Nucleic Acids Research, 1999, Vol. 27, No. 8 **1919–1925**

Human DNA topoisomerase I-mediated cleavage and recombination of duck hepatitis B virus DNA *in vitro*

Philippe Pourquier, Anne D. Jensen[1], Shih S. Gong[1], Yves Pommier and Charles E. Rogler[1,*]

Laboratory of Molecular Pharmacology, Building 37, Room 5D02, Division of Basic Sciences, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892-4255, USA and [1]Marion Bessin Liver Research Center, Department of Medicine, Jack and Pearl Resnick Campus of the Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA

Received as resubmission February 18, 1999; Revised and Accepted March 2, 1999

ABSTRACT

In this study, we report that eukaryotic topoisomerase I (top1) can linearize the open circular DNA of duck hepatitis B virus (DHBV). Using synthetic oligonucleotides mimicking the three-strand flap DR1 region of the DHBV genome, we found that top1 cleaves the DNA plus strand in a suicidal manner, which mimics the linearization of the virion DNA. We also report that top1 can cleave the DNA minus strand at specific sites and can linearize the minus strand via a non-homologous recombination reaction. These results are consistent with the possibility that top1 can act as a DNA endonuclease and strand transferase and play a role in the circularization, linearization and possibly integration of viral replication intermediates.

INTRODUCTION

Hepadnaviruses have an open circular (OC) DNA genome in which the 5′ ends of the plus and minus strands are determined by the specific replication mechanism (1–3) (Fig. 1A). Upon entry into a hepatocyte, OC DNA from the viral nucleocapsid is presumably transferred to the nucleus. Once in the nucleus, the viral DNA is found as a covalently closed circular (CCC) DNA (4,5), which serves as the template for transcription of viral messenger RNAs and pregenomic RNAs (Fig. 1A, right). The mechanism of conversion from OC DNA to CCC DNA in the nucleus is unknown. However, the conversion process can be explained by a mechanism utilizing cellular DNA modifying enzymes. The conversion would require: (i) removal of the terminal protein (TP) from the 5′ end of the minus strand; (ii) removal of one copy of the terminally redundant segment (termed ‘r’ on the minus strand DNA), followed by ligation of the minus strand; (iii) removal of the RNA primer from the 5′ end of the plus strand; and (iv) completion of the plus strand ligation (Fig. 1A).

Eukaryotic topoisomerase I (top1) is a multifunctional enzyme that regulates DNA topology during transcription and DNA replication and can act as a recombinase (6,7). Mechanistically, top1 catalyzes a change in the topological state of duplex DNA by concerted single-stranded cleavage and religation of the phosphodiester backbone (8–10). It has also been shown that top1 can irreversibly cleave DNA. In this case, the religation step of the reaction is prevented, due to dissociation of the DNA 3′ from the cleavage site. Such irreversible cleavage by top1 has been observed when the substrate is either single-stranded with the potential to base pair (11,12) or when the cleavage site on the scissile strand is located in the vicinity of a nick, gap or single-stranded branch (13–18). Such substrates that support cleavage without concomitant religation lead to aborted products also referred to as suicide products, and have proven very useful in uncoupling the cleavage and religation half reactions of top1 (15–20).

Though no absolute sequence specificity has been found for top1, preferred cleavage sites have been identified (21–24). A highly preferred cleavage site, called the hexadecameric sequence, has been found in the non-transcribed spacers flanking the extrachromosomal rRNA genes of *Tetrahymena* (21). Interestingly, a DNA sequence in the plus strand of duck hepatitis B virus (DHBV) DNA, spanning nt 2519–2531, was homologous to 13 out of 16 nt of this hexadecameric sequence (Fig. 1D). This homologous sequence partially spans the terminally redundant ‘r’ segment of the DHBV minus strand.

OC DHBV DNA represents a potential substrate for top1 which is abundant in the nucleus of the infected cell. A top1 cleavage has been previously identified in plus strand DNA opposite to the nick in the minus strand (25). Because studies utilizing synthetic DNA substrates indicate that top1 can resolve branched structures in DNA (13–15,19), we reasoned that top1 might exhibit similar activity on OC DHBV DNA. The existence of such reactions could have important implications for the processing of viral DNA into CCC DNA molecules in the nucleus of infected cells.

