE. One unit of topoisomerase activity was defined as the minimal amount of enzyme required to relax 0.4 μg of SV40 DNA in 30 min at 37 °C.

Topoisomerase II Reaction Conditions. Reactions were performed in 0.01 M Tris·Cl, pH 7.5, 0.05 M KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, and 15 μg/mL bovine serum albumin for 30 min at 37 °C, unless otherwise indicated (Pommier et al., 1987a). Purified topoisomerase was added to each reaction in 5 μL of storage buffer [40% (v/v) glycerol, 0.35 M NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM KH_2PO_4, 0.2 mM dithiothreitol, and 0.1 mM phenylmethanesulfonyl fluoride, pH 6.4]. Reaction volumes were 40 μL for topoisomerase II mediated DNA relaxation and DNA cleavage reactions (agarose gels) and 50 μL for topoisomerase II–DNA complex detection by filter binding assays.

Detection of Topoisomerase II–DNA Complexes. A filter binding assay, which has been described previously (Pommier et al., 1985; Minford et al., 1986), was used; 0.05 μg of ^{3}H-labeled SV40 DNA was reacted with topoisomerase II in the absence or presence of polyamines or amsacrine. Reactions were stopped by adding 1 mL of ice-cold 20 mM Na_2EDTA, and the solution was immediately deposited onto a protein-adsorbent filter (Gelmann Metricel DU800). Filters were rinsed with 2 mL of Na_2EDTA at 4 °C. Three milliliters of LS10 (0.2% Sarkosyl, 40 mM Na_2EDTA, and 2 M NaCl, pH 10) was then deposited onto the filter and allowed to drip out. The flow-through fractions (EDTA and LS10) were collected and counted for ^{3}H radioactivity by liquid scintillation spectrometry. Filters were counted after processing as described previously (Pommier et al., 1985; Minford et al., 1986). DNA filter binding was expressed as the fraction of the total input DNA that was bound to the filter after the EDTA and LS10 washes. Filter binding was less than 2% in the absence of enzyme.

Topoisomerase II Mediated DNA Relaxation and Cleavage. Topoisomerase II reactions were stopped by adding $NaDodSO_4$ (final concentration 1%), and DNA-bound enzyme was digested by proteinase K (0.5 mg/mL for 30 min at 37 °C). Samples were loaded into agarose gels after addition of loading buffer [10× solution consisting of 0.3% bromophenol blue, 16% Ficoll, and 0.01 M Na_2HPO_4]. Agarose gels were made in 1% agarose in TEA buffer (0.04 M Tris–acetate, pH 7.6, and 0.01 M EDTA). Gels were run at 2–3 V/cm overnight, stained with 1 μM ethidium bromide, destained with 1 mM $MgSO_4$ for 30–40 min, and then photographed under UV light with Polaroid 55 or 57 films. Densitometer scanning of the negatives of Polaroid 55 films was performed with a Beckman DU-8B spectrophotometer set at 555 nm (Figure 3) and connected to a computer in order to graph and determine the fraction of DNA present in the bands of interest (Pommier et al., 1987b) (Figure 5).

Genomic Localization of Topoisomerase II Mediated Cleavage in SV40 DNA. SV40 DNA was end-labeled at the *Ban*I or the *Hpa*II restriction sites as follows. Native SV40 DNA was linearized with *Ban*I or *Hpa*II restriction enzymes. The 5′-DNA termini were then dephosphorylated with calf alkaline phosphatase and labeled with [γ-^{32}P]ATP. A second cut was then performed with *Ban*I or with *Hpa*II in the case of *Hpa*II or *Ban*I labeling, respectively. This second cut being at approximately 50 base pairs from the labeling site, any fragment bigger than 50 base pairs could arise only from the longer DNA fragment. Thus, topoisomerase II mediated DNA cleavage sites could be localized. The DNA was phenol extracted and precipitated with cold ethanol between each step of the DNA preparation and at the end of the labeling. A similar procedure has been described previously for the analysis of the DNA cleavage sites induced by topoisomerase II in the presence of amsacrine derivatives in pBR322 DNA (Pommier et al., 1987a).

^{32}P end-labeled DNA was reacted with enzyme with and without amsacrine or teniposide and polyamines. Reactions were stopped as described above, and the DNA was extracted after proteinase K digestion with an equal volume of a 1:1 mixture of phenol–chloroform and then resuspended with 30 μL of 1× loading buffer. Samples were heated at 65 °C for approximately 2 min immediately before being loaded into 1% agarose gels in TBE buffer (Tris–borate–EDTA). Gels were run at 2–3 V/cm overnight and then dried on 3MM paper with a lyophilizer. Dried gels were autoradiographed with Kodak XAR-5 films.

Determination of the genomic localization of topoisomerase II mediated DNA breaks was performed as described previously (Pommier et al., 1987a). Autoradiography films were scanned with a DU-8B Beckman spectrophotometer set at 555 nm. The densitometer was connected to a computer in order to store, graph, and analyze the data. λ *Hind*III or λ *Hind*III–*Eco*RI markers were run in the two outer lanes of all gels in order to check the uniformity of DNA migration throughout the gels. The regression lines of the markers were then determined for each gel by calculating the regression line of the logarithm of the fragment size (in base pairs) versus the migration distance of each fragment from the reference line.

