Topoisomerase II from Chlorella Virus PBCV-1 Has an Exceptionally High DNA Cleavage Activity*

Received for publication, February 23, 2001, and in revised form, April 20, 2001
Published, JBC Papers in Press, April 25, 2001, DOI 10.1074/jbc.M101693200

John M. Fortune‡‡, Oleg V. Lavrukhin†, James R. Gurnon‡, James L. Van Etten¶, R. Stephen Lloyd‡, and Neil Osheroff***

From the Departments of *Biochemistry and **Medicine (Hematology/Oncology), Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146; the †Department of Human Biological Chemistry and Genetics and Sealy Center for Molecular Science, University of Texas Medical Branch, Galveston, Texas 77555-1071, and the ¶Department of Plant Pathology, University of Nebraska, Lincoln, Nebraska 68583-0722

Chlorella virus PBCV-1 topoisomerase II is the only functional type II enzyme known to be encoded by a virus that infects eukaryotic cells. However, it has not been established whether the protein is expressed following viral infection or whether the enzyme has any catalytic features that distinguish it from cellular type II topoisomerases. Therefore, the present study characterized the physiological expression of PBCV-1 topoisomerase II and individual reaction steps catalyzed by the enzyme. Results indicate that the topoisomerase II gene is widely distributed among Chlorella viruses and that the protein is expressed 60–90 min after viral infection of algal cells. Furthermore, the enzyme has an extremely high DNA cleavage activity that sets it apart from all known eukaryotic type II topoisomerases. Levels of DNA scission generated by the viral enzyme are 30 times greater than those observed with human topoisomerase II. The high levels of cleavage are not due to inordinately tight enzyme-DNA binding or to impaired DNA religation. Thus, they most likely reflect an elevated forward rate of scission. The robust DNA cleavage activity of PBCV-1 topoisomerase II provides a unique tool for studying the catalytic functions of type II topoisomerases.

Topoisomerases play key roles in virtually every cellular DNA process, including replication, transcription, recombination, and chromosome segregation (1–6). Although viral genomes undergo these same processes, few viruses encode their own DNA topoisomerases. Thus, it appears that most viruses rely on host enzymes to modulate the topological state of their chromosomes.

There are some notable exceptions. A few viruses with large double-stranded DNA genomes contain topoisomerase genes. For example, poxviruses (including vaccinia) encode a type I topoisomerase (7–10). The vaccinia enzyme is the smallest of three separate polypeptide subunits (17–19). This enzyme is believed to play roles in viral DNA replication and recombination (20).

For many years, no type II topoisomerases were identified in viruses that infect eukaryotic cells. However, open reading frames predicted to encode type II topoisomerases recently have been reported in three eukaryotic viral genomes: the asfarvirus African swine fever virus (21, 22), the phycodnavirus Paramecium bursaria Chlorella virus (PBCV-1) (23), and the iridovirus Chilo iridescent virus (24). Of these viruses, the only one that has been demonstrated to encode an active topoisomerase II is PBCV-1 (25).

PBCV-1 is the prototypical member of a group of viruses that infect Chlorella-like algae (26–28). 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 (29, 30). No viruses are detectable in the algae while they are living within a Paramecium; however, when algae are removed from their symbiotic partner, they serve as hosts for Chlorella viruses (26).

PBCV-1 topoisomerase II is the smallest known type II topoisomerase, with a protomer molecular mass of 120 kDa compared to 160–180 kDa for eukaryotic enzymes (1, 5, 6, 25, 31). The small size of the PBCV-1 enzyme is due to the absence of the C-terminal domain found in eukaryotic type II topoisomerases (5, 6, 25). Although this region is not highly conserved, it contains phosphorylation sites (32–34) and nuclear localization sequences (35–39). PBCV-1 topoisomerase II has high amino acid sequence identity with several other type II enzymes, including human topoisomerase IIα (46%) (23, 25). Recombinant PBCV-1 topoisomerase II (purified from yeast) displays enzymatic properties typical of eukaryotic type II enzymes; it relaxes, catenates, and decatenates double-stranded DNA substrates in an ATP-dependent manner (25).

Although PBCV-1 carries its own topoisomerase II gene, it is not known whether the protein actually is expressed following viral infection of host cells. Furthermore, it is not known whether the viral topoisomerase II has any catalytic features that distinguish it from cellular type II enzymes. To address these fundamental issues, the present study characterized the

---

* This work was supported in part by Grants GM33944 (to N. O.), ES05355 (to R. S. L.), and GM32441 (to J. L. V. E.) from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡‡ To whom correspondence should be addressed: Dept. of Biochemistry, 654 Robinson Research Bldg., Vanderbilt University School of Medicine, Nashville, TN 37232-0146. Tel.: 615-322-4338; Fax: 615-343-1166; E-mail: osheron@ctrvax.vanderbilt.edu.

