DNA Methylation Impacts the Cleavage Activity of Chlorella Virus Topoisomerase II†

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ABSTRACT: Topoisomerase II from *Paramecium bursaria* chlorella virus-1 (PBCV-1) and chlorella virus Marburg-1 (CVM-1) displays an extraordinarily high in vitro DNA cleavage activity that is 30−50 times higher than that of human topoisomerase IIα. This remarkable scission activity may reflect a unique role played by the type II enzyme during the viral life cycle that extends beyond the normal control of DNA topology. Alternatively, but not mutually exclusively, it may reflect an adaptation to some aspect of the viral environment that differs from the in vitro conditions. To this point, the genomes of many chlorella viruses contain high levels of N^6-methyladenine (6mA) and 5-methylcytosine (5mC), but the DNA employed in vitro is unmodified. Therefore, to determine whether methylation impacts the ability of chlorella virus topoisomerase II to cleave DNA, the effects of 6mA and 5mC on the PBCV-1 and CVM-1 enzymes were examined. Results indicate that 6mA strongly inhibits DNA scission mediated by both enzymes, while 5mC has relatively little effect. At levels of 6mA and 5mC methylation comparable to those found in the CVM-1 genome (10% 6mA and 42% 5mC), the level of DNA cleavage decreased ~4-fold. As determined using a novel rapid quench pre-equilibrium DNA cleavage system in conjunction with oligonucleotide binding and ligation assays, this decrease appears to be caused primarily by a slower forward rate of DNA scission. These findings suggest that the high DNA cleavage activity of chlorella virus topoisomerase II on unmodified nucleic acid substrates may reflect, at least in part, an adaptation to act on methylated genomic DNA.

Topological relationships within the double helix, including DNA under- and overwinding, knotting, and tangling, profoundly influence how the genetic material is replicated, expressed, and recombined (*1*). To modulate these relationships, cells have evolved ubiquitous enzymes known as DNA topoisomerases (*2*−*7*). Type I topoisomerases control DNA under- and overwinding by creating a transient nick on one strand of the double helix and mediating passage of the opposite strand through the break or allowing controlled rotation about the break (*4*, *6*, *8*). In contrast, type II topoisomerases function by passing an intact double helix through a transient double-stranded break that they generate in a separate segment of the DNA. As a result, these latter enzymes are able to control all aspects of DNA topology (*7*, *9*, *10*).

To regulate the opening and closing of the single- or double-stranded "DNA gate" during the control of nucleic acid topology, all topoisomerases form covalent bonds between active site tyrosyl residues and the newly generated DNA termini. These covalent enzyme−DNA complexes are known as cleavage complexes (*2*−*5*, *7*, *11*). Although the formation of cleavage complexes is required for the physiological activities of topoisomerases, their presence also poses a threat to genomic integrity (*7*, *9*, *10*). Consequently, topoisomerases normally open and close the DNA gate very rapidly. As a result, cleavage complexes usually are present at low levels and are tolerated by the cell. However, physiological conditions that increase either the lifetime or concentration of cleavage complexes convert topoisomerase I and II from important nuclear enzymes to potent cellular toxins that fragment the genetic material (*12*−*16*).

Because the DNA cleavage activities of topoisomerase I and II have the potential to destabilize the genome, these enzymes play pivotal roles in cancer. While several widely used anticancer drugs act by increasing levels of enzyme-mediated DNA scission, evidence suggests that the actions of topoisomerase II may trigger the chromosomal breaks that initiate specific types of leukemia (*13*, *14*, *17*−*30*).

Due to the potentially lethal nature of topoisomerase-mediated DNA scission, low levels of DNA cleavage are characteristic of all known eukaryotic enzymes (*2*−*5*, *7*, *11*). In contrast, topoisomerases encoded by viruses that infect eukaryotic cells appear to possess considerably higher DNA cleavage activities. Of the known viral families with eu-

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karyotic hosts, only five encode their own DNA topoisomerase (*31*, *32*). Collectively, these viruses probably have a common evolutionary ancestor and are termed nucleocytoplasmic large DNA viruses (NCLDV)[1] (*31*, *32*).

One NCLDV, Poxviridae, which contains vaccinia virus, encodes a type IB topoisomerase (*33*−*36*). Vaccinia topoisomerase is by far the smallest known type I enzyme (314 amino acids) and possesses a DNA cleavage activity that is both greater and more site-specific than that of any other characterized type IB topoisomerase (*35*−*38*).

The most recently discovered NCLDV, Mimiviridae, which includes *Acanthamoeba polyphaga* mimivirus as its only member, encodes genes for three putative topoisomerases, a type IA, a type IB, and a type II enzyme (*32*). At the present time, it is not known whether any of these genes actually are expressed or produce gene products that function as topoisomerases.