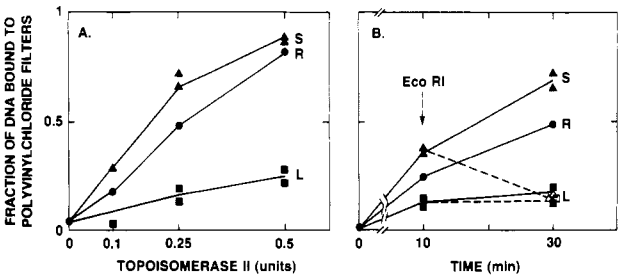

FIGURE 1: Formation of topoisomerase II–DNA complexes in supercoiled (▲) (S), circular relaxed (●) (R), and linear (■) (L) SV40 DNA. (Panel A) Dependence of DNA filter binding on amount of enzyme added to the system. (Panel B) Topoisomerase II–DNA complex production (0.25 unit of enzyme) as a function of time; dashed lines and open symbols show the effect of linearizing the DNA with *Eco*RI, which was added to the system 10 min after starting the reaction. Reactions were performed with 0.05 μg of ^{3}H-labeled SV40 DNA for 30 min at 37 °C in the absence of ATP and were loaded onto protein adsorbent filters and washed with 3 mL of EDTA, followed by 2 mL of LS10. The fraction of the DNA that was protein bound (filter bound) was determined.

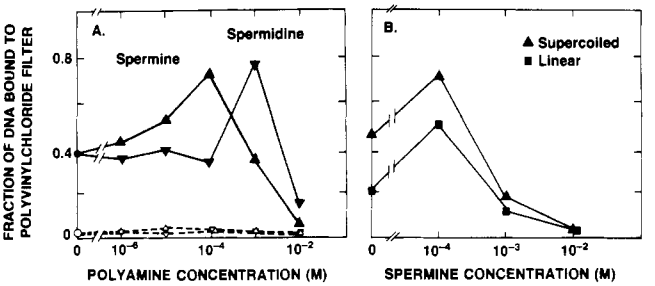

FIGURE 2: Effects of polyamines upon topoisomerase II–DNA complex formation. 0.05 μg of ^{3}H-labeled SV40 DNA was mixed with 0.25 unit of topoisomerase II and the indicated polyamine concentrations for 30 min at 37 °C in the absence of ATP (50-μL reaction volumes). (Panel A) Comparison between the effects of spermine (▲) and spermidine (▼) using supercoiled SV40 DNA; open symbols are for reactions without enzyme [spermine (△); spermidine (▽)]. (Panel B) Comparison of the effects of spermine using supercoiled (▲) or linear (■) DNA. Reaction mixtures were loaded onto protein adsorbant filters and processed as in Figure 1.

Regression coefficients were consistently near 0.99. Reaction lanes were scanned, and the size of each fragment generated by the enzyme was then determined. Fragment size determination was usually within 50 base pairs for a given fragment analyzed in different gels. A final correction was made to determine the genomic position of topoisomerase II mediated DNA breaks by taking into account the position of label relative to the conventional zero position of the SV40 genome (Fiers et al., 1978).

Results

Topological Complexes of DNA and Topoisomerase II. Supercoiled SV40 DNA was incubated with L1210 topoisomerase II without added ATP, and the mixtures were filtered through protein-adsorbing filters which were then washed extensively with Sarkosyl–NaCl solution (0.2% Sarkosyl, 2 M NaCl, and 40 mM EDTA, pH 10). DNA was found to be retained on the filters, and the amount retained increased with topoisomerase II concentration and with time of incubation (Figure 1). The DNA retention was prevented by incubation with proteinase K, supporting the presumption that the DNA retention is due to binding to the topoisomerase II protein. A peculiarity of this DNA–topoisomerase II complex was that it occurred to a much greater extent with circular (either supercoiled or relaxed) DNA than with linear DNA (Figure 1A) and that it was reversed when supercoiled DNA in the complex was linearized by means of *Eco*RI (Figure 1B). In most respects, this complex has the properties described by Osheroff (1987) for noncovalent tight complexes of *Drosophila* topoisomerase II and pBR322 DNA using a gel electrophoretic method (see Discussion). Contrary to the covalent "cleavable" complexes described by Liu et al. (1983), the present topoisomerase II–DNA complexes were not associated with a corresponding number of DNA strand breaks and were not covalent, since they were not seen when the reaction was stopped with $NaDodSO_4$ in the absence of proteinase K (Figure 3B).

Since the complexes observed in these filter binding experiments dissociate when DNA double-strand ends are present and do not require DNA supercoiling, we refer to them as "topological DNA–protein complexes".

Enhancement of the Topological Complexes by Polyamines. Addition of 0.1 mM spermine or 1 mM spermidine consistently increased the topoisomerase II induced filter binding of SV40 DNA (Figure 2A), and this increased filter binding was similar

