Site-Specific DNA Cleavage by *Chlorella* Virus Topoisomerase II†

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ABSTRACT: The DNA cleavage reaction of topoisomerase II is central to the catalytic activity of the enzyme and is the target for a number of important anticancer drugs. Unfortunately, efforts to characterize this fundamental reaction have been limited by the low levels of DNA breaks normally generated by the enzyme. Recently, however, a type II topoisomerase with an extraordinarily high intrinsic DNA cleavage activity was isolated from *Chlorella* virus PBCV-1. To further our understanding of this enzyme, the present study characterized the site-specific DNA cleavage reaction of PBCV-1 topoisomerase II. Results indicate that the viral enzyme cleaves DNA at a limited number of sites. The DNA cleavage site utilization of PBCV-1 topoisomerase II is remarkably similar to that of human topoisomerase IIα, but the viral enzyme cleaves these sites to a far greater extent. Finally, PBCV-1 topoisomerase II displays a modest sensitivity to anticancer drugs and DNA damage in a site-specific manner. These findings suggest that PBCV-1 topoisomerase II represents a unique model with which to dissect the DNA cleavage reaction of eukaryotic type II topoisomerases.

The ability to resolve knots and tangles in the genetic material is essential to the survival of all proliferating cells (*1*−*3*). This function is performed in vivo by enzymes known as type II topoisomerases (*2*−*8*). These enzymes alter the topological state of DNA by passing an intact double helix through a transient double-stranded break that they generate in a separate DNA segment (*2*, *4*−*8*). Since topoisomerase II must create a gate in the DNA in order to carry out its critical strand passage reaction (*9*, *10*), the DNA cleavage and religation events mediated by the enzyme are fundamental to its physiological activities (*11*−*13*).

Concomitant with the scission event, topoisomerase II forms covalent bonds with the 5′-termini of the cleaved DNA (*11*, *12*, *14*). These linkages prevent dissociation of the DNA when the nucleic acid gate is opened. Although the formation of covalent topoisomerase II−DNA cleavage complexes maintains the integrity of the genetic material during the strand passage reaction, their presence is potentially dangerous to the cell. When a DNA tracking enzyme, such as a polymerase or helicase, attempts to traverse one of these complexes, it often converts the transient opening in the double helix to a permanent double-stranded break (*6*, *15*, *16*). The presence of these permanent DNA breaks can initiate mutagenic recombination events and in some cases trigger cell death pathways (*16*, *17*).

This lethal aspect of topoisomerase II has been exploited for the treatment of human malignancies. Some of the most successful anticancer agents currently in clinical use kill cells by increasing the physiological concentration of topoisomerase II−DNA cleavage complexes (*5*, *6*, *16*−*19*).

Reflecting the potential danger associated with the topoisomerase II-mediated DNA scission event, equilibrium levels of cleavage complexes normally are very low. For example, under optimal conditions in vitro, less than 1% of human topoisomerase IIα is covalently complexed with its DNA substrate (*20*).

Unfortunately, the low levels of scission usually associated with enzyme activity severely limit the ability to study the critical DNA cleavage event. Recently, however, a type II topoisomerase that has an extreme proclivity to cleave DNA was isolated from *Chlorella* virus PBCV-1 (*20*−*23*). PBCV-1 is the prototypical member of a group of viruses that infects *Chlorella*-like algae (*22*, *24*, *25*). Although most members of the genus *Chlorella* are free living in nature, those that are susceptible to PBCV-1 are hereditary endosymbionts that live within *Paramecium bursaria* (*26*, *27*). PBCV-1 topoisomerase II is synthesized 60−90 min postinfection and remains throughout the life cycle of the virus. The precise role of the enzyme in viral infection is not known; however, it has been proposed to function in the late stages of viral replication, packaging, or recombination (*20*, *22*).

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In marked contrast to human topoisomerase IIα, as much as 50% of PBCV-1 topoisomerase II exists in a DNA cleavage complex at equilibrium with plasmid substrates (*20*). With the exception of its unusually high DNA cleavage activity, the catalytic properties of the viral enzyme appear to be typical of eukaryotic type II topoisomerases (*20*, *23*). Moreover, although PBCV-1 topoisomerase II is smaller than all previously described type II enzymes, it is structurally similar to eukaryotic topoisomerase II. Both its gyrB and gyrA homology domains are contained in a single polypeptide chain, and the amino acid sequence of PBCV-1 topoisomerase II is nearly 50% identical to human topoisomerase IIα (*23*).

These properties suggest that PBCV-1 topoisomerase II may provide insight into the DNA scission activity of eukaryotic type II topoisomerases. Therefore, to further characterize the viral enzyme as a potential model system, the present work compared site-specific DNA cleavage by PBCV-1 topoisomerase II to that of human topoisomerase IIα. PBCV-1 topoisomerase II cleaved DNA at a relatively restricted spectrum of sites, and its DNA site specificity was similar to that of the human enzyme. However, levels of scission at these sites were much higher with the viral enzyme than with human topoisomerase IIα. On the basis of these findings, we propose that PBCV-1 topoisomerase II represents a unique model with which to study the DNA cleavage reaction of type II topoisomerases.

EXPERIMENTAL PROCEDURES

Materials. PBCV-1 topoisomerase II was expressed in yeast (*Saccharomyces cerevisiae*) (*23*) and purified as described by Lavrukhin et al. (*23*) or Kingma et al. (*28*). Human topoisomerases IIα and II*β* were expressed in yeast (*29*) and purified by the protocol of Kingma et al. (*28*). Yeast topoisomerase II was isolated from *S. cerevisiae* by the procedure of Elsea et al. (*30*) as modified by Burden et al. (*31*). *Drosophila melanogaster* topoisomerase II was purified from embryonic Kc cells as described by Shelton et al. (*32*). Negatively supercoiled pBR322 DNA was prepared as described (*33*). A tetrahydrofuran phosphoramidite (dSpacer CE phosphoramidite) was obtained from Glen Research. Restriction endonucleases, Klenow DNA polymerase, and T4 polynucleotide kinase were from New England Biolabs. Proteinase K and [α-^{32}P]dCTP (~6000 Ci/mmol) were from Amersham. [γ-^{32}P]ATP (~6000 Ci/mmol) was from ICN. Etoposide was from Sigma, amsacrine was from Bristol-Myers Squibb, and CP-115,953 was from Pfizer. Etoposide, amsacrine, and CP-115,953 were stored at 4 °C as 10 or 20 mM stock solutions in 100% DMSO. All other chemicals were of analytical reagent grade.