In this report, we have studied the activity of top1 on purified DHBV DNA isolated from DHBV virus particles which are believed to be transported into the nucleus to establish infection. We also used synthetic oligonucleotides that mimic the three-strand flap region comprising the minus strand termini. We report that top1 can remove the three-strand flap region of viral DNA by

*To whom correspondence should be addressed. Tel: +1 718 430 2607; Fax: +1 718 430 8975; Email: crogler@aecom.yu.edu

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

cleavage and religation of the minus strand. We also show that top1 can linearize the viral DNA by suicidal cleavage of the plus strand in the three-strand flap region. The possible consequences of these opposing *in vitro* activities on viral replication are discussed.

MATERIALS AND METHODS

Cell culture

Chicken hepatoma cell line LMH-D2 was the generous gift of Drs T.-T. Wu and W. S. Mason, Fox Chase Cancer Center, Philadelphia, PA. This cell line was derived from the cell line LMH by transfection with a wild-type DHBV construct (26). The cell line was grown in DMEM-F12 medium (Gibco) supplemented with 10% fetal bovine serum and 200 µg/ml G418 to select for cell resistance to the Neo gene cotransfected with DHBV DNA.

Purification of DHBV virion DNA

DHBV virion DNA was purified from the media of LMH-D2 cells, which secrete wild-type DHBV virions (26). The procedure used was a modification of the protocol published by Pugh *et al*. (27). Briefly, medium was collected and clarified by centrifugation (2000 r.p.m., 10 min). Viral particles were precipitated with 0.35 M NaCl and 6.5% PEG_{8000} for 30 min at 4°C and collected by centrifugation (8000 r.p.m., 15 min). The viral pellet was resuspended in Tris–HCl, pH 7.9, 6 mM $MgCl_2$, and incubated with 100 µg/ml DNase I for 10 min at 37°C. Thereafter, 3 vol of SDS/Pronase lysis buffer was added (25 mM Tris–HCl, pH 7.5, 10 mM EDTA, 100 mM NaCl, 0.5% SDS, 0.5 mg/ml pronase) and incubated overnight at 37°C. After phenol extraction, viral DNA was alcohol precipitated with wheat germ RNA as carrier and dissolved in TE buffer.

Topoisomerase I-mediated cleavage of DHBV DNA purified from DHBV virions

In order to study the time-course of topoisomerase I-mediated linearization of DHBV DNA, 1 ng of DHBV DNA was mixed with 200 U human top1 (a kind gift from Ole Westergaard) in a 50 µl reaction volume, containing 10 mM Tris–HCl pH 7.5, 1 mM EDTA, 50 mM NaCl, 0.1 mM DTT, 0.1 mM spermidine, 0.1 mg/ml BSA, 10% glycerol. The samples were incubated for various times at 37°C. To determine the top1 cleavage pattern on DHBV DNA, samples of the reactions were stopped by the addition of NaCl to 0.5 M or SDS to 1%. After 30 min, SDS was added to the reactions which were initially stopped by NaCl. All samples were treated with pronase (1 mg/ml) for 1 h at 37°C, followed by alcohol precipitation and centrifugation. Viral DNAs were dissolved in TE buffer and fractionated by electrophoresis through a 1.2% agarose gel, followed by Southern blotting and hybridization with a full-length ^{32}P DHBV probe.