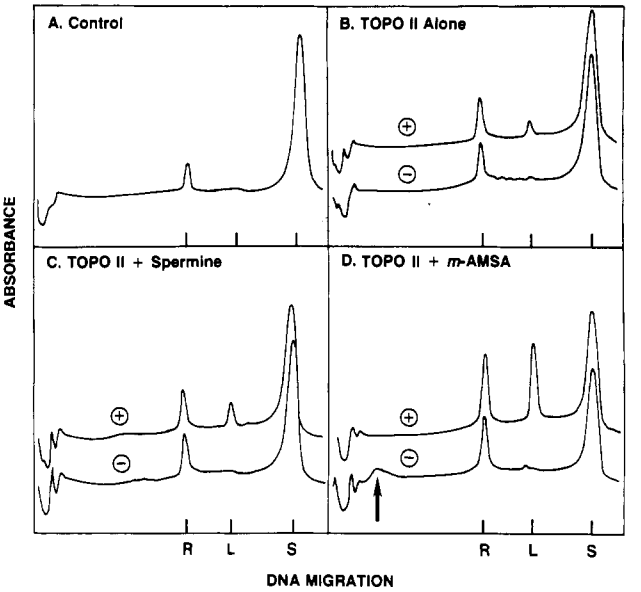

FIGURE 3: Covalent topoisomerase II–DNA complexes formed in the absence or presence of spermine or amsacrine (*m*-AMSA). 0.4 μg of SV40 DNA (panel A) was reacted with 2 units of topoisomerase II in the absence (panel B) or in the presence of 0.1 mM spermine (panel C) or 10 μM *m*-AMSA (panel D). Reactions were carried out in the absence of ATP for 30 min at 37 °C and stopped by adding 1% $NaDodSO_4$. One of the two sample was loaded directly into a 1% agarose gel in TAE buffer (−) and the other after addition of 0.5 mg/mL proteinase K and further digestion for 30 min at 37 °C (+). The gel was photographed under UV light and the Polaroid negative scanned. Each panel corresponds to the densitometer scannings. R, L, and S are the migration positions of relaxed, linear, and supercoiled SV40 DNA, respectively. The arrow in panel D corresponds to amsacrine-induced covalent enzyme–DNA complexes retained near the top of the gel.

in linear and supercoiled DNA (Figure 2B). At 10 mM, both polyamines inhibited filter binding almost completely. Neither spermidine nor spermine induced filter binding in the absence of enzyme (Figure 2A, open symbols).

The topoisomerase II–DNA complexes enhanced by polyamines, observed in the filter binding experiments, were not accompanied by any significant increase in DNA strand cleavage (Figure 3C). DNA cleavage was determined for spermine and for amsacrine at concentrations (0.1 mM and 10 μM, respectively) which increased filter binding approximately 2-fold. Whereas amsacrine produced an approximately stochiometric increase in DNA strand breaks (Figure 3D), spermine produced no significant increase in DNA cleavage

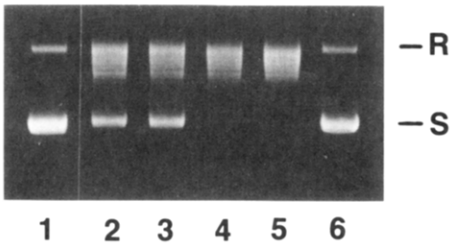

FIGURE 4: Effects of spermine upon topoisomerase II mediated DNA relaxation. 0.4 μg of native SV40 DNA (lane 1) was reacted with 0.5 unit of purified enzyme supplemented with ATP (1 mM) in the presence of increasing concentrations of spermine: 10^{-7} M (lane 2), 10^{-6} M (lane 3), 10^{-5} M (lane 4), 10^{-4} M (lane 5), or 10^{-3} M (lane 6). Reactions were performed as described in the legend of Figure 3, except for the addition of ATP.

(Figure 3C). The lack of induction of DNA cleavage by spermine was found also under the stopping conditions of the filter binding assay (40 mM Na_2EDTA, pH 10, and 0.2% Sarkosyl) (data not shown). Thus, polyamines induce the formation of a complex in which topoisomerase II is tightly bound to DNA but without DNA cleavage.

The stability of the complexes was investigated by determining their resistance to dissociation by $NaDodSO_4$ (Figure 3). SV40 DNA was reacted with topoisomerase II and spermine or amsacrine, under conditions giving similar extents of enzyme–DNA complex formation, as determined by filter binding. When the reaction products are analyzed by gel electrophoresis after treatment with $NaDodSO_4$, covalent DNA–protein complexes would be detected as a slow migrating component, as seen in the case of amsacrine (Figure 3D, arrow); proteinase K digestion showed that this component consisted of protein-linked cleaved DNA, since the DNA reverted to linear molecules (Figure 3D, upper curve). The polyamines, on the other hand, did not enhance either DNA cleavage or protein linkage (relative to that produced by the enzyme alone) since the DNA was neither cleaved nor covalently linked (compare panels C and D of Figure 3). Thus, the complexes formed by polyamines are dissociated by $NaDodSO_4$ and hence are not covalent.

Topoisomerase Activity of Polyamine-Induced Enzyme–DNA Complexes. The effects of polyamines upon topoisomerase II activity were investigated over a wide range of spermine and spermidine concentrations. Enzyme activity was assayed by measuring the ATP-dependent relaxation of supercoiled SV40 DNA. In the reactions shown in Figure 4, low enzyme concentrations were used in order to determine whether polyamines would inhibit or stimulate topoisomerase II mediated DNA relaxation. In the case of spermine, enzyme activity was stimulated at 0.01–0.1 mM and inhibited at 1 mM. In the case of spermidine, DNA relaxation was stimulated at 1 mM (not shown). Thus, polyamine concentrations which increased the extent of complex formation stimulated the topoisomerase-induced DNA relaxation. When complex formation was highest (0.1 mM spermine or 1 mM spermidine), relaxation rate was highest, and when complex formation was inhibited (1 mM spermine), relaxation was inhibited.