Plasmid DNA Cleavage. DNA cleavage reactions were based on the procedure of Fortune et al. (*20*). Each reaction contained 10 nM negatively supercoiled pBR322 DNA in a total of 20 μL of topoisomerase II reaction buffer. Reaction buffers utilized were PBCV-1 (10 mM Tris-HCl, pH 8.5, 62.5 mM KCl, 62.5 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM Na_2EDTA, and 2.5% glycerol), yeast (10 mM Tris-HCl, pH 7.9, 35 mM KCl, 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM Na_2EDTA, and 2.5% glycerol), *Drosophila* (10 mM Tris-HCl, pH 7.9, 70 mM KCl, 65 mM NaCl, 5 mM $MgCl_2$, 0.1 mM Na_2EDTA, and 2.5% glycerol), and human (10 mM Tris-HCl, pH 7.9, 135 mM KCl, 5 mM $MgCl_2$, 0.1 mM Na_2-EDTA, and 2.5% glycerol). Cleavage was initiated by the addition of topoisomerase II. Reactions were incubated for 6 min at 25 °C (PBCV-1), 28 °C (yeast), 30 °C (*Drosophila*), or 37 °C (human topoisomerase IIα or II*β*) to establish DNA cleavage/religation equilibria. Cleavage intermediates were trapped by adding 2 μL of 1.2% SDS followed by 2 μL of 120 mM Na_2EDTA, pH 8.0 (PBCV-1), or 2 μL of 5% SDS followed by 2 μL of 250 mM Na_2EDTA, pH 8.0 (all other enzymes). Proteinase K was added (2 μL of 0.8 mg/mL), and reactions were incubated for 30 min at 45 °C to digest the type II enzyme. Samples were mixed with 2 μL of 60% sucrose in 10 mM Tris-HCl, pH 7.9, heated for 2 min at 70 °C, and subjected to electrophoresis in 1% agarose gels in 40 mM Tris−acetate, pH 8.3, and 2 mM EDTA containing 0.5 μg/mL ethidium bromide. Cleavage was monitored by the conversion of negatively supercoiled plasmid DNA to linear molecules. DNA bands were visualized by UV light, photographed through Kodak 23A and 12 filters with Polaroid type 665 positive/negative film, and quantitated by scanning photographic negatives with an E-C apparatus model EC910 scanning densitometer in conjunction with Hoefer GS-370 data system software. Alternatively, DNA bands were quantitated using an Alpha Innotech digital imaging system.

Cleavage of Linear DNA Fragments. A unique ^{32}P-3′-end-labeled linear DNA substrate was prepared by digesting negatively supercoiled pBR322 DNA with *Bam*HI, labeling with [α-^{32}P]dCTP and Klenow DNA polymerase in the presence of dATP, dGTP, and dTTP, and digesting the labeled product with *Eag*I. The resulting 3797 bp *Bam*HI−*Eag*I fragment was purified by gel electrophoresis using DE81 ion-exchange paper (Whatman) (*30*). A linear 3607 bp *Eco*RI−*Pst*I fragment labeled at the *Eco*RI site was prepared by a similar protocol.

DNA cleavage reactions contained 10 nM labeled DNA substrate and 2.5−30 nM PBCV-1 topoisomerase II in 20 μL of PBCV-1 reaction buffer. Cleavage was initiated by the addition of topoisomerase II, and reactions were incubated, stopped, and processed as described above for the PBCV-1 enzyme. Reaction products were resolved by electrophoresis in a 1% agarose gel in 100 mM Tris−borate, pH 8.3, and 2 mM EDTA. The gel was dehydrated by blotting with paper towels, dried under partial vacuum at 50 °C, and visualized using a PhosphorImager (Molecular Dynamics).

Mapping of Topoisomerase II DNA Cleavage Sites. Specific sites of DNA cleavage were mapped as described by Burden et al. (*31*). A unique ^{32}P-5′-end-labeled linear DNA substrate was prepared by digesting negatively supercoiled pBR322 DNA with *Alw*NI, labeling with T4 polynucleotide kinase using [γ-^{32}P]ATP, and digesting the labeled product with *Bsa*I. The desired 549 bp fragment was purified on a nondenaturing 5% polyacrylamide gel. A 589 bp *Nde*I−*Alw*NI fragment of pBR322, ^{32}P-5′-end-labeled at the *Nde*I terminus, was generated in a similar manner.

Cleavage reactions contained 1.5 nM labeled 549 or 589 bp substrate, 1 mM ATP, and PBCV-1 topoisomerase II (4.5 nM) or human topoisomerase IIα (60 nM) in a total of 50 μL of the appropriate reaction buffer. Some reactions contained 50 μM etoposide, amsacrine, or CP-115,953, or drug solvent (all drug reactions and controls contained 1%

DMSO). Cleavage was initiated by the addition of topoisomerase II. Samples were incubated for 6 min at 25 °C (PBCV-1) or 10 min at 37 °C (human) to allow establishment of a cleavage/religation equilibrium. Cleavage intermediates were trapped by adding 5 μL of 1.2% SDS followed by 5 μL of 120 mM Na_2EDTA, pH 8.0 (PBCV-1), or 5 μL of 10% SDS followed by 3.75 μL of 250 mM Na_2EDTA, pH 8.0 (human). Samples were treated with proteinase K (5 μL of 0.8 mg/mL) for 30 min at 45 °C, precipitated twice with ethanol, resuspended in 5 μL of 40% formamide, 0.02% bromophenol blue, and 0.02% xylene cyanole FF, and subjected to electrophoresis in denaturing 7 M urea and 8% acrylamide gels in 100 mM Tris−borate, pH 8.3, and 2 mM Na_2EDTA. Gels were fixed for 5 min in 10% methanol/10% acetic acid and dried. Cleavage products were visualized using a PhosphorImager.