Topoisomerase I reactions with oligonucleotides

HPLC purified oligonucleotides were purchased from The Midland Certified Reagent Company (Midland, TX). [α-^{32}P]cordycepin 5′-triphosphate and [γ-^{32}P]ATP were purchased from New England Nuclear (Boston, MA); polyacrylamide from Bio-Rad, Inc. (Richmond, CA). 3′ labeling was performed using terminal deoxynucleotidyl transferase (Stratagene, La Jolla, CA) with [α-^{32}P]cordycepin as described previously (13). 5′ labeling or phosphorylation was performed using 10 U of T4 polynucleotide kinase from Gibco BRL (Grand Island, NY) in the presence of 1 mM ATP for 1 h at 37°C and stopped by a 10 min incubation at 70°C. Labeling mixtures were subsequently centrifuged through a G25 Sephadex column to remove unincorporated nucleotide. Radiolabeled single-stranded DNA oligonucleotides were annealed to the same concentration of unlabeled complementary strand(s) in 1× annealing buffer (10 mM Tris–HCl, pH 7.8, 100 mM NaCl, 1 mM EDTA). Annealing mixtures were heated to 95°C for 5 min and slowly chilled overnight to room temperature. DNA oligonucleotides (~50 fmol per reaction) were incubated with 5 U of human recombinant top1 (16) for 15 min (unless otherwise indicated) at 25°C with or without CPT in standard reaction buffer (10 mM Tris–HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 µg/ml BSA). Reactions were stopped by adding either SDS (final concentration 0.5%) or NaCl (0.5 M for 30 min at 25°C followed by addition of 0.5% SDS). 3.3 vol of loading buffer (98% formamide, 0.01 M EDTA, 1 mg/ml xylene cyanol and 1 mg/ml bromophenol blue) were added to the reaction mixtures before loading. 16% denaturing polyacrylamide gels (7 M urea) were run at 40 V/cm at 50°C for 2–3 h and dried on 3MM Whatman paper sheets. Imaging and quantitations were performed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Sequencing of the recombinant products was performed by using the Maxam–Gilbert purine sequencing protocol (28).

RESULTS

Hepadnavirus virion DNAs are structurally unique in many ways. The ends of the minus strand contain a tandem duplication (Fig. 1A–C). The 9 nt duplicated segment, designated ‘r’, confers a three-strand flap structure to the virion DNA in that region. This region is critical for viral replication since it contains the site for base pairing of minus strand DNA primers (which are initiated within epsilon and translocated to DR1) and elongation of minus strands from those primers (29). Due to the limited coding capacity of the hepadnavirus genome, the virus depends on cellular enzymes for the multiple structural modifications needed for replication. Therefore, our identification of a nucleotide sequence in the ‘r’ region of DHBV DNA with sequence homology to the highly preferred top1 ‘hexadecameric’ cleavage site (21), suggested that DHBV might utilize top1 to carry out structural alterations in the DR1/r region.

Linearization of DHBV virion DNA by mammalian topoisomerase I

The potential top1 cleavage site in the hexadecameric sequence corresponds to cleavage on the 3′ side of DHBV nt 2528 of the plus strand (Fig. 1). Since nt 2528 is opposite to the nick in the minus strand, cleavage of plus strands at 2528 could lead to linearization of the viral DNA. Alternatively, cleavage at the same site after CCC DNA formation would relax the CCC DNA molecule.

In order to determine the overall effect of top1 cleavage on DHBV virion DNA, we incubated DNA isolated from DHBV virions with eukaryotic top1. Virion DNA preparation included a pronase step to remove the terminal protein bound to the 5′ end of the minus strand. After incubation for various times with human recombinant top1, aliquots were removed and the reaction was stopped by the addition of 1% SDS or 0.5 M NaCl (final concentrations), followed by an additional pronase treatment to