† The abbreviations used are: PBCV-1, Paramecium bursaria Chlorella virus; kb, kilobase(s).
physiological expression of PBCV-1 topoisomerase II and examined individual reaction steps catalyzed by the enzyme. Results indicate that PBCV-1 topoisomerase II is synthesized in vivo post-infection. Moreover, the enzyme has an extremely high DNA cleavage activity that sets it apart from all known eukaryotic type II topoisomerases.

**EXPERIMENTAL PROCEDURES**

**Materials**—PBCV-1 topoisomerase II was purified using a Saccharomyces cerevisiae overexpression system as described by Lavrutkin et al. (25). Human topoisomerase IIIs also was expressed in yeast (40) and was purified by the protocol of Kingma et al. (41). Negatively supercoiled pBR322 DNA was prepared as described previously (42). [γ-32P]ATP (3000 Ci/mmol) was obtained from Amersham Pharmacia Biotech, etoposide was from Sigma Chemical Co., amascrine was from Bristol-Myers Squibb, CP-115,953 was from Pfizer, and genistein was from ICN. Etoposide, amascrine, CP-115,953, and genistein were stored from 0.1 mM NaEDTA, and 2.5% glycerol). Reactions were initiated by the addition of PBCV-1 topoisomerase II or human topoisomerase II and allowed to proceed until cleavage reactions were complete. Apparent first order religation rates were determined by converting the photographs of the ethidium bromide-stained membranes to digital images using a Hewlett-Packard ScanJet 4C scanner and analyzing the images with ImageQuaNT software. To monitor possible loading differences between samples, the relative amount of the 3.6-kb rRNA in each lane was determined using a Storm 840 PhosphorImager and ImageQuaNT software. ATPase assays were performed as described by Osheroff et al. (48). Reaction mixtures contained 20 nM PBCV-1 topoisomerase II, 300 nM negatively supercoiled pBR322 DNA, and 1 mM [γ-32P]ATP (8000 Ci/mmol) in a total of 20 μl of PBCV-1 topoisomerase II reaction buffer (10 mM Tris-Cl, pH 7.5, 62.5 mM KCl, 62.5 mM NaCl, 2.5 mM MgCl₂, 0.1 mM NaEDTA, and 2.5% glycerol). Reactions were initiated by the addition of PBCV-1 topoisomerase II and allowed to proceed until cleavage reactions were complete. Apparent first order religation rates were determined by converting the photographs of the ethidium bromide-stained membranes to digital images using a Hewlett-Packard ScanJet 4C scanner and analyzing the images with ImageQuaNT software.

To follow synthesis of PBCV-1 topoisomerase II protein, Chlorella NC64A cells (1 × 10⁹ cells/sample) were collected by centrifugation at various times after PBCV-1 infection, frozen in liquid nitrogen, and stored at −80 °C. Cells were suspended in TRIzol reagent (Life Technologies, Inc.) and disrupted by vortexing with glass beads (0.25–0.30 mm in diameter) at high speed for 5–20 min with intermittent cooling. Total RNA was subjected to electrophoresis on 1.5% agarose/formaldehyde-denaturing gels, stained with ethidium bromide, and transferred to nylon membranes (Micron Separations), fixed by UV cross-linking, and hybridized with a γ-32P-labeled PBCV-1 topoisomerase II full-length gene probe as described previously (44).

**Physiological Expression of PBCV-1 Topoisomerase II**—Transcription of the PBCV-1 topoisomerase II gene was monitored by the procedure of Sun et al. (45). Briefly, Chlorella NC64A cells (1 × 10⁶ cells/sample) were collected by centrifugation at various times after PBCV-1 infection, frozen in liquid nitrogen, and stored at −80 °C. Cells were suspended in TRIzol reagent (Life Technologies, Inc.) and disrupted by vortexing with glass beads (0.25–0.30 mm in diameter) at high speed for 5–20 min with intermittent cooling. Total RNA was subjected to electrophoresis on 1.5% agarose/formaldehyde-denaturing gels, stained with ethidium bromide, and transferred to nylon membranes (Micro Separation). Membranes subsequently were photographed under UV illumination to visualize transferred RNA. The RNA was hybridized with a γ-32P-labeled PBCV-1 topoisomerase II full-length gene probe as described (45), and radioactivity bound to the membranes was visualized using a Storm 840 PhosphorImager and ImageQuant software (Molecular Dynamics). To monitor possible loading differences between samples, the relative amount of the 3.6-kb rRNA in each lane was determined by converting the photographs of the ethidium bromide-stained membranes to digital images using a Hewlett-Packard ScanJet 4C scanner and analyzing the images with ImageQuant software.