Cleavage of Oligonucleotide Substrates. Oligonucleotides were generated that corresponded to segments of pBR322 plasmid DNA that contained strong cleavage sites for topoisomerase II. An 80-mer double-stranded oligonucleotide (residues 2968−3047 of pBR322, containing cleavage site 2) was formed by annealing the single-stranded oligonucleotides 5′-CTAGAAGGACAGTATTTGGTATCTGCGCTCTG-CTGAAGCC↓AGTTACCTTCGGAAAAAGAGTTGGTAGCTC-TTGATCCGGC-3′ and 5′-GCCGGATCAAGAGCTACCAA-CTCTTTTTCCGAAGGT↓AACTGGCTTCAGCAGAGCG-CAGATACCAAATACTGTCCTTCTAG-3′. In addition, a series of nested 60-, 50-, 40-, and 30-mer double-stranded oligonucleotides were generated. Single-stranded oligonucleotides for the shorter substrates were as for the 80-mer, except that they were shorter on each end by an equal number of bases to give the desired length. A second 40-mer (residues 2409−2448 of pBR322, containing cleavage site 1) was generated by annealing the single-stranded oligonucleotides 5′-GTATCAGCTCACTCAAAGGC↓GGTAATACGGTTAT-CCACAG-3′ and 5′-CTGTGGATAACCGTAT↓TACCGCCTTT-GAGTGAGCTGATAC-3′. In some cases, oligonucleotides contained a tetrahydrofuran abasic site analogue at a single position instead of the normal nucleotide. Cleavage sites in the above sequences are denoted by arrows. All oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer.

DNA cleavage reactions were based on the procedure of Kingma et al. (*28*). Each reaction contained 100 nM oligonucleotide in a total of 20 μL of the appropriate reaction buffer. Some reactions contained 50 μM etoposide, amsacrine, or CP-115,953. All drug reactions and controls contained 1% DMSO (drug solvent). Cleavage was initiated by the addition of PBCV-1 topoisomerase II or human topoisomerase IIα (enzyme concentration was 200 nM unless otherwise indicated). Reactions were incubated, terminated, and treated with proteinase K (2 μL of 1.6 mg/mL) as described above for plasmid DNA cleavage. Proteinase K treatment was omitted in experiments that analyzed the enzyme−DNA covalent linkage. Samples were precipitated twice with ethanol, resuspended in 5 μL of 90% formamide, 6% sucrose, 1 mM Tris-HCl, pH 7.9, 0.05% bromophenol blue, and 0.05% xylene cyanole FF, and subjected to electrophoresis in denaturing 7 M urea and 14% acrylamide gels in 100 mM Tris−borate, pH 8.3, and 2 mM Na_2EDTA. Gels were fixed, dried, and analyzed as described in the preceding section.

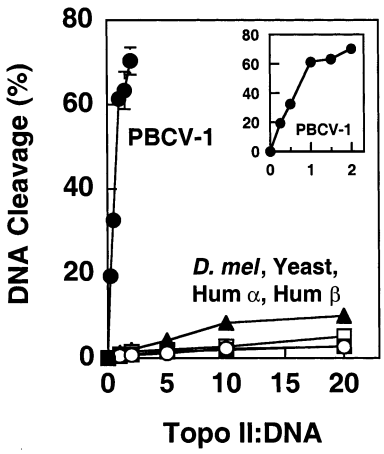

FIGURE 1: DNA cleavage by type II topoisomerases. The ability of several type II topoisomerases to cleave negatively supercoiled pBR322 plasmid DNA was determined. A range of enzyme:DNA ratios was employed (at a constant DNA concentration of 10 nM). Results are shown for PBCV-1 (●), yeast (□), and *Drosophila* (▲) topoisomerase II and human topoisomerase IIα (○) and IIβ (■). PBCV-1 topoisomerase II DNA cleavage also is shown in the inset. Cleavage is expressed as the percentage of DNA substrate in a topoisomerase II−DNA cleavage complex at equilibrium. Error bars represent the standard errors of the mean for two independent experiments.

RESULTS

DNA Cleavage Mediated by PBCV-1 Topoisomerase II. A previous study demonstrated that the DNA cleavage activity of *Chlorella* virus PBCV-1 topoisomerase II was dramatically higher than that of human topoisomerase IIα (*20*). To extend this finding, the ability of the viral enzyme to cleave negatively supercoiled pBR322 plasmid DNA was compared to that of every well-characterized eukaryotic type II topoisomerase including *Drosophila* and yeast topoisomerase II and human topoisomerase IIα and topoisomerase IIβ. As seen in Figure 1, ~50% of the DNA was cleaved at a 1:1 ratio of viral enzyme to plasmid. In fact, scission could not be measured accurately at enzyme:plasmid ratios higher than 2:1, due to the formation of multiple cleavage complexes per plasmid and the subsequent generation of linear molecules that were less than unit length.

In marked contrast to PBCV-1 topoisomerase II, none of the other enzymes cleaved more than 1.3% of the supercoiled DNA substrate at an enzyme:plasmid ratio of 1:1. Even at an enzyme:plasmid ratio of 20:1, *Drosophila* topoisomerase II was the only other enzyme that cleaved as much as 10% of the plasmid. Thus, the DNA cleavage activity of PBCV-1 topoisomerase II clearly distinguishes the viral enzyme from every well-characterized eukaryotic type II topoisomerase.

Site-Specific DNA Cleavage by PBCV-1 Topoisomerase II. Despite the fact that PBCV-1 topoisomerase II cleaves DNA much better than does human topoisomerase IIα, it displays a similar binding affinity for plasmid DNA (*20*). Moreover, rates of DNA religation mediated by the viral enzyme are faster than those observed for the human enzyme (*20*). These findings suggest that the high levels of DNA scission with PBCV-1 topoisomerase II are not due to inordinately tight DNA binding or impaired religation. Thus, it appears that they reflect a greatly enhanced ability of the bound enzyme to form DNA cleavage complexes.