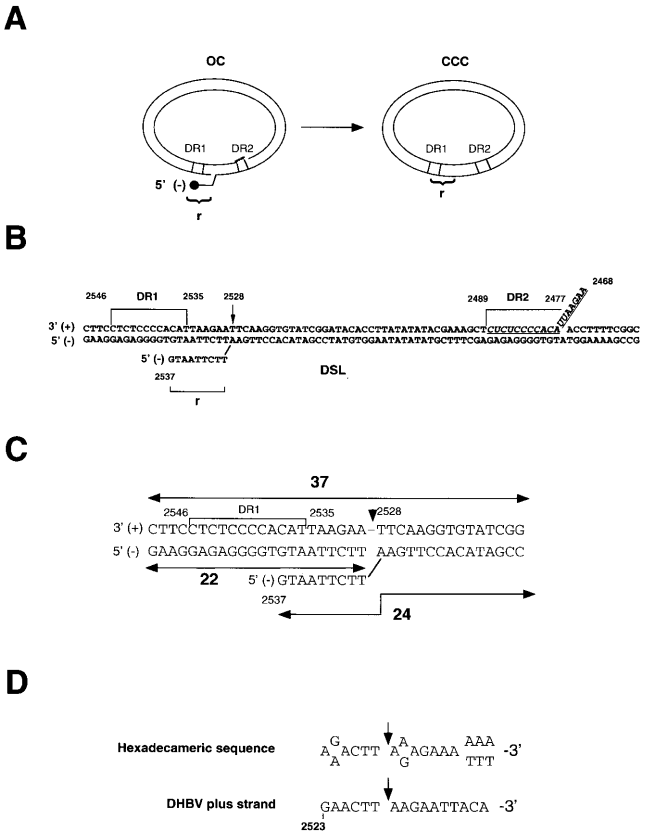

Figure 1. Structures of DHBV DNA molecules and nucleotide sequence between DR1 and DR2, including the 'r' region. (**A**) (OC) Structure of the DHBV OC DNA molecule (not to scale). The virion DNA shows the tandem duplication at the ends of the minus strand 'r' and the covalently bound terminal protein/reverse transcriptase (filled circle) at nt 2537. The broken line at DR2 represents the RNA primer at the 5′ end of the plus strand at position 2468. Both minus and plus strands are discontinuous in the OC form. (CCC) The CCC form of DHBV DNA that is required for transcription of the RNA pregenome. DR1 and DR2 represent the two direct repeat sequences. (**B**) DHBV DNA nucleotide sequence between DR1 and DR2. 'r' is the 9 bp tandem duplication in the DHBV DNA minus strand. Sequence in italics is the plus strand RNA primer. Nucleotide sequence numbers refer to the *Eco*RI site as 1. (**C**) Sequences of the synthetic oligonucleotides used in this study to mimic the region across the DR1 and 'r' region of the DHBV virion DNA. Length of the oligonucleotides is indicated by bold numbers. (**D**) Sequence homology between the specific top1 cleavage site of the hexadecameric sequence of *Tetrahymena* ribosomal DNA and the DHBV plus strand DNA region flanking nt 2528. Arrows indicate top1 cleavage sites.

remove any top1 molecules covalently bound to DHBV DNA. Reaction products were then analyzed by Southern blot (Fig. 2). While addition of a denaturant such as SDS stops the reaction by 'freezing' top1 cleavage complexes (11), high salt concentration (0.5 M NaCl) is known to reverse top1-mediated cleavage by inducing the religation step of the reaction (24,30,31). Formation of top1-linked DNA intermediates, such as double-stranded linear forms of the DHBV DNA, can be evidenced by an absence of reversal in the presence of high salt concentration, due to the inability of such aborted (or suicide) products to undergo religation.

Densitometric analysis of the top1 reaction products revealed the rapid production of a single major species of DNA that comigrated with the double-stranded linear (DSL) DHBV DNA (Fig. 2). The time-course of appearance of the linear DHBV DNA

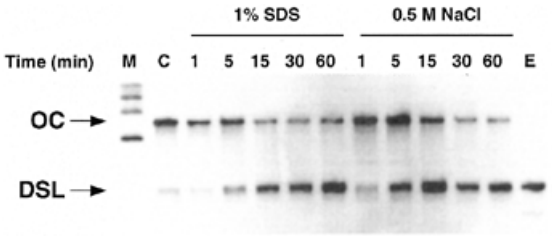

Figure 2. Cleavage of DHBV virion DNA by top1. Purified DHBV virion DNA was incubated with 200 U of human top1. Aliquots of the reaction were removed at the indicated times and reactions stopped by the addition of NaCl to 0.5 M or SDS to 1% (final concentration) as labeled. Lane M, λ *Hin*dIII digested DNA marker. Lane C, purified DHBV virion DNA incubated for 1 h. Lane E, *Eco*RI digested DHBV virion DNA. All samples were deproteinized with pronase and then fractionated by agarose gel electrophoresis (1.2%) followed Southern blot analysis with ^{32}P DHBV whole genome probe. OC, OC DHBV DNA; DSL, DSL DHBV DNA.

species was basically the same whether 0.5 M NaCl or 1% SDS was used to stop the reactions (Fig. 2). As seen in Figure 2, ~60% of the OC DHBV molecules were linearized after 30 min and no further linerization was detected, even after 3 h of incubation with top1 (data not shown). We reasoned that accumulation of the DSL species of DHBV DNA in the reactions stopped with 0.5 M NaCl (Fig. 2) was due to cleavage of OC molecules that were not readily religated due to their linearization.