To follow synthesis of PBCV-1 topoisomerase II protein, Chlorella NC64A cells (1 × 10⁶ cells/sample) were collected by centrifugation at various times after viral infection, resuspended in lysis buffer (10 mM Tris, pH 8.0, 140 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 0.5% plant protease inhibitor mixture (Sigma), and stored at −80 °C (45). Samples were disrupted by vortexing with glass beads as described above and clarified by centrifugation at 16,000 × g for 15 min, at 4 °C. Proteins were immunoprecipitated from equal numbers of cells with 2 μl of anti-PBCV-1 topoisomerase II antibodies (45), and equal numbers of samples were resolved by electrophoresis on a denaturing polyacrylamide gel (46). Protein gels were stained with Coomassie Brilliant Blue R or transferred to nylon membranes and reacted with anti-PBCV-1 topoisomerase II antibodies (diluted 1:200). Protein concentrations were measured by the method of Bradford (47) with bovine serum albumin as a standard.

**ATP Hydrolysis**—ATPase assays were performed as described by Osheroff et al. (48). Reaction mixtures contained 20 nM PBCV-1 topoisomerase II, 300 nM negatively supercoiled pBR322 DNA, and 1 mM [γ-32P]ATP (8000 Ci/mmol) in a total of 20 μl of PBCV-1 topoisomerase II reaction buffer (10 mM Tris-Cl, pH 7.5, 62.5 mM KCl, 62.5 mM NaCl, 2.5 mM MgCl₂, 0.1 mM NaEDTA, and 2.5% glycerol). Reactions were initiated by the addition of PBCV-1 topoisomerase II and allowed to proceed until cleavage reactions were complete. Apparent first order religation rates were determined by converting the photographs of the ethidium bromide-stained membranes to digital images using a Hewlett-Packard ScanJet 4C scanner and analyzing the images with ImageQuaNT software. ATP hydrolysis was monitored by the release of free phosphate.

**DNA Cleavage**—DNA cleavage reactions were performed on the procedure of Fortune and Osheroff (49). Each reaction contained 10 nM negatively supercoiled pBR322 DNA in a total of 20 μl of PBCV-1 topoisomerase II reaction buffer. Reactions were initiated by the addition of PBCV-1 topoisomerase II reaction buffer (10 mM Tris-Cl, pH 7.5, 135 mM KCl, 5 mM MgCl₂, 0.1 mM NaEDTA, and 2.5% glycerol). Some reactions included 1 mM ATP. In certain reactions, 1 mM MgCl₂ was used or replaced by 1 mM CaCl₂, MnCl₂, CoCl₂, SrCl₂, BaCl₂, CuCl₂, ZnCl₂, or CdCl₂. Some reactions contained etoposide, amascrine, CP-115,953, genistein, or drug solvent (water or 100% Me₂SO). Drug concentrations were 50 μM unless otherwise indicated. Cleavage was initiated by the addition of PBCV-1 topoisomerase II or human topoisomerase II (enzyme concentrations were 20 nM for PBCV-1 and 200 nM for human unless stated otherwise). Reactions were incubated for 6 min at 25 °C (PBCV-1) or 37 °C (human) to allow establishment of a cleavage/religation equilibrium. Cleavage intermediates were trapped by adding 2 μl of 12% SDS and 2 μl of 120 mM NaEDTA, pH 8.0 (PBCV-1), or 2 μl of 5% SDS and 2 μl of 250 mM NaEDTA, pH 8.0 (human). Proteinase K was added (2 μl of 0.8 mg/ml), and reactions were incubated 30 min at 45 °C to digest the type II enzyme. Samples were mixed with 2 μl of agarose gel loading buffer (60% sucrose in 10 mM Tris-Cl, pH 7.9), heated for 2 min at 70 °C, and subjected to electrophoresis in 1% agarose. Membranes subsequently were photographed under UV illumination to visualize transferred RNA. The RNA was hybridized with the images with ImageQuant software.

In reactions that determined whether DNA cleavage by PBCV-1 topoisomerase II was reversible, NaCl (an additional 250 mM) or EDTA (10 mM final concentration, in lieu of post-SDS addition) was added prior to treatment with SDS. To determine whether cleavage was protein-linked, proteinase K treatment was omitted.

**DNA Binding**—To characterize topoisomerase II-DNA binding, an electrophoretic mobility shift assay was carried out according to the procedure of Osheroff (50). Binding mixtures contained 10 nM negatively supercoiled pBR322 DNA and PBCV-1 topoisomerase II or human topoisomerase IIIs in 20 μl of the appropriate reaction buffer. For these assays, PS was omitted from these buffers to eliminate DNA cleavage and the resulting covalent enzyme-DNA interaction. Samples were incubated at 25 °C (PBCV-1) or 37 °C (human) for 6 min, loaded directly onto a 1% agarose gel, and subjected to electrophoresis in TAE buffer containing 0.5 μg/ml ethidium bromide. DNA bands were visualized and photographed as described in the preceding section.