The effects of spermine and spermidine upon the kinetics of DNA relaxation were further examined by using higher enzyme concentrations (Figure 5). Enzyme kinetics were quantified by scanning photographic negatives of the gels. The relaxation kinetics were approximately first order both in the absence and in the presence of polyamines. At the optimum polyamine concentrations (0.1 mM spermine or 1 mM spermidine), the relaxation rates were increased approximately 3-fold. On the other hand, 1 mM spermine (which inhibited

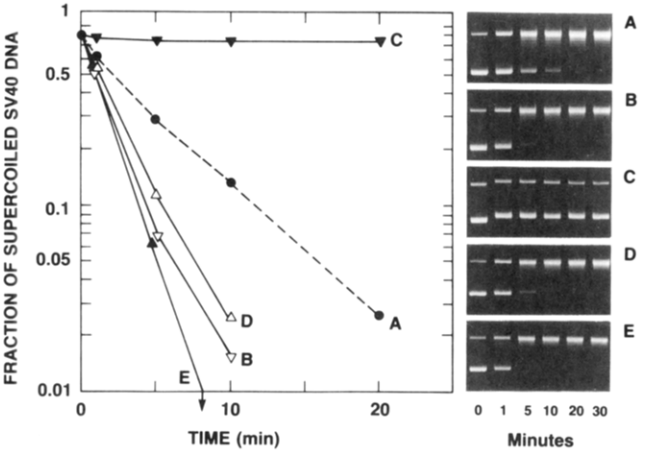

FIGURE 5: Kinetics of topoisomerase II mediated DNA relaxation in the absence or presence of spermine or spermidine. 0.4 μg of native SV40 DNA was reacted with 2 units of enzyme in the presence of ATP (1 mM) under the following conditions: no polyamine added in panel A and curve A; 10^{-4} M spermine in panel B and curve B; 10^{-3} M spermine in panel C and curve C; 10^{-4} M spermidine in panel D and curve D; and 10^{-3} M spermidine in panel E and curve E. Reactions were performed as described in the legend of Figure 3. The right panels show the agarose gel electrophoresis pictures. The left panel shows the quantification of the effects of polyamines after scanning the negatives of the agarose gel pictures. DNA relaxation half-times were 8.5 min for (A), 3 min for (B), 3.5 min for (D), and 2.5 min for (E).

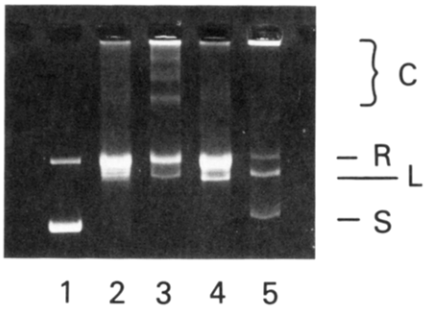

FIGURE 6: Effects of 1 mM spermine upon topoisomerase II mediated DNA topoisomerization reactions. 0.4 μg of native SV40 DNA (lane 1) was reacted with 5 units of enzyme in the presence of 1 mM ATP at 37 °C. After 15 min, an aliquot of the reaction mixture was stopped as described in the legend of Figure 3 (lane 2). The remaining part of the reaction mixture was divided into two tubes, one containing 1 mM spermine (final concentration) (lane 3) and the other without spermine (lane 4). Reactions were performed for an additional 30 min at 37 °C. Lane 5 shows the reaction products in the case where spermine (1 mM) was added from the beginning of the reaction (45-min total incubation time). C, R, L, and S indicate the migration positions of catenated, relaxed, linear, and supercoiled SV40 DNA, respectively.

filter binding) completely inhibited the relaxation reaction.

The apparent inhibitory effect of 1 mM spermine upon DNA relaxation was further investigated in order to determine whether spermine may stimulate an active supercoiling process by the enzyme (Figure 6). Topoisomerase II alone induced DNA relaxation and catenation within 15 min of reaction (lane 2). Incubation in the absence of spermine for an additional 30 min did not increase catenation further, but produced DNA cleavage, which can be seen as the appearance of linear DNA molecules (lane 4); 1 mM spermine increased markedly the formation of DNA catenanes without increasing DNA cleavage or inducing DNA supercoiling (lane 3). The DNA catenation was greater in this case than when spermine was added from the beginning of the reaction (compare lanes 3 and 5 of Figure 6), which is consistent with previously published results (Holden & Low, 1985). Hence, the effects of

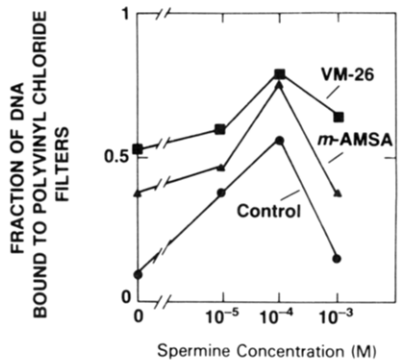

FIGURE 7: Effects of polyamines upon drug-induced topoisomerase II–DNA complex formation. 0.05 μg of ^{3}H-labeled SV40 DNA was reacted with 0.1 unit of topoisomerase II and the indicated polyamine concentrations for 30 min at 37 °C in the absence of ATP (50-μL reaction volumes). (●) Reactions in the absence of drug; (▲) reactions in the presence of 10 μM amsacrine (*m*-AMSA); (■) reactions in the presence of 10 μM teniposide (VM-26).