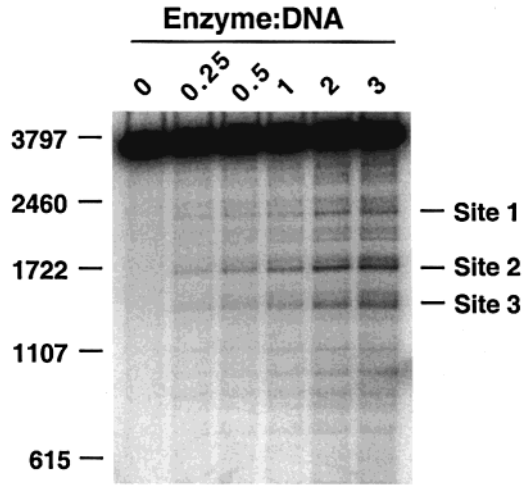

FIGURE 2: Cleavage of linear DNA fragments by PBCV-1 topoisomerase II. An agarose gel is shown. A topoisomerase II titration was carried out such that enzyme:DNA ratios ranged from 0.25 to 3 (at a constant DNA concentration of 10 nM). A linear 3.8 kb *Eag*I−*Bam*HI fragment of pBR322 DNA, labeled at the *Bam*HI terminus, was utilized as the cleavage substrate. The three strongest cleavage bands are labeled sites 1−3. Molecular weight standards (bp) are indicated on the left.

Two scenarios can be envisioned that are consistent with this conclusion. First, PBCV-1 topoisomerase II may interact with a limited number of DNA sites, but the forward rate of scission at these sites is much higher than typically observed with other type II enzymes. Alternatively, the rate of scission at any given DNA sequence may be similar to that of other type II topoisomerases, but the viral enzyme utilizes a broader spectrum of sites. At the present time, no assays are available that can monitor the forward rate of topoisomerase II-mediated DNA cleavage independently from DNA binding and religation. Therefore, the DNA site specificity of PBCV-1 topoisomerase II was examined to help to distinguish between these two possibilities.

First, sites of DNA cleaved by the viral enzyme were analyzed at the resolution of agarose gels. Two overlapping linear fragments (3.6−3.8 kb in length) that encompassed all of pBR322 were employed as substrates. Data for a fragment that extended from the *Eag*I site to the *Bam*HI site are shown in Figure 2. PBCV-1 topoisomerase II cleaved at a relatively small number of sites. Approximately 15 bands were seen within the 3.8 kb fragment, the three most prominent of which are labeled (top to bottom) 1 through 3. These results suggest that PBCV-1 topoisomerase II is not a promiscuous enzyme that cleaves DNA at a large number of sites.

Second, sites of PBCV-1 topoisomerase II-mediated DNA cleavage were mapped in a series of shorter DNA fragments (500−600 bp in length) and compared to those cleaved by human topoisomerase IIα.[1] These fragments contained the three strongest cleavage sites in pBR322 identified in the agarose gel experiments. Maps were analyzed by polyacrylamide gel electrophoresis, allowing sites to be characterized at the nucleotide level.

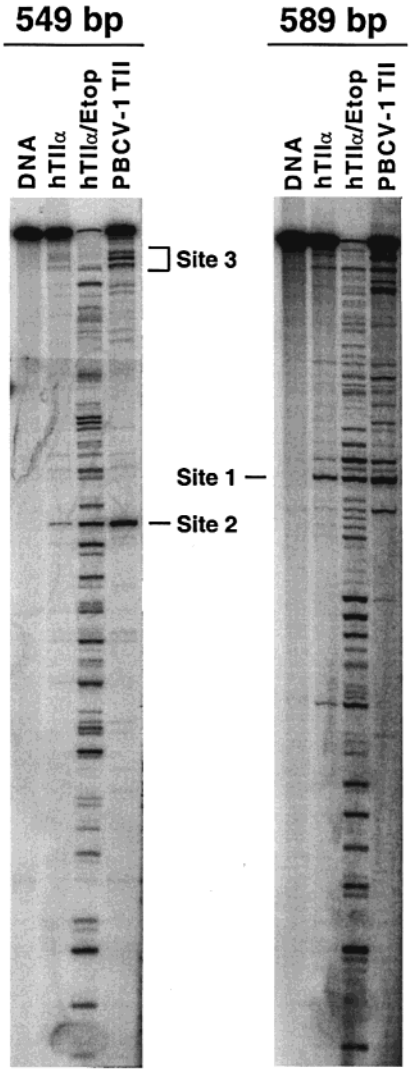

FIGURE 3: PBCV-1 topoisomerase II DNA cleavage map. A polyacrylamide gel is shown. Two linear substrates derived from pBR322 plasmid DNA were used in cleavage reactions: a 549 bp *Alw*NI−*Bsa*I fragment, labeled at the *Alw*NI terminus (left panel), and a 589 bp *Nde*I−*Alw*NI fragment, labeled at the *Nde*I terminus (right panel). Controls that lacked enzyme were included (DNA). Cleavage reactions contained human topoisomerase IIα in the absence (hTIIα) or presence of 50 μM etoposide (hTIIα/Etop) or PBCV-1 topoisomerase II (PBCV-1 TII). The enzyme:DNA ratio was 40:1 for human and 3:1 for PBCV-1 reactions. Cleavage sites 1−3 are indicated.

Maps of a 549 bp fragment (containing sites 2 and 3) and a 589 bp fragment (containing site 1) are shown in Figure 3. Consistent with the agarose gel results, sites corresponding to bands 1, 2, and 3 were among the strongest observed. In addition, the site originally designated as band 3 separated into three bands at the higher resolution of the polyacrylamide gel. This explains why band 3 is somewhat diffuse on the agarose gel (see Figure 2). Finally, a 564 bp *Bam*HI−*Eag*I fragment that corresponded to a portion of the agarose map that was devoid of PBCV-1 topoisomerase II DNA scission showed no strong sites of cleavage (not shown).

Even at the nucleotide level, a limited number of cleavage sites were seen with PBCV-1 topoisomerase II. For example,

[1] It should be noted that levels of DNA cleavage generated by PBCV-1 topoisomerase II with linear DNA molecules were lower than those observed when negatively supercoiled substrates were employed. This effect of DNA topology on enzyme-mediated scission is commonly seen with eukaryotic type II topoisomerases (*5*, *6*, *9*). Even with linear DNA substrates, however, levels of cleavage generated by the viral enzyme were dramatically higher than those produced by human topoisomerase IIα (see Figures 3 and 4).