The inability of top1 to fully convert OC DHBV DNA into linear molecules could be due to the rapid loss of enzyme activity or to top1-mediated modification of OC DNA, making it no longer susceptible to linearization. To address this question, a further 200 U of top1 were added to additional reaction mixes after the 30 min of incubation. Linearization of DHBV DNA remained incomplete after 60 min incubation (data not shown). These data supported the hypothesis that top1 could modify OC DHBV molecules in such a way that they were no longer susceptible to linearization. One mechanism for this effect would be circularization of minus strand DNA. Experiments to map the plus strand top1 cleavage site in complete DHBV virion DNA and also test for minus strand linkage are in progress, and will be reported elsewhere.

A topoisomerase I cleavage site on the plus strand maps to the 3′ side of DHBV nt 2528

We designed synthetic oligonucleotides to test whether top1 cleavage occurred at the predicted site in the hexadecameric sequence of the DHBV DNA plus strand. We constructed two sets of oligonucleotides to mimic either the structure of the region in OC virion DNA (Fig. 3A), or in CCC DHBV DNA (Fig. 3B). We labeled the 3′ end of the plus strand in each construct and carried out reactions in the presence or absence of high NaCl, or camptothecin (CPT), or both. CPT specifically inhibits the religation step of the top1 nicking–closing reaction by binding to the DNA–top1 cleavage intermediate (32).

These experiments enabled us to map the plus strand top1 cleavage site in both constructs tested (Fig. 3A and B). The size of the unique reaction product was 23 bp and this mapped to cleavage on the 3′ side of DHBV nt 2528. This corresponds exactly to the expected cleavage site, by analogy with the original hexadecameric sequence (Fig. 1D). In the OC substrate in Figure 3A, plus strand cleavage would lead to spontaneous separation of the reaction intermediates (as it would in intact

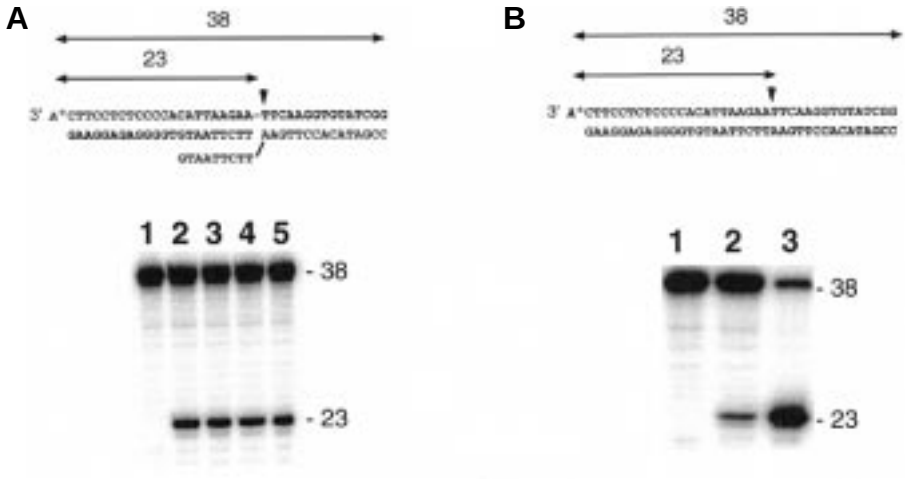

Figure 3. Topoisomerase I-mediated cleavage of the DHBV plus strand in oligonucleotides mimicking virion DNA across the DR1–'r' region. (**A**) The oligonucleotide was labeled at the 3′ end of the plus strand with [α-^{32}P]cordycepin (*A**). Lane 1, DNA alone; lanes 2 and 3, + top1; lanes 4 and 5, + top1 + 10 µM CPT. Reactions were performed at 25°C for 15 min and stopped either immediately with 0.5% SDS (lanes 2 and 4) or first treated with 0.5 M NaCl (final concentration) for an additional 30 min at 25°C before addition of 0.5% SDS (lanes 3 and 5). (**B**) Oligonucleotide mimicking a full duplex DHBV virion DNA across the DR1–r region. Lane 1, DNA alone; lane 2, + top1; lane 3, + top1 + 10 µM CPT. Reactions were stopped with 0.5% SDS after 15 min incubation with top1 at 25°C. Numbers indicate product sizes in nucleotides.

DHBV DNA), which further prevent religation in the presence of salt as shown in Figure 3A (compare lanes 2 and 3). The addition of CPT did not enhance the cleavage reaction, as would be expected for suicide reactions (Fig. 3A, lanes 4 and 5).