The three remaining NCLDV families, including Phycodnaviridae (which contains the chlorella viruses) (*39*−*41*), Asfarviridae (which contains African swine fever virus) (*42*, *43*), and Iridoviridae (which contains Chilo iridescent virus) (*44*), encode reading frames that are homologous to type II topoisomerases (*31*). Of these, only chlorella virus has been demonstrated to express a functional type II enzyme. Topoisomerase II has been isolated and characterized from two distantly related chlorella viruses, *Paramecium bursaria* chlorella virus-1 (PBCV-1) and chlorella virus Marburg-1 (CVM-1) (*45*−*49*). Both enzymes are significantly smaller (1063 and 1059 amino acids, respectively) than their eukaryotic counterparts due to a large C-terminal deletion, but otherwise display high levels of amino acid sequence identity (∼46%) to human topoisomerase IIα. The DNA cleavage activities of these type II topoisomerases are 30−50 times higher than that of the human enzyme. However, in all other respects, the catalytic reactions of PBCV-1 and CVM-1 topoisomerase II are typical of eukaryotic type II enzymes (*45*−*49*).

It is not known why certain viral families encode their own DNA topoisomerases. Unlike other viruses, all of the NCLDVs possess large double-stranded DNA genomes that are characterized by either hairpin telomeres or circularly permuted ends (*32*, *50*−*54*). In addition, they encode many of the proteins required for viral replication and replicate in part or entirely in the host cytoplasm (*39*−*41*, *52*, *53*, *55*, *56*). The high DNA cleavage activities of vaccinia topoisomerase I and chlorella virus topoisomerase II suggests that these enzymes play alternative roles in the viral life cycle that extend beyond the normal control of DNA topology. A second, but not mutually exclusive, possibility is that the robust DNA cleavage observed in vitro may reflect an adaptation to some unique aspect of the viral environment.

In this regard, many chlorella virus genomes are highly methylated (*41*, *57*−*61*). For example, the CVM-1 genome contains 10% 6mA and 42% 5mC (*57*). The methylated bases occur as a result of virally encoded site-specific DNA methyltransferases (*59*, *60*). These methyltransferases are part of a viral DNA restriction modification system, similar to that of bacteria. The chlorella viruses, including strains that infect chlorella NC64A (such as PBCV-1) and Pbi (such as CVM-1), encode a minimum of 20 distinct DNA methyltransferases (*57*). This methylation is different from that found in eukaryotes. For example, mammalian genomes contain only 5mC, which plays a role in chromatin structure and the regulation of transcription (*62*−*64*).

To determine whether methylation affects the ability of chlorella virus topoisomerase II to cleave DNA, the effects of 6mA and 5mC on the PBCV-1 and CVM-1 enzymes were examined. Results indicate that 6mA decreases the level of DNA scission mediated by these enzymes, while high levels of 5mC have relatively little effect. At a percentage of 6mA and 5mC methylation comparable to that observed for the CVM-1 genome in vivo, the level of DNA cleavage decreased ∼4-fold. As determined directly by rapid quench kinetic analysis, this decrease appears to be caused primarily by a slower forward rate of scission. These findings suggest that the high DNA cleavage activity of chlorella virus topoisomerase II on unmodified nucleic acid substrates may reflect, at least in part, the potential to act on heavily methylated genomic DNA.

EXPERIMENTAL PROCEDURES

Enzymes and Materials. Topoisomerase II from PBCV-1 and that from CVM-1 were expressed in *Saccharomyces cerevisiae* JEL-1Δ*top1* and purified by a modification (*49*) of the procedure of Lavrukhin et al. (*45*). [γ-^{32}P]ATP (∼5000 Ci/mmol) was obtained from ICN. 6mA and 5mC phosphoramidites were from Glen Research. All other chemicals were analytical reagent grade.

Equilibrium DNA Cleavage. Equilibrium DNA cleavage mediated by PBCV-1 and CVM-1 topoisomerase II was assessed as described previously (*47*, *65*). A 50-mer oligonucleotide and its complementary strand were prepared on an Applied Biosystems DNA synthesizer. The sequences of the top and bottom strands were 5′-TTGGTATCTGCGCTCTGCTGAAGCC↓AGTTACCTTCGGAAAAAGAGTTGGT-3′ and 5′-ACCAACTCTTTTTCCGAAGGT↓AACTGGCTTCAGCAGAGCGCAGATACCAA-3′, respectively. This substrate contains a single cleavage site for topoisomerase II that has been characterized previously (*47*). Scissile bonds are denoted with arrows.

6mA and 5mC were inserted into the oligonucleotide at both specific and random positions using phosphoramidite chemistry. Oligonucleotides were labeled on the 5′-termini using T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP, and gel-purified as described previously (*65*). Double-stranded DNA substrates were generated by annealing equimolar amounts of complementary oligonucleotides at 70 °C for 10 min and cooling to 25 °C.