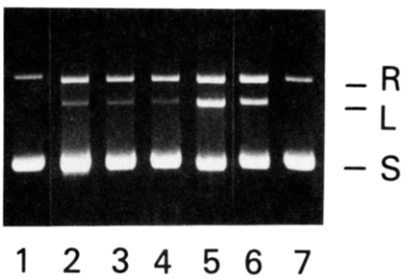

FIGURE 8: Effects of spermine upon amsacrine-induced topoisomerase II mediated DNA cleavage. 0.4 μg of native SV40 DNA (lane 1) was reacted with 2 units of topoisomerase II in the absence of amsacrine (lanes 2–4) or in the presence of 10 μM amsacrine (lanes 5–7). 10^{-4} M spermine was present in lanes 3 and 6 and 10^{-3} M spermine in lanes 4 and 7. Reactions and electrophoresis were carried out as described in the legend of Figure 3.

polyamines are not attributable merely to changes in DNA linking number.

Interaction between Polyamines and Drug-Induced Complexes. Amsacrine and teniposide are known to stabilize topoisomerase II–DNA "cleavable complexes". These complexes can be detected also by filter binding assay (Figure 7). However, they differ from those induced by polyamines because they are associated with DNA cleavage (lane 5, Figure 8) and enzyme inhibition (lane 4, Figure 9); this has been demonstrated over a range of concentrations of amsacrine or teniposide. Amsacrine or teniposide, when combined with spermine, produced essentially additive extents of filter-trappable DNA–topoisomerase complexes (Figure 7).

The next question was whether the enhanced filter binding was due to "cleavable complexes" or "topological complexes"; 10^{-4} M spermine, which produced a nearly 2-fold enhancement of filter binding compared with drug alone, produced no further enhancement of overall DNA cleavage (Figure 8, compare lanes 5 and 6) and prevented the inhibition by amsacrine of DNA relaxation (Figure 9, lane 5). (Gel electrophoresis in the presence of chloroquine showed that the band at position R in lane 5 is not due to nicked DNA but to relaxed topoisomers whose positions are shifted due to amsacrine intercalation during the relaxation reaction.) Thus, the same type of functionally active topological enzyme–DNA complexes was enhanced by spermine in the absence or presence of topoisomerase II inhibitory drugs.

Effects of Polyamines on the Location of Topoisomerase II–DNA Cleavage Sites. The location of topoisomerase II–DNA cleavage complexes was mapped in the SV40 genome

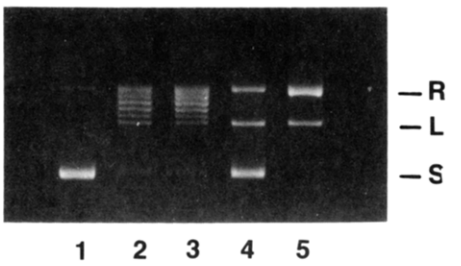

FIGURE 9: Effect of spermine on amsacrine-induced inhibition of topoisomerase II mediated DNA relaxation. Reactions were performed as described in the legend of Figure 8, except for the addition of 1 mM ATP. Lane 1, untreated DNA; lane 2, DNA reacted with enzyme alone; lane 3, same plus 10^{-4} M spermine; lane 4, DNA reacted with enzyme and 10 μM amsacrine; lane 5, same plus 10^{-4} M spermine.

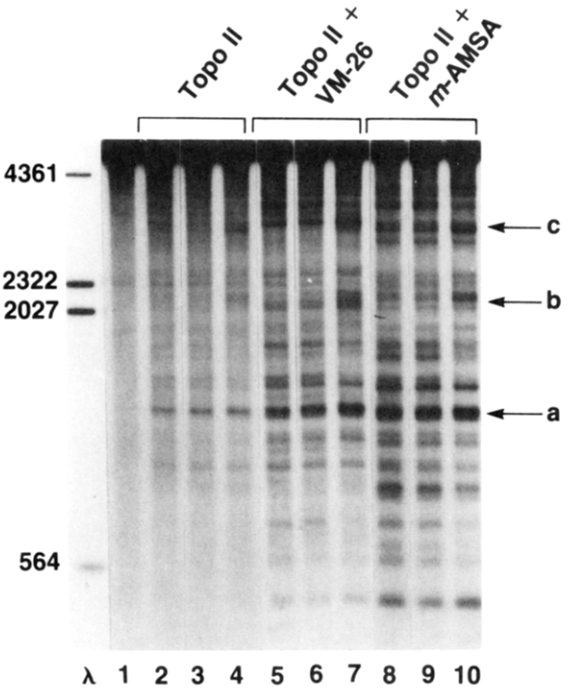

FIGURE 10: Effects of spermine upon the genomic distribution of topoisomerase II induced breaks in linear SV40 DNA. ^{32}P end-labeled SV40 DNA (lane 1) was reacted with purified enzyme in the absence (lanes 2–4) or presence of 10 μM teniposide (VM-26) (lanes 5–7) or amsacrine (*m*-AMSA) (lanes 8–10). Spermine concentrations were 10^{-4} M in lanes 3, 6, and 9 and 10^{-3} M in lanes 4, 7, and 10. Reactions were stopped after 30-min incubation at 37 °C by adding 1% Na-DodSO$_4$ and 0.5 mg/mL proteinase K (final concentrations). After an additional 30-min incubation at 37 °C, the DNA was extracted with phenol–chloroform and loaded into a 1% agarose gel in TBE buffer. ^{32}P end-labeled *Hind*III restrictiono fragments of λ DNA (λ) were used as markers. The numbers on the left correspond to the size of these markers (in bp). Sites "a", "b", and "c" are discussed in the text.