In contrast, top1 cleavage of the fully duplex oligonucleotide mimicking the CCC DNA substrate across the same region was greatly enhanced by CPT and was reversible in the presence of high salt as previously shown for equivalent substrates (13,24,31). This result is consistent with established mechanisms in which CPT stacks into the DNA at top1 nicking sites and inhibits the religation reaction (33–35).

Mapping of topoisomerase I cleavage sites in oligonucleotides mimicking the DHBV minus strand

In order to test whether top1 could be involved in the linearization of the minus strand, due to its recombinase activity, we first investigated whether top1 was able to cleave the DHBV DNA minus strand. To map these potential top1 cleavage sites, we separately labeled both strands (22mer and 24mer in Fig. 1C) with [α-^{32}P]cordycepin on the 3′ end. No cleavage was detected on the 24mer minus strand (data not shown). However, analysis of the top1 cleavage sites in the 22mer (23mer with the 3′-label) minus strand revealed five bands in the 3′ copy of 'r' (Fig. 4A). These positions correspond to the sites marked with five arrows in Figure 5B.

We also conducted experiments to determine whether top1 could cleave the same sites in the minus strand when the sequence was in the fully double-stranded conformation. We did not observe cleavage under such conditions even upon addition of CPT (data not shown). These results indicated that top1 can cleave the minus strand both within and in the close vicinity of the 'r' region.

Topoisomerase I-mediated recombination of the DHBV minus strands

The failure of top1 to completely linearize OC DHBV DNA (Fig. 2) led us to investigate other possible top1-mediated structural changes in DHBV DNA. One mechanism by which top1 could render OC DHBV DNA resistant to linearization would be by removing the terminal redundancy, 'r', in the minus strand and producing an intact minus strand across the region via a religation/recombination reaction. To test this possibility, we labeled the 5′ end of the 22mer minus strand oligonucleotide and reacted this three-strand flap substrate (Fig. 4B) with top1. To investigate which termini could be involved in the top1-mediated recombination reaction, we placed either a hydroxyl (OH) or a phosphate (P) at the 5′ ends of the fragments at the X or Y positions shown in Figure 4B. Analysis of the reaction products revealed the production of four recombinant molecules only when the X moiety was a 5′-OH (Fig. 4B, arrows 1–4). Bands 1 and 4 represented the major recombinant products (45 and 36%, respectively) whereas both bands 2 and 3 (wild-type sequence) represented ~10% of the recombinant products. As expected, migration of recombination products was not affected by proteinase K treatment conversely to the top1-linked intermediates which disappeared from the wells after top1 digestion by proteinase K (Fig. 4B, compare lanes 2 and 3). This demonstrates that the presence of the 5′-OH terminus of the 'r' sequence is required for top1-mediated recombination and confirms that top1 cleaves at specific sites in the 3′ end of the minus strand. The four recombinant DNA molecules were designated 1–4 from the smallest to the largest. Their length varied between 37 and 40 nt (Fig. 4B).

Sequence analysis of minus strand recombination products

To analyze the top1-mediated recombination products of the minus strands, we excised the bands from the gel shown in Figure 4B and directly sequenced each of these products using the Maxam–Gilbert purine sequencing technique (Fig. 5A). As a control, a 5′-labeled full-length minus strand was sequenced in the same conditions (Fig. 5A, lane C). This analysis showed that the smallest 35 bp recombinant band contained a minus strand with a 2 bp deletion (Fig. 5A, lane 4). The 37 nt middle band contained a wild-type DHBV sequence, indicating cleavage of