**DNA Religation**—Religation reactions were based on the procedure of Robinson and Osheroff (51). DNA cleavage/religation equilibria were established as described above for PBCV-1 topoisomerase II and human topoisomerase IIIs, except that CaCl₂ was substituted for MgCl₂ in the reaction buffers. After the 6-min incubation, 10 mM NaEDTA, pH 8.0, was added to trap the DNA cleavage complexes and 250 mM NaCl was added to prevent re-cleavage. Religation was initiated by the addition of 2 mM MgCl₂. Reactions were stopped at different time points by adding SDS followed by NaEDTA, pH 8.0, as described for topoisomerase II-mediated DNA cleavage. Samples were processed and analyzed as described for cleavage reactions. Apparent first order religation rates were determined by quantitating the loss of linear DNA.

**RESULTS**

**Distribution of the Topoisomerase II Gene among Chlorella Viruses**—The Chlorella virus PBCV-1 genome contains an open reading frame whose sequence is homologous to eukaryotic type II topoisomerases (23). When expressed in yeast, this gene was found to be highly expressed in yeast (24). Human topoisomerase II was purified by the protocol of Kingma et al. (25). When expressed in yeast, this gene was found to be highly expressed in yeast (24). Human topoisomerase II was purified by the protocol of Kingma et al. (25).
High DNA Cleavage Activity of PBCV-1 Topoisomerase II

Because PBCV-1 topoisomerase II requires ATP to relax, catenate, or decatenate DNA substrates (25), the first reaction step that was characterized was ATP hydrolysis. As seen in Fig. 3, the viral enzyme displayed an intrinsic ATPase activity that was stimulated 3- to 5-fold by DNA. These properties are similar to those of previously characterized cellular type II topoisomerases from eukaryotic sources (48, 52–54).

**DNA Cleavage**—To interconvert different topological forms of DNA, topoisomerase II must first create a transient double-stranded break in the sugar-phosphate backbone of the double helix (55, 56). During this scission event, topoisomerase II forms covalent bonds with the 5'-termini of the newly cleaved DNA (1, 2, 5, 6, 55). These covalent protein-DNA linkages maintain genomic integrity and help align the DNA termini for religation (1, 6, 57). The DNA cleavage/religation reaction mediated by topoisomerase II is fundamental to every essential cellular function catalyzed by the enzyme. In addition, it is the target for several widely used anticancer drugs that kill cells by inducing high levels of topoisomerase II-mediated DNA breaks (5, 6, 58, 59).

Because of the central importance of the scission event, the ability of PBCV-1 topoisomerase II to cleave DNA was characterized (Fig. 4). As determined by conversion of negatively supercoiled circular plasmid to linear molecules, the enzyme generated double-stranded breaks in its DNA substrate. DNA scission was reversed when salt or EDTA was added to the reaction prior to topoisomerase II denaturation, suggesting that the enzyme does not release the cleaved DNA intermediate. The covalent enzyme-DNA linkage was confirmed by omitting the proteinase K treatment. In the absence of protease digestion, the electrophoretic mobility of cleaved (i.e. linear) DNA decreased and the band broadened significantly.

The qualitative nature of DNA cleavage mediated by PBCV-1 topoisomerase II was similar to those of cellular type II enzymes. However, the quantitative aspect of the reaction was markedly different; levels of DNA scission observed in the presence of the viral enzyme were exceptionally high. Because PBCV-1 topoisomerase II was compared with that of human topoisomerase IIa (Fig. 5). Optimal catalytic conditions for the individual enzymes were used for these experiments. Levels of DNA cleavage were determined at a series of enzyme:plasmid...
The ATPase activity of PBCV-1 topoisomerase II was determined by monitoring the release of free phosphate from [γ-32P]ATP. Reactions were carried out in the absence (○) or presence (●) of DNA. Error bars represent the standard error of the mean for two independent experiments.

High DNA Cleavage Activity of PBCV-1 Topoisomerase II

ratios in the absence or presence of ATP. In the absence of the nucleotide triphosphate, scission corresponds to equilibrium levels of the topoisomerase II-cleaved DNA intermediate (i.e., cleavage complex) formed prior to DNA strand passage (50). In the presence of ATP, levels of scission correspond to the steady-state concentration of pre- and post-strand passage DNA cleavage intermediates generated during ongoing enzyme catalysis (50, 60). DNA cleavage often is higher in the presence of ATP (50, 55, 60).

The ability of human topoisomerase IIα to cleave DNA is comparable to that observed for many eukaryotic type II topoisomerases. At an enzyme:DNA ratio of 20:1 (in the absence of ATP), ~10% of the DNA was in a covalent cleavage complex with the enzyme (left panel). Even in the presence of ATP, only 20% of the DNA was cleaved at this enzyme:plasmid ratio (right panel). These results suggest that ~1% of human topoisomerase IIα is covalently linked to DNA in a cleavage complex.

FIG. 3. ATP hydrolysis catalyzed by PBCV-1 topoisomerase II. The ATPase activity of PBCV-1 topoisomerase II was determined by monitoring the release of free phosphate from [γ-32P]ATP. Reactions were carried out in the absence (○) or presence (●) of DNA. Error bars represent the standard error of the mean for two independent experiments.