DNA cleavage reaction mixtures contained 300 nM PBCV-1 or CVM-1 topoisomerase II and 100 nM double-stranded oligonucleotide in 20 μL of cleavage buffer [PBCV-1 topoisomerase II, 10 mM Tris-HCl (pH 8.5), 62.5 mM NaCl, 62.5 mM KCl, 0.1 mM NaEDTA, 2.5 mM $MgCl_2$, and 2.5% glycerol (v/v); CVM-1 topoisomerase II, 10 mM Tris-HCl (pH 8.5), 120 mM KCl, 0.1 mM NaEDTA, 15 mM $MgCl_2$, and 2.5% glycerol (v/v)]. Reactions were initiated by the addition of enzyme, and mixtures were incubated at 25 °C (PBCV-1) or 30 °C (CVM-1) for 10 min. The reaction conditions described above represent the optimal parameters

[1] Abbreviations: NCLDV, nucleocytoplasmic large DNA viruses; PBCV-1, *Paramecium bursaria* chlorella virus-1; CVM-1, chlorella virus Marburg-1; 6mA, N^6-methyladenine; 5mC, 5-methylcytosine.

for DNA cleavage by the two chlorella enzymes. DNA cleavage products were trapped by the addition of 2 μL of 1.15% SDS followed by 2 μL of 115 mM NaEDTA (pH 8.0). Samples were digested with proteinase K (2 μL of a 0.8 mg/mL solution) for 30 min at 37 °C, precipitated twice with 100% ethanol, rinsed once with 70% ethanol, dried, and resuspended in 40% formamide (v/v), 8.4 mM EDTA, 0.02% bromophenol blue (w/v), and 0.02% xylene cyanole FF (w/v). DNA cleavage products were resolved by electrophoresis in 7 M urea and 14% polyacrylamide gels in 100 mM Tris-borate (pH 8.3) and 2 mM NaEDTA, and were visualized and quantified using a Bio-Rad FX molecular imager.

When only one strand of the oligonucleotide was modified with methylated DNA bases, DNA cleavage was monitored on the opposite, unmodified strand to maintain a consistent level of labeling from experiment to experiment.

DNA Ligation. DNA ligation assays were carried out by a modification of the procedure of Kingma et al. (*65*). DNA cleavage−ligation equilibria were established in cleavage buffer as described in the preceding section except that $MgCl_2$ in the reaction buffer was replaced with 5 mM $CaCl_2$ for both PBCV-1 and CVM-1 topoisomerase II. Topoisomerase II−DNA cleavage complexes were trapped by the addition of EDTA (pH 8.0) to a final concentration of 6 mM. NaCl was added to a final concentration of 500 mM to prevent recleavage of the DNA substrate. Ligation was initiated by the addition of $MgCl_2$ at a final concentration of 0.1 mM and terminated at times up to 60 s by the addition of 2 μL of 1.15% SDS. Samples were processed and analyzed as described above. The percent DNA cleavage at time zero was set to 100%, and the rate of ligation was determined by quantifying the loss of the cleaved DNA over time.

Topoisomerase II−DNA Binding. The effects of DNA methylation on the affinity of PBCV-1 and CVM-1 topoisomerase II for DNA were monitored by a competitive nitrocellulose filter binding assay (*66*). Nitrocellulose filters (0.45 μm, Millipore) were equilibrated in binding buffer [10 mM Tris-HCl (pH 8.5), 30 mM KCl, 0.1 mM NaEDTA, and 2.5% glycerol (v/v)]. Assays were performed in the absence of divalent cation to prevent topoisomerase II-mediated DNA cleavage. Binding mixtures contained 300 nM PBCV-1 or CVM-1 topoisomerase II in 20 μL of binding buffer that contained 100 nM ^{32}P-labeled unmodified oligonucleotide and 0−400 nM cold competitor oligonucleotide. Binding equilibria were established by incubating samples at 25 °C for 10 min. Samples were transferred to nitrocellulose filters, and washed three times with binding buffer in vacuo. Filters were placed in 8 mL of Econo-Safe scintillation fluid (Research Products International), and the amount of ^{32}P-labeled unmodified oligonucleotide that remained bound to the filter was quantified using a Beckman LS 5000TD scintillation counter.

Pre-Equilibrium DNA Cleavage Kinetics. Pre-equilibrium DNA cleavage was performed using a KinTek (Austin, TX) model RQF-3 quench flow apparatus. Cleavage was initiated by rapid mixing of two independent solutions. The first contained a noncovalent complex formed between 600 nM PBCV-1 or CVM-1 topoisomerase II and 200 nM ^{32}P-labeled oligonucleotide (methylated or unmodified) in cleavage buffer that lacked $MgCl_2$. The second solution contained cleavage buffer in which the $MgCl_2$ concentration was 2 times higher than normal (5 mM for PBCV-1 or 30 mM for CVM-1). Equal volumes of the two solutions were mixed for times varying from 5 ms to 5 s, and DNA cleavage was quenched with equal volumes of 1% SDS (v/v). Products were processed and analyzed as described in the DNA cleavage section. The rate of DNA cleavage was determined by a fit of the data to the equation $y = a + b(1 - e^{kX})$, where a is the y intercept, b is the amplitude, k is the rate, X is the time, and y is the amount of DNA cleavage relative to equilibrium.