(Figures 10 and 11). In the case of topoisomerase II alone, many minor sites were detected, the most prominent of which (marked "a") was estimated to be at nucleotide position 1562 ± 20. Both teniposide and amsacrine stimulated topoisomerase II mediated DNA cleavage. Many of the cleavage sites appeared at similar locations, but with different relative intensities for the two drugs, and were common to the sites seen in the absence of drug (compare lanes 2, 5, and 8, Figure 10).

Low concentrations of spermine (10^{-4} M and below) did not affect the distribution of cleavage sites (Figure 10, lanes 6 and 9). However, 10^{-3} M spermine (lanes 7 and 10) decreased drug-induced DNA cleavage globally and increased specifically DNA cleavage in two regions, marked "b" and "c". These two sites of enhancement by spermine were also present, although weak, in the absence of amsacrine or teniposide (lane 4).

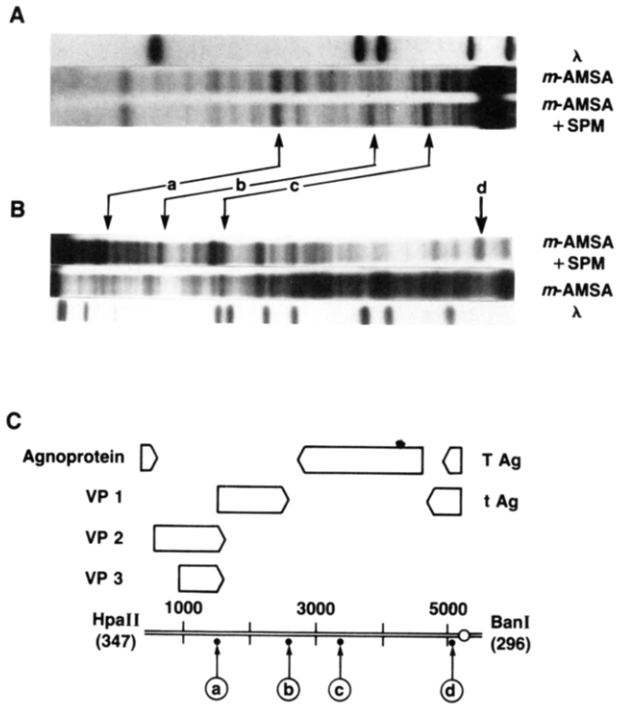

FIGURE 11: Genomic distribution of the sites of enhancement of topoisomerase II mediated DNA cleavage by 1 mM spermine. ^{32}P end-labeled SV40 DNA was reacted with purified enzyme and 10 μM amsacrine (*m*-AMSA) without or with 10^{-3} M spermine. Reactions, electrophoresis, and autoradiographies were performed as described in the legend of Figure 10. In panel A, DNA was labeled at the *Hpa*II restriction site of SV40, electrophoretic migration was from right to left, and *Hind*III restriction fragments of λ DNA were used (λ). In panel B, DNA was radiolabeled at the *Ban*I restriction site, reactions were performed in the presence of ATP, electrophoretic migration was from left to right, and *Hind*III–*Eco*RI restriction fragments of λ DNA were used as DNA migration markers (λ). Panel C shows the genomic location of the DNA cleavage sites stimulated by spermine.

Additional experiments were performed with amsacrine in order to map the spermine enhancement sites over most of the SV40 genome (Figure 11). The strong band at site "a" at position 1562 ± 20 is a major cutting site of topoisomerase II either in the absence or in the presence of amsacrine or teniposide. It is located in the common coding sequence for the three structural proteins VP 1, VP 2, and VP 3 (Figure 11, panel C). Site "b" at position 2620 ± 50 is midway between the early and late transcription origins and corresponds to the termination region of both (Figure 11, panel C). It is characterized by a DNA sequence particularly rich in A and T and by a particular spacing of these residues corresponding to the helical pitch (10.5 nucleotides). Site "c" corresponds to two bands, whose locations were estimated at positions 3400 and 3500 ± 50. This region is located in the middle of the coding sequence of exon 2 of the large T antigen. Site "d" is located near genomic position 5015, at the beginning of the coding sequence of exon 1 of the large T antigen and of the coding sequence of the small T antigen. It was only detected in the *Ban*I restriction fragment of SV40 (Figure 11, panel B); although the band is not very intense on the photograph, longer exposures of the autoradiogram showed it more clearly.

Discussion

Topoisomerase II changes the topology of DNA by catalyzing the passage of one double-stranded segment of DNA through a double-strand break in another segment. Topoisomerase II catalyzed DNA strand passage reactions are known to proceed by way of an intermediate complex in which one or both strands of the DNA are cleaved, and in which each 5′ terminus of the DNA strand breaks is covalently linked to a tyrosine residue on the enzyme (Sander & Hsieh, 1983; Liu et al., 1983). These complexes are stabilized by certain DNA intercalating agents, including amsacrine (Nelson et al., 1984; Pommier et al., 1985; Minford et al., 1986), and by epipodophyllotoxins such as teniposide (Long et al., 1984; Chen et al., 1985).