Table 1: DNA Sequences Cleaved by PBCV-1 Topoisomerase II

Cleavage Site[a]	% DNA Cleavage
GCC↓AGTT ACC[b] CGG TCAA↑TGG	11.6
GGC↓GGTA ATA[c] CCG CCAT↑TAT	7.6
GAC↓AGTT ACC[d] CTG TCAA↑TGG	2.7
GTG↓AGGC ACC[d] CAC TCCG↑TGG	1.9
TAC↓GGTT ATC ATG CCAA↑TAG	1.6
GCT↓CACT CAA CGA GTGA↑GTT	1.4
AGT↓ATAT ATG[d] TCA TATA↑TAC	1.3
AGG↓CCAG GAA TCC GGTC↑CTT	1.3
TAT↓ATGA GTA ATA TACT↑CAT	1.0
ATG↓CTTA ATC TAC GAAT↑TAG	1.0

[a] Arrows denote the points of DNA cleavage. [b] Denoted as site 2 in Figures 2 and 3. [c] Denoted as site 1 in Figures 2 and 3. [d] Denoted as site 3 in Figures 2 and 3.

only four prominent cleavage sites (accounting for ~75% of the total cleavage) were observed in the 549 bp fragment (Figure 3). These results are comparable to those seen with human topoisomerase IIα in the absence of anticancer drugs. In contrast, when the DNA cleavage activity of the human enzyme was increased by the addition of 50 μM etoposide, more than 25 sites were observed in this fragment. Taken together, these findings strongly suggest that the viral enzyme cleaves DNA at a limited number of sites, but does so at a rate that is much higher than typically observed with eukaryotic type II topoisomerases.

A second feature of the polyacrylamide DNA cleavage maps was striking; most of the sites cleaved by PBCV-1 topoisomerase II had counterparts in the human map (Figure 3). However, cleavage levels at each of these sites were considerably higher with the viral enzyme. [Most of the sites cleaved by human topoisomerase IIα were difficult to detect in the absence of drugs. To observe these sites, longer exposures of the gels were required (not shown).] Sequence data for two very strong and eight strong PBCV-1 topoisomerase II DNA cleavage sites are listed in Table 1. The two very strong sites are indicated as sites 2 and 1 in Figure 3 and represent sequences at which the viral enzyme cleaved ~12% and 8% of the DNA substrate, respectively. The eight strong sites include the three bands indicated as site 3 and were defined as sequences at which the enzyme cleaved between 1% and 5% of the substrate.

Consistent with the DNA cleavage gels shown in Figure 3, the sequence preference of PBCV-1 topoisomerase II (Table 1) is similar to that described previously for human

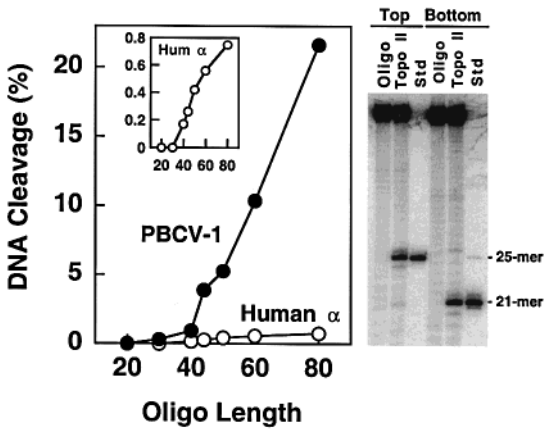

FIGURE 4: Effects of substrate length on topoisomerase II-mediated DNA cleavage. Oligonucleotide substrates ranging from a 20-mer to an 80-mer were employed in cleavage assays with PBCV-1 topoisomerase II (●) or human topoisomerase IIα (○). All oligonucleotides contained cleavage site 2. Substrates shorter than the 80-mer were identical to the 80-mer except that they were decreased in length by an equal number of base pairs on each terminus. The percentage of the DNA substrate that was cleaved is plotted versus oligonucleotide length. DNA cleavage mediated by human topoisomerase IIα (Hum α) also is shown in the inset. Error bars represent the standard deviations for at least three independent experiments. An autoradiogram of a gel containing DNA cleavage products of a 50-mer substrate is shown at the right. The 5′-terminus of the top or bottom strand of the oligonucleotide was labeled as indicated. Samples were oligonucleotide controls (Oligo), DNA cleavage samples (Topo II), and DNA standards corresponding to the expected cleavage products (Std; 25-mer and 21-mer, top and bottom strands, respectively).

topoisomerase IIα (*34*). Two of the most biased positional specificities for the human enzyme are C, no A at the −1 position and no T at the +5 position. Although the viral enzyme displayed a slightly higher propensity for T over C at the −1 position, six of the ten viral sites contained a C at this position on one of the two strands. Moreover, none of the very strong or strong sites cleaved by PBCV-1 topoisomerase II contained either an A at the −1 position or a T at the +5 position.

Finally, as seen with eukaryotic type II topoisomerases (*11*, *12*, *14*, *35*), the viral enzyme covalently attaches to the 5′ DNA termini during scission (data not shown) and generates cleavage products that contain four-base 5′-overhanging ends (Figure 4).

Effects of Substrate Length on DNA Cleavage. In cleavage reactions that contained the 549 bp fragment of pBR322 DNA (see Figure 3), PBCV-1 topoisomerase II cut ~12% of the substrate at site 2. This value should be considered a lower limit for the intrinsic affinity of the viral enzyme for site 2, due to the presence of competing cleavage sequences in the 549 bp fragment.

To investigate the effects of length on DNA scission mediated by PBCV-1 topoisomerase II, a nested series of double-stranded oligonucleotide substrates that corresponded to the DNA sequence surrounding cleavage site 2 (see Figure 3) was synthesized. Site 2 was chosen for these studies because it is a very strong site and the region containing this site lacks competing cleavage sequences.