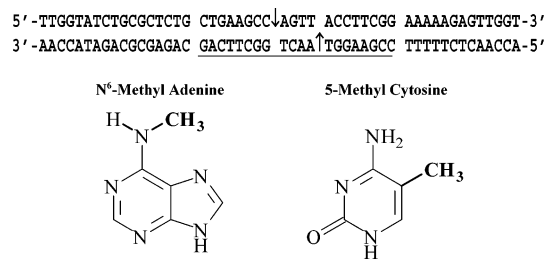

FIGURE 1: 6mA and 5mC placement in the oligonucleotide substrate. A 50-mer oligonucleotide substrate (top) derived from pBR322 (*47*) was employed. In assays that examined the effects of 6mA (bottom left) or 5mC (bottom right) on topoisomerase II-mediated DNA cleavage, all of the adenines or cytosines within the central underlined region were substituted with the appropriate methylated base during synthesis. In assays that examined physiological levels of DNA methylation, 10% of the adenine residues and 42% of the cytosine residues throughout the 50-mer substrate were randomly substituted on both strands with 6mA and 5mC during synthesis.

RESULTS

Effects of DNA Methylation on DNA Cleavage Mediated by Chlorella Virus Topoisomerase II. Topoisomerase II from two chlorella viruses, PBCV-1 and CVM-1, displays an extraordinarily high DNA cleavage activity in vitro (*45*−*47*). Using plasmid substrates, the type II enzyme from PBCV-1 or CVM-1 cleaves 30−50 times more DNA than human topoisomerase IIα in comparable experiments. This remarkable scission activity may reflect a unique role played by the type II enzyme during the viral life cycle that extends beyond the normal control of DNA topology. Alternatively, the robust DNA cleavage may reflect a fundamental difference between in vitro conditions and the viral milieu. These two possibilities are not mutually exclusive.

One major difference between the DNA substrates employed for in vitro experiments and those found within the viral environment is the level of methylation. Whereas in vitro substrates contain no methylated bases, the genomes of many chlorella viruses are highly modified (*41*, *57*−*61*). In contrast to mammalian DNA, which contains a small percentage of 5mC, some chlorella virus genomes contain as much as 37% 6mA and 45% 5mC (*57*). The CVM-1 genome, which encodes one of the type II topoisomerases used in this study, contains 10% 6mA and 42% 5mC (*57*).

To determine whether DNA methylation affects DNA cleavage mediated by chlorella virus topoisomerase II, an oligonucleotide system containing a strong site for enzyme action was employed (Figure 1). This site was identified during the characterization of the PBCV-1 enzyme and is utilized by both PBCV-1 and CVM-1 topoisomerase II (*47*). As observed previously, both viral enzymes cut the oligonucleotide substrate very well. However, PBCV-1 topoi-

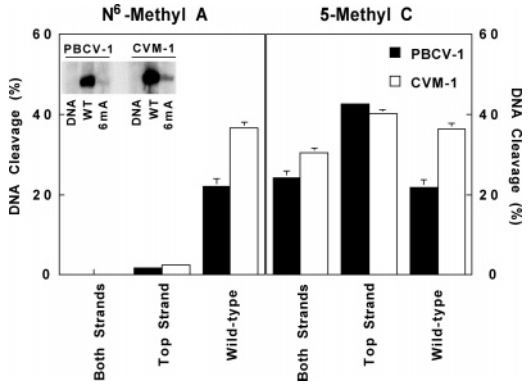

FIGURE 2: Effects of 6mA and 5mC on the DNA cleavage activity of chlorella virus topoisomerase II. The effects of modifying either the top strand or both strands of the oligonucleotide substrate with 6mA (left) or 5mC (right) were determined. In these experiments, all of the adenine or cytosine residues within the central 20 bp surrounding the topoisomerase II DNA cleavage site were modified (see the underlined region in Figure 1). Results are shown for DNA cleavage mediated by PBCV-1 (black bars) or CVM-1 (white bars) topoisomerase II. Data for cleavage of the wild-type unmodified substrate (Wild-type) are shown for comparison. Error bars represent the standard deviation of three independent experiments. The inset depicts a representative autoradiogram showing DNA cleavage products from reactions with the wild-type unmodified substrate (WT) or the substrate modified on the top strand with 6mA (6mA). A DNA control in the absence of enzyme is shown (DNA).

somerase II generates somewhat less cleaved DNA than does CVM-1 topoisomerase II (22 and 36% cleavage, respectively, at a 3:1 enzyme:oligonucleotide ratio) (Figure 2). By comparison, human topoisomerase IIα cleaves only 1.8% of the substrate under optimal conditions (data not shown).

Initial experiments were designed to gauge the maximal potential effects of the two methylated bases on DNA scission mediated by the two viral enzymes. To this end, all of the adenines or cytosines within the central 20 bp region of the oligonucleotide substrate were converted to either 6mA or 5mC, respectively. This region of the substrate is underlined in Figure 1 and spans nearly one turn of the helix on either side of the scissile bonds, which are denoted by arrows. The top strand contains four adenine and six cytosine residues, and the bottom strand contains five adenine and five cytosine residues within the underlined sequence. The site specificity of DNA cleavage mediated by either PBCV-1 or CVM-1 topoisomerase II was not altered by the presence of 6mA (inset of Figure 2) or 5mC (not shown).