A different type of complex, tight but noncovalent and induced by nonhydrolyzable ATP analogues, has been described by Osheroff (1987) between topoisomerase II from *Drosophila melanogaster* and supercoiled pBR322 DNA; this complex formed with supercoiled and nicked circular DNA much more than with linear DNA. Using a filter binding method, we find evidence for a complex between topoisomerase II from mouse L1210 cells and SV40 DNA that appears to be similar to that described by Osheroff (Figure 1). The complex in our experiments was tight in that it was not dissociated by washing with Sarkosyl–NaCl, but it was noncovalent because it was dissociated by $NaDodSO_4$, and it was not associated with DNA strand scission. The complex formed to a greater extent with circular DNA than with linear DNA. Relaxed circular double-stranded DNA was only slightly less effective in forming the complex than was the native supercoiled DNA; thus, the smaller extent of complex formation with linear DNA is probably not due primarily to differences in DNA conformation. As proposed by Osheroff, this behavior suggests that the enzyme is mobile along the DNA and can dissociate when it encounters a double-strand end. We refer to this type of complex as a "topological" or "donut" complex (Figure 12).

The topological complex in our system can form in the absence of ATP or analogue cofactor. This and other evidence can most simply be accommodated by the assumption that the initial "donut complex" is with the passing DNA strand rather than with the cleavage strand (Figure 12).

The polyamines, spermine and spermidine, were found to enhance the formation of tight noncovalent topoisomerase II–DNA complexes having all of the properties described above for topological complexes. The simplest interpretation is that the polyamines restrict the mobility of donut complexes along the DNA, and thus reduce the rate at which these complexes reach sites at which the enzyme can dissociate from the DNA (Figure 12). For linear DNA, dissociation would most often occur at the DNA ends. We find, however, that the polyamines also enhance the complexes with circular DNA; hence, we must assume that dissociation can also occur, albeit at a lower rate, at a few specific sites in closed circular DNA.

Polyamines were found to increase the rate of topoisomerase II mediated relaxation of supercoils in native SV40 DNA (Figure 5). This suggests that topological complexes can be rate-limiting intermediates in strand passage reactions, as suggested in the hypothesis illustrated in Figure 12. The stimulation of DNA catenation by polyamines (Figure 6; Marini et al., 1980) could also be due to the increased concentration of topological complexes, as well as the neutralization of DNA negative charge.

Topoisomerase II has been reported to be a major component of the nuclear matrix and has been proposed to serve as DNA attachment points (Earnshaw et al., 1985). Mobile topological complexes would be an appropriate mechanism by which topoisomerase II may attach DNA to the nuclear matrix, since it would circumvent the problem of how transcription or replication would be able to bypass fixed attachment points. Such a role fits naturally into the scheme proposed in Figure

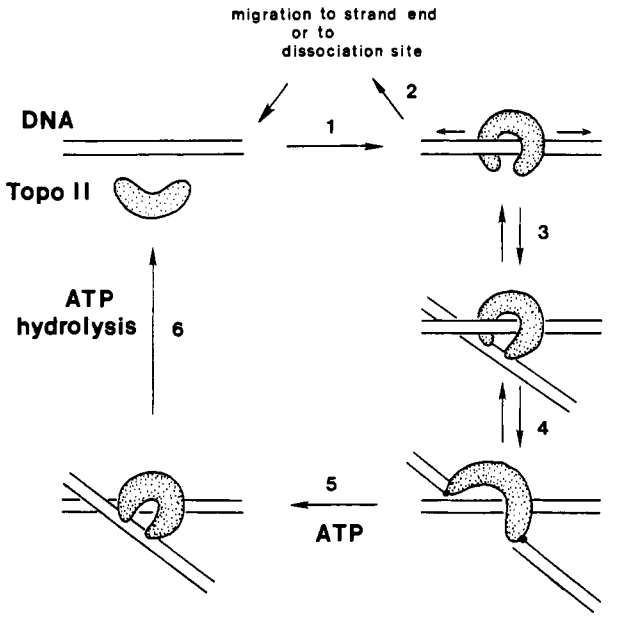

FIGURE 12: Hypothesis for the strand passage process by topoisomerase II and the effects of polyamines. The process begins with the free enzyme wrapping around a DNA double helix (step 1) to form a noncovalent topological "donut" complex which can migrate along the DNA. If the migration leads to a DNA end, then there is a high probability of dissociation, but if a specific dissociation site is encountered, then dissociation can occur at that site with low probability (step 2). The binding of polyamines to DNA impedes the migration (step 2) and thereby reduces the dissociation rate and increases the local concentration of topological complexes. Topological complexes interact reversibly with a second DNA strand segment which becomes bound to the two cleavage sites of the enzyme (step 3). The DNA strands are then cleaved, and the 5′ strand termini are covalently linked to the enzyme (step 4). The cleavage complex can close in two ways: (1) it can reverse in the absence of ATP, but this requires that a DNA double strand must occupy the interior of the protein, and therefore this path does not normally produce strand passage (reverse step 4); or (2) occupancy of a receptor site by ATP or a nonhydrolyzable analogue can force closure, and strand passage (step 5). ATP hydrolysis regenerates the enzyme (step 6).