FIG. 4. DNA cleavage mediated by PBCV-1 topoisomerase II is protein-linked and reversible. The ability of PBCV-1 topoisomerase II to cleave negatively supercoiled DNA was examined. An agarose gel stained with ethidium bromide is shown. A DNA control (DNA Std) and a DNA cleavage sample (Topo II) are shown. To determine whether the observed DNA cleavage was protein-linked, proteinase K treatment was omitted (-Pro K). Reversibility of the cleavage reaction was determined by adding salt or EDTA prior to SDS. Double-stranded DNA cleavage converts negatively supercoiled plasmid (form I, FI) to linear molecules (form III, FIII). The position of nicked circular DNA (form II, FII) also is indicated.

FIG. 5. Comparison of DNA cleavage mediated by PBCV-1 topoisomerase II and human topoisomerase IIα. Cleavage of negatively supercoiled plasmid DNA by PBCV-1 topoisomerase II (●) and human topoisomerase IIα (○) was determined over a range of enzyme:plasmid ratios (at a constant plasmid concentration of 10 nM). Enzyme concentrations were varied from 5 to 30 nM for PBCV-1 topoisomerase II, and from 10 to 200 nM for human topoisomerase IIα. DNA cleavage was examined both in the absence (left panel) and presence (right panel) of ATP. Levels of DNA cleavage are expressed as the percentage of plasmid substrate that was cleaved. The insets show the linear DNA cleavage bands observed with PBCV-1 topoisomerase II at an enzyme:DNA ratio of 2:1 and with human topoisomerase IIα at an enzyme:DNA ratio of 20:1. Data are representative of four independent experiments.

Higher levels of DNA cleavage intermediates generated by the viral enzyme may result from alterations in three separate reaction steps (5, 6). They may reflect 1) a higher affinity of the enzyme for its DNA substrate, 2) an increase in the forward rate of DNA scission, or 3) a decrease in the rate of DNA religation. Unfortunately, no assay has been developed that is capable of monitoring the forward rate of DNA scission independently from the rates of DNA binding and religation. Consequently, the ability of PBCV-1 topoisomerase II to bind its DNA substrate and to religate cleaved DNA was examined.

DNA Binding—The binding of PBCV-1 topoisomerase II to DNA was monitored by an electrophoretic gel mobility shift assay (Fig. 6). Assays utilized negatively supercoiled plasmid DNA and were carried out in the absence of divalent cation to prevent DNA cleavage (and the resulting covalent protein-DNA interactions) (61, 62). Results were compared with those obtained with human topoisomerase IIα.

Electrophoretic mobility shifts observed at low concentrations of PBCV-1 topoisomerase II and human topoisomerase IIα were similar (Fig. 6). Furthermore, initial upshifts were observed at the same concentration for both enzymes. These

2 Scission mediated by the viral enzyme could not be measured accurately at enzyme:plasmid ratios higher than 3:1, due to the formation of multiple cleavage complexes per plasmid and the subsequent formation of linear molecules that were less than unit length.

3 At high enzyme concentrations, characteristics of the DNA electrophoretic mobility shift differed for PBCV-1 topoisomerase II and human topoisomerase IIα. While the plasmid continued to shift upwards in a stepwise manner with increasing concentrations of PBCV-1 topoisomerase II, the DNA shifted to the origin at high concentrations of the human enzyme. Similar species-specific variations among eukaryotic type II topoisomerases have been observed previously (49, 50, 63).
findings suggest the DNA binding affinity of the viral enzyme is similar to that of human topoisomerase IIα. This conclusion is supported by the fact that both enzymes display optimal catalytic DNA relaxation activity at comparable ionic strengths (~150 mM) and convert from a processive to a distributive reaction at ~190 mM salt (Ref. 25, data not shown). If the binding affinity of PBCV-1 topoisomerase II were considerably higher, one would expect that the ionic strengths required for maximal catalytic activity and the processive-to-distributive transition would be much greater than those observed for the human enzyme.

DNA Religation—The next reaction step that was examined was DNA religation. As seen in Fig. 7, the PBCV-1 enzyme religated its cleaved DNA intermediate with an apparent first order rate that was ~2-fold faster than that observed for human topoisomerase IIα (0.071 s⁻¹ versus 0.034 s⁻¹, respectively). Therefore, the high levels of cleavage complexes observed with the PBCV-1 enzyme cannot be explained by a low rate of DNA religation. Together, the DNA binding and religation results suggest that the robust cleavage observed with PBCV-1 topoisomerase II reflects a high rate of DNA scission rather than inordinately tight DNA binding or impaired religation.

Divalent Cations—The only cofactor required for topoisomerase II-mediated DNA cleavage is a divalent cation (61). The ability of different divalent cations to support DNA scission by the viral enzyme. In addition, DNA cleavage is not typically observed at Mg²⁺ (the least effective cofactor that supported scission) at 1 µM, and the apparent first order rate of religation was determined from the disappearance of cleaved DNA. The amount of DNA cleavage observed at equilibrium for each enzyme was set to 100% at time zero. Data are representative of two independent experiments.