As seen in Figure 2, 6mA had a large inhibitory effect on the ability of PBCV-1 and CVM-1 topoisomerase II to cleave the oligonucleotide. Conversion of the adenine residues on the top strand to 6mA decreased the level of scission mediated by either enzyme ~15-fold (to ~2% cleavage). A comparable inhibition of DNA cleavage was observed when adenine residues on the bottom strand were converted to 6mA (data not shown). Furthermore, concurrent modification of the adenine residues on both the top and bottom strands abolished DNA scission.

In contrast to the adenine modification, cytosine methylation had virtually no inhibitory effect on DNA cleavage. Conversion of the cytosine residues on the top strand to 5mC actually increased the level of scission as much as 2-fold, and concurrent modification on both strands had only marginal effects on DNA cleavage with either enzyme. Taken together, these data indicate that 6mA has a much more

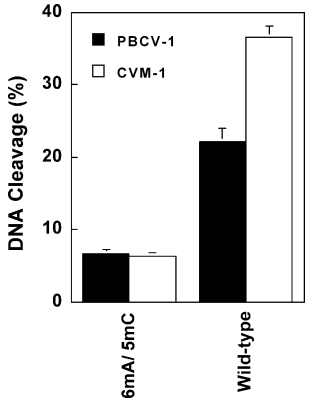

FIGURE 3: Effects of physiological levels of DNA methylation on topoisomerase II-mediated DNA cleavage. A 50-mer oligonucleotide substrate was synthesized such that 10% of all adenines were 6mA and 42% of all cytosines were 5mC. This percentage represents that found in the CVM-1 genome (*57*). The ability of PBCV-1 (black bars) and CVM-1 (white bars) topoisomerase II to cleave the mixed methyl substrate (6mA/5mC) or the wild-type unmodified oligonucleotide (Wild-type) is shown. Error bars represent the standard deviation of three independent experiments.

profound effect on chlorella virus topoisomerase II than does 5mC and significantly inhibits the ability of the PBCV-1 and CVM-1 enzymes to cut DNA.

At least 13 distinct consensus methylation sequences have been identified in different chlorella viruses (*57*). Generally, chlorella virus DNA methyltransferases have short recognition sequences that range from two to four bases in length. In addition, several of the sequences are highly degenerate. Consequently, methylation is widespread throughout the viral genome (*41*, *57*−*61*).

Therefore, as a first attempt to approximate the physiological levels and distribution of DNA methylation sites found in the CVM-1 genome, while still maintaining a site-specific system, 10% of the adenine residues and 42% of the cytosine residues of the 50-mer substrate were randomly substituted on both strands with 6mA and 5mC during synthesis. On the basis of the known consensus methylation sequences identified in DNAs from different chlorella viruses, this substrate contains a minimum of 23 potential sites of modification that span the oligonucleotide (*57*).

Cleavage of the randomly modified 6mA/5mC substrate is shown in Figure 3. Mixed methylation substantially reduced the level of scission by both chlorella virus enzymes, producing ~7% cleaved oligonucleotides as compared to the wild-type substrate (~22−37%). This decrease in the level of scission is less dramatic than that obtained with substrates whose entire central region was converted to 6mA on one or both strands. However, these results suggest that physiological levels of modification can profoundly influence the activity of chlorella virus topoisomerase II.

Mechanistic Basis for the Methylation-Dependent Decrease in the Level of Topoisomerase II-Mediated DNA Cleavage. Three reaction steps together regulate the levels of DNA cleavage mediated by topoisomerase II: enzyme−DNA binding, the forward rate of DNA scission, and the reverse rate of DNA ligation (*7*, *9*, *10*). A series of experiments was carried out to determine which of these steps was primarily responsible for the methylation-dependent decrease in the level of chlorella virus topoisomerase II-mediated DNA cleavage. The mixed 6mA/5mC (10%/

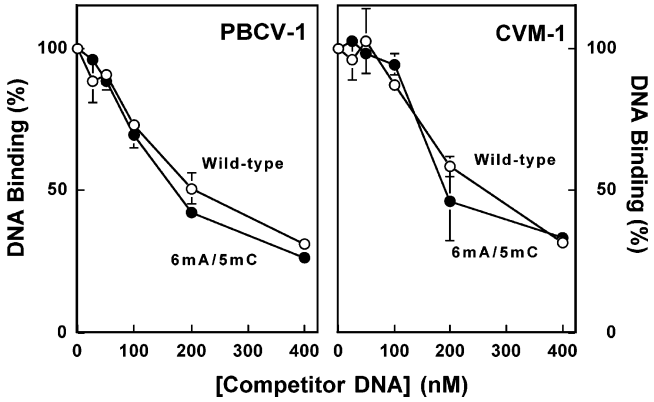

FIGURE 4: Ability of PBCV-1 and CVM-1 topoisomerase II to bind methylated or unmodified oligonucleotide. Results from a competitive nitrocellulose filter binding assay are shown. Unmodified and 6mA/5mC oligonucleotide substrates are as in Figure 3. Radioactively labeled unmodified oligonucleotide substrate (100 nM) and 0−400 nM unmodified (Wild-type, ○) or 6mA/5mC mixed methyl oligonucleotide (6mA/5mC, ●) were incubated with PBCV-1 (left) or CVM-1 (right) topoisomerase II. Error bars represent the standard deviation of three independent experiments.