PBCV-1 topoisomerase II cut less than 6% of the substrate when it was a 50-mer oligonucleotide (see Figures 4 and 5). This finding suggests that the enzyme can recognize the

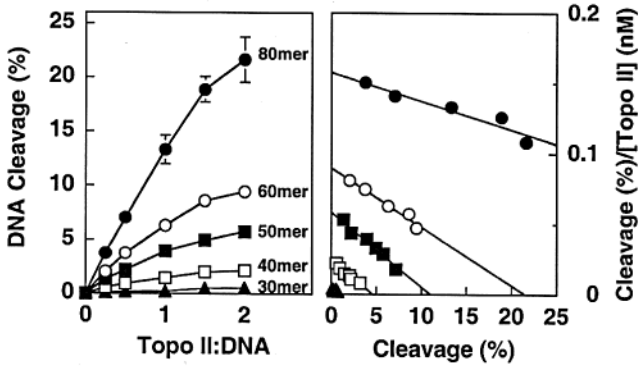

FIGURE 5: Kinetic analysis of DNA cleavage with different length substrates. Cleavage of oligonucleotides by PBCV-1 topoisomerase II was examined for the 80-mer (●), 60-mer (○), 50-mer (■), 40-mer (□), and 30-mer (▲) substrates containing cleavage site 2. A topoisomerase II titration was carried out for each oligonucleotide at a constant substrate concentration of 10 nM. The percentage of cleavage observed for each substate is shown in the left panel. In addition, these data were transformed as Eadie−Hofstee plots (right panel). Error bars represent the standard errors of the mean for two or three independent experiments.

length of its DNA substrate and has a preference for molecules that are longer than 50 bp. Therefore, the ability of PBCV-1 topoisomerase II to cleave site 2-containing oligonucleotides ranging in size from 20 to 80 bp was determined (Figure 4). Levels of DNA cleavage were greatly dependent on substrate length. The highest levels of cleavage, which were observed with the 80-mer (~22%), were even higher than those observed for site 2 in the 549 bp fragment. In contrast, levels of cleavage were barely detectable when the 30-mer was employed.

PBCV-1 topoisomerase II lacks the C-terminal region of eukaryotic type II enzymes and is significantly smaller than human topoisomerase IIα (~120 vs ~170 kDa protomer molecular mass) (*23*). Because of its smaller size, it is possible that the viral enzyme has a different DNA target size than the human enzyme. Therefore, the effects of oligonucleotide length on DNA cleavage mediated by human topoisomerase IIα were compared to those on the viral enzyme (Figure 4).

Like the viral enzyme, human topoisomerase IIα cleaved the nested oligonucleotides specifically at site 2. Although levels of scission were uniformly lower than observed with PBCV-1 topoisomerase II, the length dependence of cleavage was similar to that of the viral enzyme. This finding suggests that the DNA target size of PBCV-1 topoisomerase II is comparable to that of eukaryotic type II enzymes.

There are two possible reasons why type II enzymes prefer to cleave longer DNA substrates. First, they may bind to the longer oligonucleotides with higher affinity. Second, the presence of distal nucleotides may have little effect on substrate binding but may alter a chemical or conformational step that is required for enzyme-mediated DNA cleavage.

A kinetic approach was utilized to distinguish between these possibilities. The concentrations of the 30-, 40-, 50-, 60-, and 80-mer cleavage substrates were held constant, while the concentration of PBCV-1 topoisomerase II was varied over an enzyme:oligonucleotide ratio of 0.25:1 to 2:1. Data were plotted as percent DNA cleavage vs the topoisomerase II:DNA ratio (Figure 5, left panel) and transformed as Eadie−Hofstee plots (right panel). Data also were analyzed as double reciprocal plots (not shown). In all cases, absolute levels of DNA cleavage as well as calculated maximal levels of cleavage increased with oligonucleotide length. Values for the 80-mer were ~50 times higher than those observed with the 30-mer. In contrast, the kinetic affinity of oligonucleotides for the enzyme was similar for the different length substrates (~200−500 nM topoisomerase II), and there was no correlation between calculated affinities and substrate length. Thus, the higher levels of scission observed with longer DNA substrates are not due to their greater affinity for topoisomerase II. Rather, they suggest that sequences distal to the cleavage site alter the enzyme−DNA complex in a manner that promotes the scission event.

Effects of Topoisomerase II-Targeted Drugs on DNA Cleavage. A number of anticancer agents kill cells by increasing levels of DNA cleavage mediated by topoisomerase II (*5*, *6*, *16*−*19*). A previous study demonstrated that several of these drugs had little effect on the global ability of PBCV-1 topoisomerase II to cleave negatively supercoiled plasmid molecules (*20*). However, this study could not discount the possibility that these drugs actually affected scission, but did so only at a limited number of DNA sites. Therefore, the effects of etoposide, amsacrine, and the quinolone CP-115,953 on the site-specific cleavage of DNA by PBCV-1 topoisomerase II were compared to those observed for human topoisomerase IIα.

The first experiment used the 549 and 584 bp pBR322 fragments as substrates (Figure 6). Drugs had a dramatic effect on the human enzyme. In all cases, the overall level of cleavage was increased greatly, and scission was induced at a multitude of sequences that were not apparent in the absence of drugs. An effect on the viral enzyme also was observed, but drug actions were more limited in scope. Cleavage at a few established sites increased (or decreased), and occasionally a site was observed only in the presence of drugs.

To further characterize the drug sensitivity of PBCV-1 topoisomerase II, the effects of these agents on cleavage at isolated sites were determined. A 50-mer oligonucleotide containing site 2 and a 40-mer containing site 1 were used for these studies. The shorter substrate was used to examine site 1, because additional cleavage sequences for the viral enzyme were present in longer oligonucleotides.

The impact of drugs on cleavage at site 2 by PBCV-1 topoisomerase II was modest (Figure 7, top left). Etoposide and CP-115,953 had little impact on scission; however, amsacrine tripled levels of cleavage at this site. The amsacrine effect was not as pronounced in the 549 bp fragment (see Figure 6), possibly due to the influence of competing sites within the longer DNA fragment. In contrast to the limited consequences of drug seen with the viral enzyme at site 2, a much larger effect was observed with human topoisomerase IIα (Figure 7, top right). Levels of cleavage doubled in the presence of etoposide and went up 10- or 20-fold, respectively, when amsacrine or CP-115,953 was included in reactions.