Because the high DNA cleavage activity of PBCV-1 topoisomerase II is atypical of eukaryotic type II enzymes, the requirement for a divalent cation in this process was examined (Fig. 8). As expected, DNA scission mediated by PBCV-1 topoisomerase II required the presence of a divalent cation. No cleavage was observed at Mg²⁺ concentrations ≤ 100 µM, and optimal activity plateaued by 1 mM (not shown). The highest levels of cleaved DNA were observed in the presence of Mg²⁺ or Ca²⁺. Cleavage also was supported by Mn²⁺, albeit to a lesser extent. No DNA scission was observed in the presence of Zn²⁺, Cd²⁺, or Ba²⁺. However, in marked contrast to eukaryotic type II topoisomerases, the viral enzyme was able to use several additional divalent cations for DNA cleavage. Substantial levels of scission were observed in the presence of Co²⁺, Sr²⁺, or Ba²⁺, ranging from ~9 to 18% DNA cleavage (compared with ~50% cleavage observed with Mg²⁺). To put these values into perspective, levels of DNA cleavage generated in the presence of Ba²⁺ (the least effective cofactor that supported scission) at a 1:1 ratio of PBCV-1 topoisomerase II:plasmid were comparable to those generated at a 20:1 ratio for human topoisomerase IIα in the presence of Ca²⁺ (see Fig. 5, left panel).

The DNA cleavage observed in reactions containing Co²⁺, Sr²⁺, or Ba²⁺ cannot be explained by the presence of contaminating divalent cations. The salt preparations employed contained less than 0.005% Mg²⁺ or Ca²⁺. This would lead to the presence of ~50 mM contaminants in reaction mixtures, and as shown in Fig. 8, 100 µM divalent cation does not sustain DNA scission by the viral enzyme. In addition, DNA cleavage is not
due to an enzyme-independent reaction, because no DNA scission was observed when plasmid substrates were incubated with Co²⁺, S²⁻, or Ba²⁺ in the absence of PBCV-1 topoisomerase II (not shown).

These data demonstrate that the active site of PBCV-1 topoisomerase II is able to accommodate a much broader spectrum of divalent cations than other type II enzymes.

Anticancer Drugs—Topoisomerase II is the target for several important drugs that are used to treat many different human cancers (5, 6, 58, 59). Although these drugs are structurally diverse, they act by a common mode of action. While they inhibit overall catalytic activity of the enzyme, these compounds kill cells by increasing levels of covalent topoisomerase II-DNA cleavage complexes (5, 6, 64, 65). Because the concentration of DNA cleavage intermediates is already very high for PBCV-1 topoisomerase II (not shown).

The effects of etoposide, amsacrine, CP-115,953, or genistein on PBCV-1 topoisomerase II-mediated DNA cleavage were examined (Fig. 9). No significant enhancement of DNA cleavage was observed with any of the compounds. Under similar conditions, all of these drugs enhanced cleavage with human to-

DISCUSSION

Viruses that infect eukaryotic cells commonly rely on host proteins to support fundamental DNA processes. Consequently, it is rare that they encode their own DNA topoisomerases. Nonetheless, poxviruses encode a type I topoisomerase (7–10), and three other viruses, African swine fever virus (21, 22), P. bursaria Chlorella virus (23), and Chilo iridescent virus (24), contain open reading frames that are predicted to encode type II topoisomerases. One common feature of these viruses is that they have large double-stranded DNA genomes, ranging from 170 kb in African swine fever virus (69) to 330 kb in PBCV-1 (70). Furthermore, these have a similar genome structure; poxviruses (71), African swine fever virus (72, 73), and PBCV-1 (70, 74) contain linear nonpermuted double-stranded DNA genomes with inverted repeat regions adjacent to covalently closed hairpin termini. In contrast, the Chilo iridescent virus genome is circularly permuted and terminally redundant (75–77).

Of the three viruses with predicted topoisomerase II genes, only PBCV-1 has been shown to encode an active type II enzyme (25). The original study on PBCV-1 topoisomerase II demonstrated that the enzyme could be produced in a yeast recombinant system (25) but did not determine whether the protein actually is expressed following viral infection of algal cells. As determined in the present work, the PBCV-1 topoisomerase II gene is transcribed shortly after infection. The protein is synthesized 60–90 min post-infection and remains throughout the life cycle of the virus. Thus, PBCV-1 topoisomerase II is the first viral type II enzyme found to be expressed during the life cycle of a virus that infects eukaryotic cells.