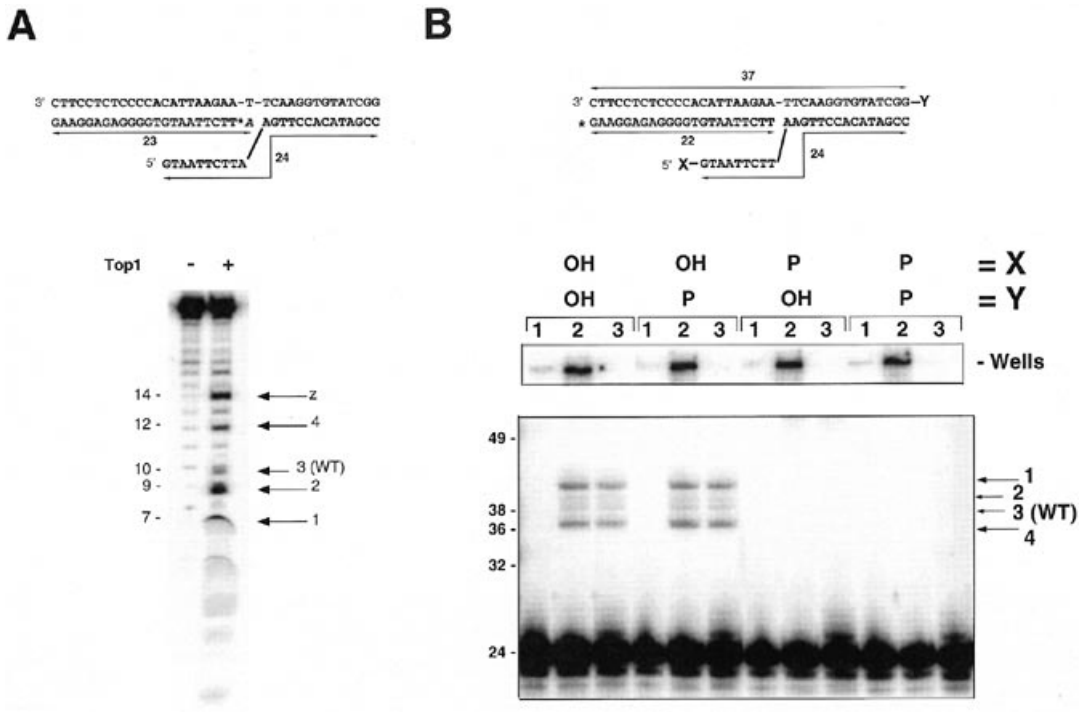

Figure 4. Topoisomerase I-mediated cleavage of the DHBV minus strand in oligonucleotides mimicking virion DNA across the DR1–r region. Oligonucleotides were labeled at the 3′ end with [α-^{32}P]cordycepin (*A*) or the 5′ end with [γ-^{32}P]ATP (*) of the minus strand as indicated at the top of each panel. (**A**) Top1-mediated cleavage of the 3′ end of the DHBV minus strand. Oligonucleotides labeled at the 3′ end were reacted with top1 for 15 min at 25°C. Reactions were stopped with 0.5% SDS, and products were separated on a 25% polyacrylamide gel. Sizes of the cleavage products are indicated on the left (in nucleotides) and numbering on the right corresponds to the recombination sites according to Figure 5B. z represents an additional cleavage site that should not lead to circularization of the DHBV minus strand DNA. (**B**) Top1-mediated cleavage of the 5′-end of the DHBV minus strand. X and Y represent the 5′ termini of the tandem repeat (r) and the plus strand, respectively. Top1 reactions were performed with the indicated substrates for 30 min at 25°C. Reactions were stopped either by 0.5% SDS (lanes 2) or 0.5 mg/ml Proteinase K (lanes 3). Lanes 1, DNA alone. Numbers on the right correspond to the four top1-mediated recombination products shown in Figure 5.

the 3′ ‘r’ of the minus strand between nt 2537 and 2538 (position 3, Fig. 5B) and ligation to the 5′-OH on nt 2537 of the 5′ end of the minus strand (Fig. 5A, lane 3, and B, arrow 3). The largest 40 bp band contained a 3 nt insertion within the ‘r’ region, and the 38 bp fragment contained a 1 bp insertion. We propose that these recombination products result from an initial top1 suicide cleavage at the 3′ end of the minus strand between nt 2537/2536 and 2534/2533 (arrows 1 and 2 in Fig. 5B, respectively). Religation of these suicide intermediates with the 5′ end of the minus strand would generate the recombination products that would correspond to a circularization of the minus strand in virion DNA.