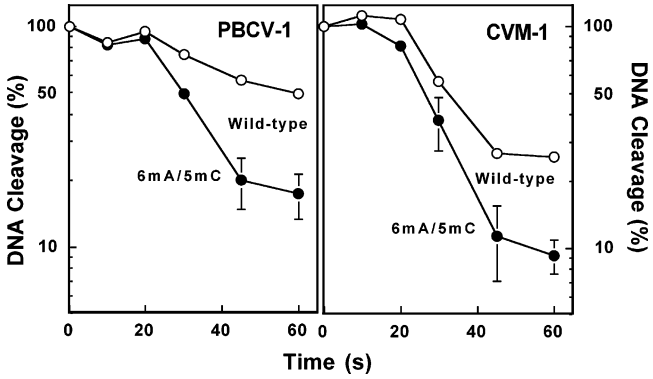

FIGURE 5: Ability of PBCV-1 and CVM-1 topoisomerase II to ligate the methylated or unmodified oligonucleotide substrate. Unmodified and 6mA/5mC oligonucleotide substrates are as in Figure 3. The effects of methylation on DNA ligation mediated by PBCV-1 (left) and CVM-1 (right) topoisomerase II are shown. The amount of DNA cleavage observed at equilibrium for unmodified (Wild-type, ○) or methylated (6mA/5mC, ●) oligonucleotide was set to 100% at time zero. DNA ligation was quantified by the loss of cleaved molecules. Error bars represent the standard deviation of three independent experiments.

42%) 50-mer was used for all of the mechanistic experiments described below. This oligonucleotide was chosen because it most closely approximates the physiological modification of genomic CVM-1 DNA.

DNA methylation is known to alter the ability of numerous proteins to bind the double helix (*62*). Therefore, the first set of experiments assessed the effects of the modification on topoisomerase II−DNA binding. A competition assay was used to determine the ability of unlabeled wild-type or methylated 50-mer to compete with radioactively labeled wild-type oligonucleotide. On the basis of levels of competitor DNA required to weaken binding of the radioactive oligonucleotide by 50%, PBCV-1 and CVM-1 topoisomerase II display similar affinities for the wild-type and methylated substrate (Figure 4). Thus, it appears that a decreased level of cleavage of the modified oligonucleotide is not related to a decreased level of enzyme−DNA binding.

Several anticancer drugs that increase levels of topoisomerase II-generated DNA strand breaks act specifically by inhibiting the ability of the enzyme to ligate cleaved DNA molecules (*7*, *9*, *10*). A ligation assay was utilized to determine whether methylation, which decreases levels of cleaved DNA, acts by enhancing the rate of DNA ligation. As seen in Figure 5, rates of ligation with the methylated substrate were slightly higher than that observed with the wild-type oligonucleotide. Although increases in ligation rates cannot account for the 3−5-fold decrease produced by methylation, they may contribute to the overall effect of DNA modification.

Previously, no assay has been available to monitor the forward rate of DNA scission mediated by topoisomerase II directly without using suicide substrates. These substrates always include single-stranded DNA breaks or double-stranded−single-stranded junctions proximal to the scissile bonds (*67*). Although suicide substrates have been valuable tools for comparative experiments, they often are cleaved with slow reaction kinetics that do not reflect those of the enzyme with intact double-stranded DNA substrates.

Therefore, to determine the effects of DNA methylation on the forward reaction kinetics of chlorella virus topoisomerase II, a rapid quench pre-equilibrium DNA cleavage assay was established. In this system, a noncovalent complex between viral topoisomerase II and end-labeled oligonucleotide substrate was pre-formed in the absence of Mg^{2+}. Since the enzyme requires a divalent cation to cleave and ligate DNA, only a binding complex is produced under these conditions (*68*). Under the conditions that were used (3:1 enzyme:oligonucleotide), ~90% of the DNA substrate was bound (data not shown). DNA scission was initiated by the addition of Mg^{2+}, and the reaction was terminated at time points ranging from 5 ms to 5 s by the addition of SDS. Reaction products were resolved on denaturing polyacrylamide gels and visualized by digital autoradiography.