It is not clear why PBCV-1 carries its own topoisomerase II gene. The fact that type II topoisomerases are uncommon among viruses implies that the enzyme plays a unique and important role. This hypothesis is supported by the finding that the topoisomerase II gene is widely distributed among Chlorella viruses. At the present time, the physiological functions of the viral enzyme have not been defined. Circumstantial evidence indicates that initial replication of the PBCV-1 chromosome probably takes place in the nucleus (28). The lack of an obvious nuclear localization signal in the viral enzyme (due to the C-terminal truncation) (25), together with the fact that replication begins ~60 min following infection (28) (just as synthesis of PBCV-1 topoisomerase II is beginning), makes it doubtful that the type II enzyme is required for the early stages of this process. More likely, PBCV-1 topoisomerase II functions
in the late stages of viral replication or packaging, both of which take place in the cytoplasm (28). Finally, the high DNA cleavage activity of the enzyme suggests a potential role for PBCV-1 topoisomerase II in viral recombination. Unfortunately, until molecular techniques become available that allow specific manipulation of the PBCV-1 genome, the precise role(s) of the enzyme in the virus life cycle will remain unknown.

Because viral enzymes often display unique properties, the present study characterized individual reaction steps of the PBCV-1 topoisomerase II catalytic cycle to determine whether the protein has features that differentiate it from cellular type II enzymes. One striking difference was observed. The concentration of DNA cleavage complexes formed by PBCV-1 topoisomerase II is dramatically higher than typically observed with eukaryotic type II enzymes. Compared with human topoisomerase IIa, the viral enzyme generates levels of DNA cleavage that are ~30 times higher. This increased scission is not due to an excessively tight enzyme-DNA binding or to an impaired DNA relaxation activity. Moreover, preliminary studies indicate that the viral enzyme does not cleave DNA at a wider distribution of sites than human topoisomerase IIa.4 These findings suggest that the high level of scission generated by PBCV-1 topoisomerase II reflects an exceptionally robust forward DNA cleavage reaction.

The molecular basis for the high DNA cleavage activity of PBCV-1 topoisomerase II is not known. It may be related to the C-terminal truncation of the viral enzyme. This highly charged region of eukaryotic type II topoisomerases has been proposed to play an autoinhibitory function (78, 79). Alternatively, increased DNA scission may result from subtle differences in the active site of PBCV-1 topoisomerase II. Evidence for such a difference comes from divergent cation experiments. The DNA cleavage reaction of the viral enzyme is supported by a broader spectrum of divalent cations than previously observed for any eukaryotic type II topoisomerase.

Amino acid sequence alterations that may contribute to increased cleavage are not obvious. The primary structure of PBCV-1 topoisomerase II is ~45% identical to a variety of eukaryotic type II enzymes, including budding yeast, fission yeast, and Drosophila topoisomerase II, as well as human topoisomerase IIa and IIβ (23, 25). Most of the highly conserved charged residues in the DNA binding/cleavage domain of eukaryotic type II enzymes are present in the viral enzyme. However, several positions that contain hydrophobic or neutral amino acids in most eukaryotic enzymes are replaced by charged residues in PBCV-1 topoisomerase II.

In summary, PBCV-1 topoisomerase II is the first viral type II enzyme found to be expressed following infection of a eukaryotic host. Furthermore, levels of DNA scission generated by this enzyme are significantly greater than previously observed for eukaryotic type II topoisomerases. The extraordinary DNA cleavage activity of PBCV-1 topoisomerase II provides a unique model in which to study the catalytic functions of type II enzymes.

Acknowledgments—We are grateful to M. Sabourin and A. M. Wiletsman for critical reading of the manuscript.