12, because topological enzyme complexes could form easily and directly without commitment to further reactions, and dissociation would ordinarily be a rare event. Since polyamines bind selectively to AT-rich regions of DNA, our conjecture that polyamines restrict the mobility of "donut complexes" would suggest that AT-rich regions may act as polyamine-modulated insulators between different DNA attachment loops.

Spermine (1 mM) enhanced the intensity of topoisomerase II induced cleavage at a few specific sites on the SV40 genome (Figures 10 and 11). In the presence of amsacrine or teniposide, each of which enhanced cleavage at a distinctive pattern of sites, spermine nevertheless enhanced cleavage at the same few sites that it enhanced in the absence of the drugs (Figure 10). It is of interest that the specific sites of spermine-enhanced cleavage were in regions of possible physiological significance (Figure 11). This is particularly striking for site "b" in Figure 12 which is in the termination region of both the early and late transcription. A closer examination of the DNA sequence in the four regions of enhancement of topoisomerase II mediated DNA cleavage showed that these regions had the following in common: (1) several stretches of consecutive adenine or thymine nucleotides, which could form DNA bends or adopt an A-DNA conformation (Wu et al., 1981; Griffith et al., 1986); (2) diad axis of symmetry sequences (palindromes), which could form DNA cruciforms (Sullivan & Lilley, 1986); and (3) stretches of alternating purine-pyrimidine, which could form Z-DNA (Nordheim & Rich, 1983). Spermidine and spermine have been reported to induce alternate conformations of DNA, such as A- or Z-DNA [reviewed by Marton and Morris (1987)], to stabilize double-stranded DNA (Srivenugopal et al., 1987), and to bend the DNA helix by binding into the major groove (Feuerstein et al., 1986). Hence, the effect of 1 mM spermine may be due to local alterations in DNA conformation or to the redistribution of mobile topoisomerase complexes along the DNA.

Since polyamines and topoisomerase II are normal chromatin constituents, it is tempting to suggest that polyamines play a role in the regulation of topoisomerase II and that this may bear on the known effects of polyamines on cells (Pegg, 1986; Marton & Morris, 1987).

Registry No. *m*-AMSA, 51264-14-3; VM-26, 29767-20-2; spermine, 71-44-3; spermidine, 124-20-9; topoisomerase, 80449-01-0.

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In Vitro Assembly of 30S and 70S Bacterial Ribosomes from 16S RNA Containing Single Base Substitutions, Insertions, and Deletions around the Decoding Site (C1400)

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ABSTRACT: An in vitro system developed for the site-specific mutagenesis of 16S RNA of *Escherichia coli* ribosomes [Krzyzosiak et al. (1987) *Biochemistry 26*, 2353-2364] was used to make 10 single base changes around C1400, a residue known to be at the decoding site. C1400 was replaced by U, A, or G, five single base deletions at and to either side of C1400 were made, and C or U was inserted next to C1400. Another mutant possessed seven additional nucleotides at the 3′ end of the 16S RNA such that a stem and loop involving the anti-Shine–Dalgarno sequence could form. Each of the mutant RNAs was reconstituted with a complete mixture of 30S proteins to yield 30S ribosomes. Modified in vitro reconstitution conditions were required to obtain assembly of all of the synthetic ribosomes. Quantitative HPLC analysis of the protein content of each mutant showed that all of the proteins were present. The ability of synthetic 30S to form 70S particles under functional assay conditions was about 75% that of natural 30S and was unchanged by any of the mutations except for the deletion of G1401, which decreased the association activity under the standard conditions to 35–40% of synthetic 30S. That part of the ribosomal P site which interacts with the anticodon loop of tRNA was investigated by near-UV (>300 nm) induced cross-linking of AcVal-tRNA. Cross-linking depended on both 30S subunits and the correct codon. The cross-linking yield of all mutants with a pyrimidine at position 1400 was equal to control isolated 30S, and the first-order rate constants for cross-linking of those mutants tested were like reconstituted natural 30S. The site of cross-linking for mutants with a C or U insertion between C1400 and G1401 was shifted to the inserted residue. Cross-linking to the base 5′ to G1401 rather than to the residue 3′ to C1399 indicates that G1401 is an important structural determinant of the P site.

In a previous publication, we described a novel system for creating and studying *Escherichia coli* ribosomes mutant in their RNA moiety (Krzyzosiak et al., 1987). This was accomplished by modifying a 16S RNA gene to allow synthesis in vitro by T7 RNA polymerase. The transcription product contained three additional 5′-G residues with a presumed 5′-triphosphate terminus, a base change from A → G at position 2, and, in 20% of the molecules, an additional residue at the 3′ end. Otherwise, the transcript was a faithful copy of the 1542 nucleotides of 16S RNA, except that the 10 modified bases known to be present in that RNA (Noller, 1984) were lacking. The RNA could be reconstituted into 30S particles by using a modification of the procedures described by Held et al. (1973). The particles were morphologically indistinguishable from 30S ribosomes and were shown to possess considerable tRNA binding activity.

Two mutant ribosomes, A1400 and G1400, were also described in that work. This position, normally a C residue, was chosen for initial study because a considerable body of evidence from our laboratory had implicated this position in the decoding site function of the ribosome (Ehresmann & Ofengand, 1984; Gornicki et al., 1984; Ciesiolka et al., 1985; Nurse et al., 1987). We have now made eight additional mutations in the vicinity of C1400 plus one at the 3′ end and have studied

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