Previous nonsuicide systems initiated DNA cleavage by the addition of topoisomerase II to DNA buffers that contained a divalent cation (*7*, *9*, *10*). Consequently, cleavage rates reflected both the rate of enzyme−DNA binding and the forward rate of scission. Furthermore, since DNA cleavage in most of these systems was assessed after the DNA cleavage−ligation equilibrium was established, levels of cleaved DNA also reflected the relative ratio of the rates of scission and ligation. The rapid quench assay circumvents these deficiencies. By forming a noncovalent enzyme−DNA complex prior to cleavage, the rate of binding is eliminated from consideration. Moreover, because the rapid quench assay monitors pre-equilibrium levels of DNA cleavage, the rate of ligation is immaterial. Finally, since the entry of Mg^{2+} into the enzyme−DNA complex is diffusion-controlled, reaction kinetics generated in this system should reflect the actual forward rate of DNA scission mediated by topoisomerase II.

Results of the rapid quench DNA cleavage assay are shown in Figure 6. In all cases, final levels of DNA cleavage were set to 1.0 to allow direct comparisons between different enzymes and substrates. Actual levels of DNA cleavage at equilibrium were similar to those shown in Figure 3. PBCV-1 and CVM-1 topoisomerase II both cut the wild-type oligonucleotide rapidly. The DNA cleavge−ligation equilibrium

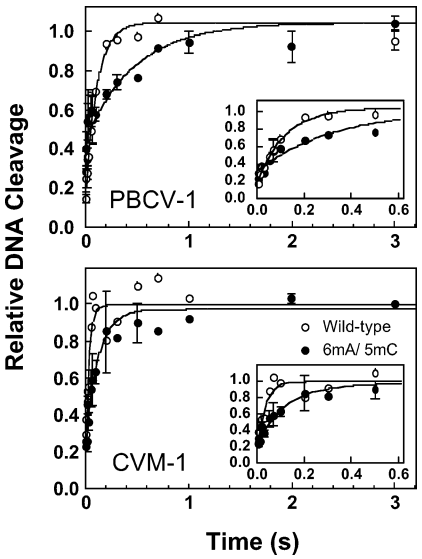

FIGURE 6: Pre-equilibrium DNA cleavage of the methylated or unmodified oligonucleotide by PBCV-1 and CVM-1 topoisomerase II. Unmodified and 6mA/5mC oligonucleotide substrates are as in Figure 3. Pre-equilibrium DNA cleavage for PBCV-1 (top) and CVM-1 (bottom) was monitored using a novel rapid quench kinetic system. No DNA cleavage was observed in the absence of Mg^{2+}. Data are shown for cleavage of the unmodified DNA substrate (Wild-type, ○) and 6mA/5mC mixed methyl oligonucleotide (6mA/5mC, ●). Expanded time courses for DNA cleavage, including time points up to 0.6 s, are shown in the insets. Error bars represent the standard error of the mean of at least two independent experiments.

was established within 1−2 s. However, PBCV-1 topoisomerase II, which produces lower levels of cleaved DNA at equilibrium than the CVM-1 enzyme (see Figures 2 and 3), displayed a slower forward rate of scission (8.92 and 30.32 s^{-1}, respectively). In addition, the two viral enzymes cleaved the methylated substrate at rates that were ∼4-fold slower (2.07 and 8.08 s^{-1}, respectively) than that observed with the wild-type oligonucleotide. Taken together, these results strongly suggest that methylation decreases levels of DNA cleavage mediated by chlorella virus topoisomerase II primarily by decreasing the forward rate of DNA scission. However, enhanced rates of DNA ligation may also play a role in mediating the effects of the DNA modification.

DISCUSSION

Whereas the vast majority of viruses rely on their host cell topoisomerases to replicate, some members of the NCLDV families encode their own type II topoisomerases (*31*, *32*, *39*−*44*). Only two of these enzymes, from chlorella viruses PBCV-1 and CVM-1, have been characterized (*45*−*47*, *49*). While similar to eukaryotic topoisomerase II in most respects, the two viral enzymes display two attributes that are strikingly different from those of other known type II topoisomerases. First, they are considerably smaller than their eukaryotic counterparts and lack the entire C-terminal domain. Second, they display a robust in vitro DNA cleavage activity with plasmid substrates that is 30−50 times greater than that of eukaryotic topoisomerase II (*45*−*47*, *49*). The mechanistic basis for this high DNA cleavage activity is not known. However, the absence of the C-terminal domain contributes to the increased level of DNA scission (*69*).

Less clear than the mechanistic issue is the physiological rationale for the high DNA cleavage activity of PBCV-1 and CVM-1 topoisomerase II. Two possibilities exist that are not mutually exclusive. Elevated levels of DNA scission may reflect an important physiological requirement for the enzyme that extends beyond the normal control of DNA topology. Alternatively, the high DNA cleavage observed in vitro may reflect an adaptation that chlorella virus topoisomerase II has made to a specific aspect of the viral milieu. Since the chlorella virus genome is heavily methylated and includes both 6mA and 5mC (*41*, *57*−*61*), we examined the effects of these two DNA modifications on the cleavage activity of PBCV-1 and CVM-1 topoisomerase II. While the presence of 5mC had relatively little effect on either enzyme, 6mA impaired the ability of both to cleave an oligonucleotide substrate. At a percent methylation eqivalent to that present in the CVM-1 genome, 10% 6mA and 42% 5mC, the level of cleavage mediated by both enzymes was decreased 3−5-fold. Therefore, it appears that the robust activity of viral topoisomerase II observed in vitro reflects, at least in part, the fact that unmodified DNA is not its native substrate.