The effects of drugs on cleavage at site 1 (Figure 7, bottom left) by PBCV-1 topoisomerase II were larger than observed with site 2. Levels of scission doubled in the presence of etoposide or CP-115,953 and went up ~4-fold with amsacrine. Similar effects were observed with the human enzyme

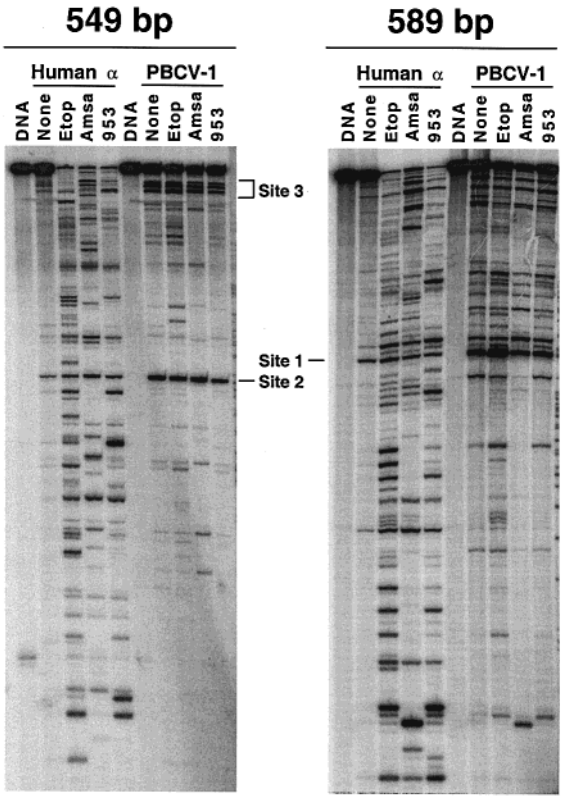

FIGURE 6: Site-specific cleavage by topoisomerase II in the presence of anticancer drugs. The effects of anticancer drugs on site-specific DNA cleavage by PBCV-1 topoisomerase II (PBCV-1) and human topoisomerase IIα (Human α) were determined. A polyacrylamide gel is shown. The 549 bp (left panel) and 589 bp (right panel) fragments of pBR322 were used as substrates (see Figure 3). Cleavage reactions were carried out with topoisomerase II in the absence of drug (None) or in the presence of 50 μM etoposide (Etop), amsacrine (Amsa), or CP-115,953 (953). The enzyme:DNA ratio was 3:1 for PBCV-1 reactions and 40:1 for human reactions. A control that lacked enzyme also is included (DNA). Cleavage sites 1−3 are indicated.

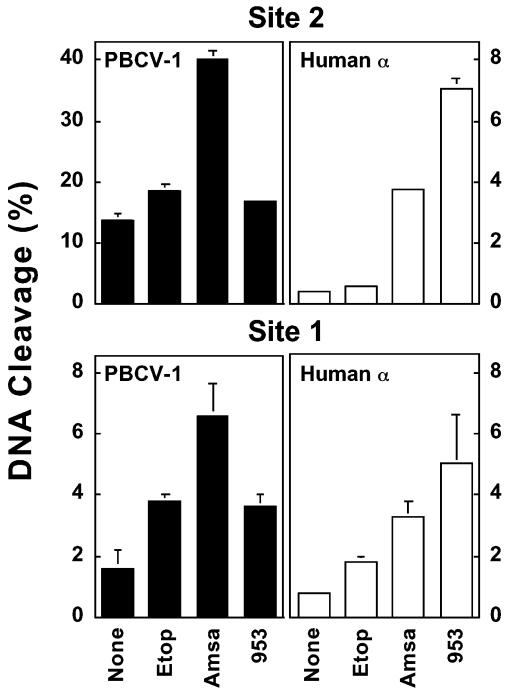

FIGURE 7: Effects of anticancer drugs on DNA cleavage at isolated sites. Two oligonucleotide substrates were employed: a 50-mer that contained cleavage site 2 (top panels) and a 40-mer that contained cleavage site 1 (bottom panels). Cleavage reactions were carried out with either PBCV-1 topoisomerase II (left panels) or human topoisomerase IIα (right panels) and contained no drug (None) or 50 μM etoposide (Etop), amsacrine (Amsa), or CP-115,953 (953). The enzyme:DNA ratio was 2:1 for all reactions (100 nM oligonucleotide). Error bars represent the standard deviations for three independent experiments.

for etoposide and amsacrine (Figure 7, bottom right); however, CP-115,953 stimulated scission to a greater extent (~6-fold).

These findings demonstrate that PBCV-1 topoisomerase II is susceptible to anticancer drugs. Although drug effects generally are smaller than those seen with human topoisomerase IIα, cleavage enhancement is observed at specific sequences.

Effects of Abasic Sites on DNA Cleavage. In addition to anticancer drugs, a variety of DNA lesions increase levels of topoisomerase II-mediated DNA scission (*6*, *28*, *36*−*41*). These lesions act in a position-specific manner, enhancing scission only when they are located within the four-base cleavage stagger (*38*). (Positions are numbered 5′ → 3′, such that the DNA is cleaved between −1 and +1, and positions +1 through +4 on each strand are located between the points of scission.) Of the lesions examined to date, abasic sites appear to stimulate cleavage to the greatest extent (*40*). Therefore, the sensitivity of PBCV-1 topoisomerase II to abasic sites was compared to that of human topoisomerase IIα (Figure 8). The two oligonucleotide cleavage substrates described in the previous section were used for these studies.

Relative cleavage enhancement by abasic lesions generally was greater for human topoisomerase IIα. However, the patterns of DNA cleavage enhancement obtained for the two enzymes were remarkably similar. Abasic lesions located at the +4 position of either cleavage site stimulated DNA scission to the greatest extent. Significant effects also were observed when the lesion was located at the +1 position. Thus, the recognition of DNA damage by PBCV-1 topoisomerase II appears to be similar to that of human topoisomerase IIα.

DISCUSSION

The DNA cleavage reaction mediated by topoisomerase II is central to all of the critical cellular events catalyzed by the enzyme (*2*, *4*−*8*). This reaction also is the target for a broad spectrum of clinically relevant anticancer drugs (*5*, *6*, *16*−*19*). Thus, it is important to understand how type II topoisomerases cleave their DNA substrates.

Unfortunately, efforts to characterize this fundamental aspect of topoisomerase II function have been hampered by the low levels of DNA breaks generated by these enzymes. Typically, in any given mixture of enzyme and plasmid, less than 1% of the topoisomerase II population is found in a cleavage complex (*20*).