REFERENCES

1. Wang, J. C. (1996) Annu. Rev. Biochem. 65, 635–692
2. Wang, J. C. (1998) Q. Rev. Biophys. 3, 107–144
3. Nittis, J. L. (1998) Biochim. Biophys. Acta 1400, 63–81
4. Pommier, Y., Fourquier, P., Fan, Y., and Strumbeg, D. (1998) Biochim. Biophys. Acta 1400, 83–106
5. Burden, D. A., and Osheroff, N. (1998) Biochim. Biophys. Acta 1400, 139–154
6. Fortune, J. M., and Osheroff, N. (2000) Prog. Nucleic Acid Res. Mol. Biol. 64, 221–353
7. Bauer, W. R., Resner, E. C., Kates, J., and Patatke, J. V. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 1841–1845
8. Shaffer, R., and Traktman, P. (1987) J. Biol. Chem. 262, 9309–9315
9. Shuman, S., and Moss, B. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 7478–7482
10. Shuman, S. (1998) Biochim. Biophys. Acta 1400, 321–337
11. Shuman, S., and Prescott, J. (1990) J. Biol. Chem. 265, 17826–17836
12. Shuman, S. (1991) J. Biol. Chem. 266, 1786–1803
13. Shuman, S., Goldner, M., and Moss, B. (1987) Virology 170, 302–306
14. Shuman, S. (1992) J. Biol. Chem. 267, 8620–8627
15. Shuman, S. (1992) J. Biol. Chem. 267, 16755–16758
16. Shuman, S., Ayres, J., and Osheroff, S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 785–789
17. Liu, L. F., Liu, C. C., and Alberts, B. M. (1979) Nature 281, 456–461
18. Stetler, G. L., King, G. J., and Huang, W. M. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 3737–3741
19. Kreutzer, K. N., and Huang, W. M. (1983) in Bacteriophage T4 (Matthews, K. C., Kutter, E. M., Mosig, and Berget, P. B., eds) pp. 90–96, American Society for Microbiology, Washington, DC
20. Mosig, G. (1988) Annu. Rev. Genet. 32, 379–413
21. Garcia-Beato, R., Freije, J. M., Lopez-Otin, C., Blasco, R., Vinuela, E., and Salas, M. L. (1992) Virology 188, 838–847
22. Baylin, S. A., Dixon, L. K., Vyledelimg, S., and Smith, G. L. (1992) J. Mol. Biol. 228, 1003–1010
23. Li, Y., Lu, Z., Sun, L., Ropp, S., Kushtis, G. F., Rock, D., and Van Etten, J. L. (1997) Virology 237, 360–377
24. Muller, K., Tidona, C. A., and Darai, G. (1999) Virus Genes 18, 243–264
25. Lavrukhin, O. V., Fortune, J. M., Wood, T. G., Burbank, D. E., Van Etten, J. L., Osheroff, N., and Lloyd, R. S. (2000) J. Biol. Chem. 275, 6915–6921
26. Van Etten, J. L., Meints, R. H., Kuczynski, J. B., Burbank, D. E., and Lee, K. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 3867–3871
27. These findings suggest that the high level of scission generated by PBCV-1 topoisomerase II reflects an exceptionally robust forward DNA cleavage reaction.
28. The molecular basis for the high DNA cleavage activity of PBCV-1 topoisomerase II is not known. It may be related to the C-terminal truncation of the viral enzyme. This highly charged region of eukaryotic type II topoisomerases has been proposed to play an autoinhibitory function (78, 79). Alternatively, increased DNA scission may result from subtle differences in the active site of PBCV-1 topoisomerase II. Evidence for such a difference comes from divergent cation experiments. The DNA cleavage reaction of the viral enzyme is supported by a broader spectrum of divalent cations than previously observed for any eukaryotic type II topoisomerase.
29. Amino acid sequence alterations that may contribute to increased cleavage are not obvious. The primary structure of PBCV-1 topoisomerase II is ~45% identical to a variety of eukaryotic type II enzymes, including budding yeast, fission yeast, and Drosophila topoisomerase II, as well as human topoisomerase IIa and IIβ (23, 25). Most of the highly conserved charged residues in the DNA binding/cleavage domain of eukaryotic type II enzymes are present in the viral enzyme. However, several positions that contain hydrophobic or neutral amino acids in most eukaryotic enzymes are replaced by charged residues in PBCV-1 topoisomerase II.
30. In summary, PBCV-1 topoisomerase II is the first viral type II enzyme found to be expressed following infection of a eukaryotic host. Furthermore, levels of DNA scission generated by this enzyme are significantly greater than previously observed for eukaryotic type II topoisomerases. The extraordinary DNA cleavage activity of PBCV-1 topoisomerase II provides a unique model in which to study the catalytic functions of type II enzymes.
31. Acknowledgments—We are grateful to M. Sabourin and A. M. Wiletsman for critical reading of the manuscript.

4 J. M. Fortune and N. Osheroff, unpublished data.
69. Yanez, R. J., Rodriguez, J. M., Nogal, M. L., Yuste, L., Enriquez, C., Rodriguez, J. F., and Vinuela, E. (1995) Virology 208, 249–278
70. Rohozinski, J., Girton, L. E., and Van Etten, J. L. (1989) Virology 168, 363–369
71. Geshein, P., and Berns, K. I. (1974) J. Mol. Biol. 88, 785–796
72. Ortin, J., Enjuanes, L., and Vinuela, E. (1979) J. Virol. 31, 579–583
73. Gonzalez, A., Talavera, A., Almendral, J. M., and Vinuela, E. (1986) Nucleic Acids Res. 14, 6835–6844
74. Zhang, Y., Strasser, P., Grabherr, R., and Van Etten, J. L. (1994) Virology 202, 1079–1082
75. Delius, H., Darai, G., and Flugel, R. M. (1984) J. Virol. 49, 609–614
76. Schnitzler, P., Soltau, J. B., Fischer, M., Reisner, H., Scholz, J., Delius, H., and Darai, G. (1987) Virology 160, 66–74
77. Soltau, J. B., Fischer, M., Schnitzler, P., Scholz, J., and Darai, G. (1987) J. Gen. Virol. 68, 2717–2722
78. Corbett, A. H., Fernald, A. W., and Osheroff, N. (1993) Biochemistry 32, 2090–2097
79. Cardenas, M. E., Walter, R., Hanna, D., and Gasser, S. M. (1993) J. Cell Sci. 104, 533–543