Chlorella virus genomes are methylated by virally encoded DNA methyltransferases (*59*, *60*). However, levels of genomic methylation vary widely among the chlorella viruses (*57*). CVM-1 DNA was employed as the representative model for this study for two reasons. First, topoisomerase II from this virus strain is one of only two viral type II enzymes that have been characterized, and it was desirable to examine levels of methylation that might be encountered by one of these enzymes in vivo. Second, levels of genomic methylation in CVM-1 are intermediate among those of characterized chlorella viruses. It is notable that the level of methylation of the PBCV-1 genome (1.5% 6mA and 2% 5mC) is significantly lower than that found in CVM-1 (*57*). The fact that both PBCV-1 and CVM-1 topoisomerase II respond in a fashion similar to DNA methylation implies that the ability to function on a methylated genome is ancestrally encoded within the chlorella virus type II enzyme. Furthermore, despite lower levels of global DNA methylation, it is possible that critical sites of topoisomerase II action are modified within the PBCV-1 genome.

The trends observed for chlorella virus topoisomerase II also were observed for human topoisomerase IIα (data not shown). For example, while the presence of 5mC in both strands of the 50-mer substrate increased the level of cleavage slightly (from ∼1.8 to 2.3%), the inclusion of 6mA abolished scission. In addition, levels of DNA cleavage observed with the mixed 6mA/5mC substrate were ∼4.5-fold lower (∼0.4%) than that observed with unmodified DNA. Thus, it appears that the response of type II topoisomerases to DNA methylation crosses species lines. One significant difference between the human and viral enzymes should be noted, however. In contrast to the poor ability of topoisomerase IIα to cut the mixed 6mA/5mC oligonucleotide, chlorella virus topoisomerase II maintained a relatively high cleavage activity with this substrate. Presumably, this residual activity would allow the viral enzymes to function properly, even on heavily methylated genomes. Finally, while the level of DNA cleavage observed for the chlorella virus enzymes with the mixed 6mA/5mC substrate (∼7%) was decreased as compared to that seen with the wild-type substrate (∼22−37%), it was still considerably higher than that observed for human topoisomerase IIα with the unmodified oligonucleotide (∼1.8%). This finding leaves open the possibility that

other aspects of the viral environment further mute topoisomerase II cleavage activity in vivo. However, it cannot rule out the alternative that an elevated level of topoisomerase II-mediated DNA cleavage, even within a heavily methylated viral genome, still plays a vital role in the life cycle of chlorella virus.

It is not known why 6mA has such a profound effect on chlorella virus topoisomerase II, while the influence of 5mC is modest. However, the two modifications have different effects on DNA structure. The methyl group of 6mA can exist in a form either trans or cis to N1 (*70*). In the trans configuration, the methyl group protrudes into the major groove of B-form DNA and does not perturb hydrogen bonding with the opposite thymine (*70*). However, in the cis configuration, the methyl group interferes with Watson−Crick base pairing (*70*, *71*). Thus, the presence of 6mA destabilizes helix stability (*72*). In contrast, the effects of 5mC on DNA structure are variable and poorly understood. While some studies indicate that 5mC can induce intermediate structures between B- and A-form DNA (*73*, *74*), others reported no major affect on DNA structure (*75*−*77*). The presence of 5mC does appear to restrict the dynamics and bending flexibility of DNA and also may alter sugar puckering modes (*78*, *79*). With regard to this last finding, it should be noted that the presence of modified sugar rings in DNA can lead to a modest increase in the level of DNA cleavage mediated by human topoisomerase IIα (*66*, *80*, *81*).

An interesting issue that could not be addressed in this study is the effect of DNA methylation on the overall catalytic activity (DNA relaxation, decatenation, etc.) of chlorella virus topoisomerase II. While it is presumed that decreased rates of DNA cleavage would predict losses in catalytic function, the answer is not known at this time. To investigate this issue, it would be necessary to generate a circular DNA substrate that contained appropriate levels of 6mA and 5mC. Unfortunately, the methyltransferases necessary to adequately modify an existing plasmid such as pBR322 are not available. In addition, the chlorella virus genomes are not circular, and their sizes (330 to 370 kb) preclude the facile isolation of intact genomic DNAs suitable for in vitro studies.

Finally, the use of a novel rapid quench pre-equilibrium DNA cleavage system, together with existing DNA binding and ligation assays, allowed the mechanistic basis for the inhibition of DNA cleavage by methylation to be characterized. Results indicate that the primary effect of methylation on the cleavage activity of chlorella virus topoisomerase II is a decrease in the forward rate of enzyme-mediated DNA scission. While previous studies have implied an effect of anticancer drugs and DNA lesions on the forward scission reaction, it has not been possible to demonstrate this directly (*7*, *9*, *10*). The development of the rapid quench system will provide an important tool for the future study of DNA cleavage mediated by topoisomerase II.

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