To boost levels of DNA scission, it has been common to add anticancer drugs to cleavage reactions (*5*, *6*). However, these agents often alter fundamental properties of DNA scission and religation. For example, many drugs increase the concentration of cleavage complexes by inhibiting the ability of topoisomerase II to religate DNA (*5*, *6*, *42*−*45*). Furthermore, all drugs have dramatic effects on the array of

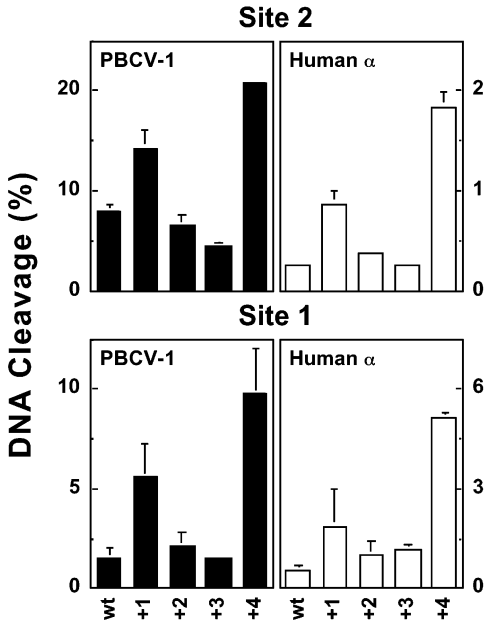

FIGURE 8: Effects of abasic lesions on DNA cleavage at isolated sites. A 50-mer that contained cleavage site 2 (top panels) and a 40-mer that contained cleavage site 1 (bottom panels) were used as cleavage substrates. Position-specific abasic lesions were incorporated into the bottom strand at the +1, +2, +3, or +4 position. The positions are numbered 5′ → 3′, such that the DNA strands are cleaved between the −1 and +1 positions, and the +1 through +4 positions on each strand are located between the points of scission (see Figure 4). Oligonucleotides without abasic lesions were designated wild type (wt). Cleavage reactions were carried out with either PBCV-1 topoisomerase II (left panels) or human topoisomerase IIα (right panels). The enzyme:DNA ratio was 2:1 for all reactions (100 nM oligonucleotide). Error bars represent the standard deviations for three independent experiments.

clevage sites utilized by the enzyme (*34*, *46*, *47*). Thus, it is not clear that the addition of drugs adequately models the intrinsic DNA cleavage activity of eukaryotic topoisomerase II.

Recently, a type II topoisomerase with an extraordinarily high intrinsic DNA cleavage activity was isolated from *Chlorella* virus PBCV-1 (*20*, *23*). This enzyme shares amino acid sequence homology with human topoisomerase IIα and exhibits catalytic properties typical of eukaryotic topoisomerase II (*20*, *23*). The high levels of DNA cleavage generated by PBCV-1 topoisomerase II do not result from either proteolysis of the enzyme or an artifact of overexpression in yeast. Recombinant PBCV-1 topoisomerase II is identical in size to enzyme expressed in *Chlorella* following viral infection (*20*). Additionally, the properties of virally expressed topoisomerase II appear to be indistinguishable from those of the recombinant enzyme (*23*), and no topoisomerase II proteolysis is observed in DNA cleavage assays (data not shown). In light of these characteristics, the viral enzyme has the potential to act as a unique model for topoisomerase II-mediated DNA scission. Consequently, the present study characterized the site-specific DNA cleavage reaction of PBCV-1 topoisomerase II.

Despite its robust cleavage activity, the viral enzyme cut DNA at a limited number of sites. This finding suggests that the rate of DNA scission by PBCV-1 topoisomerase II at these sites must be extremely high in comparison to that of human topoisomerase IIα. It also stands in marked contrast to the multitude of sites cleaved by the human enzyme in reactions that contained anticancer agents.

The DNA site utilization and cleavage specificity of PBCV-1 topoisomerase II are remarkably similar to those of the human enzyme in the absence of drugs. Thus, these two enzymes appear to differ primarily in their rates of DNA scission, rather than in their recognition of cleavage sites. This conclusion is supported by the finding that the DNA target size for PBCV-1 topoisomerase II, as determined by the effect of substrate length on cleavage, is similar to that of human topoisomerase IIα.

We previously reported that anticancer drugs had little effect on the ability of PBCV-1 topoisomerase II to cleave a supercoiled plasmid substrate (*20*). The present study examined drug sensitivity more closely by characterizing the influence of anticancer agents on site-specific DNA cleavage. Modest drug effects were observed at the nucleotide level. Subtle differences in site utilization by the viral enzyme were observed, and cleavage enhancement as high as 4-fold was seen when isolated cleavage sites were analyzed. One notable difference between PBCV-1 topoisomerase II and human topoisomerase IIα was the sensitivity toward CP-115,953. While most prokaryotic and eukaryotic type II topoisomerases are highly susceptible to this quinolone (*48*−*51*), the viral enzyme appeared to be relatively insensitive.

The limited sensitivity of PBCV-1 topoisomerase II to anticancer drugs may simply reflect the remarkably high baseline cleavage activity of the viral enzyme. However, two specific amino acid residues in the viral enzyme may contribute to this effect. First, residue 403 (which corresponds to arginine 450 in the human sequence) is an asparagine. Mutation of this arginine to a glutamine in human topoisomerase IIα renders the enzyme highly resistant to a broad spectrum of anticancer drugs (*52*, *53*). Second, residue 723 (which corresponds to glutamine 785 in the human sequence) is a leucine. Mutation of the corresponding glutamine in the bacterial type II topoisomerase DNA gyrase (glutamine 104 in gyrA) results in a 4−10-fold resistance to quinolone antibacterials (*54*).

In summary, PBCV-1 topoisomerase II generates extraordinarily high levels of DNA scission. However, in all other respects, the cleavage activity of the enzyme resembles that of human topoisomerase IIα. Furthermore, at least at specific sequences, cleavage mediated by the viral enzyme is stimulated by anticancer drugs and DNA damage. On the basis of these findings, we propose that PBCV-1 topoisomerase II represents an intriguing model with which to dissect the DNA cleavage reaction of eukaryotic type II topoisomerases.

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