DISCUSSION

Minus strand circularization

In this report we have characterized *in vitro* enzymatic activities of top1 on DHBV DNA. The *in vitro* top1 activities carry out structural alterations that could have major regulatory consequences for viral DNA replication if they occur *in vivo*. An early major structural alteration in DHBV DNA during infection is covalent circularization of minus and plus strands. For minus strands, this cannot occur unless the terminal protein, covalently bound to the 5′ end of the minus strand, is removed. *In vivo* data on the circularization of DHBV DNAs demonstrated that the required enzymatic activity for removing the terminal protein is present in hepatocytes (36). Our work allows us to think that top1 is one potential cellular enzyme candidate that could carry out the minus strand circularization once the terminal protein is removed. This does not exclude, however, that other DNA processing enzymes such as FEN-1 (flap recombinase) in combination with a ligase could also be responsible for DHBV three-strand flap processing *in vivo*.

The essential features of the top1 reactions include cleavage of the 3′ end of the minus strand at one of several positions spanning nt 2542–2535 downstream and within the ‘r’ (Fig. 5B). These cleavages would produce short, 6–13 bp 3′-end minus strand oligonucleotides that must be removed before ligation with the 5′ end can occur. In our experiments, a 5′-OH at the end of the minus strand (nt 2537) served as the acceptor for the 3′-covalently-bound top1 located at the newly cleaved 3′ ends of the minus strand. The ligation reactions produced wild-type recombinants as well as mutants with either 1 or 3 bp duplications or a 2 bp deletion within the ‘r’ and DR1 (Fig. 5).

Interestingly, a precedent exists for high frequency non-homologous recombination in the ‘r’ region of DHBV. Using a mutant DHBV, which synthesizes only DSL DHBV viral DNA molecules, Yang and Summer (36) observed the circularization of DSL DHBV DNA molecules leading to the production of wild-type and mutant CCC DHBV DNAs in a process called ‘illegitimate replication’ in primary duck hepatocytes. One general finding of their work was that the 5′ end of the minus strand (2537) was present in 34% the recombinant DHBV DNA molecules. This is consistent with our finding that the presence of a 5′ hydroxyl at

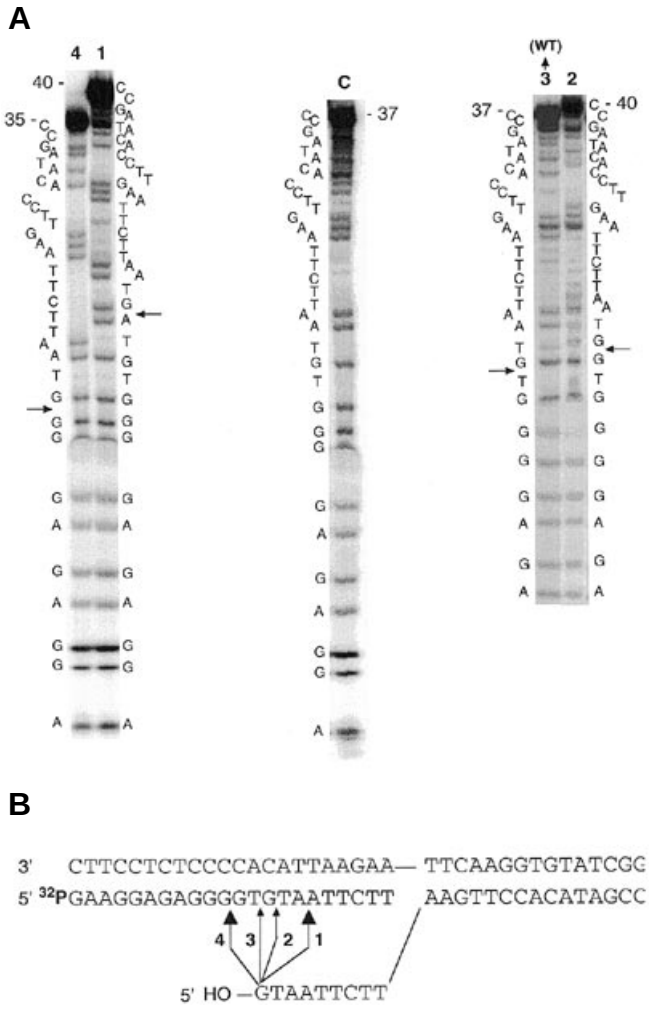

Figure 5. Sequence analysis of the top1-mediated recombination products of the DHBV minus strand DNA corresponding to potential minus strand religation. (**A**) G/A sequence ladders for each top1-mediated recombination product shown in Figure 4B. Four bands corresponding to #1 = 39 bp (3 bp duplication), #2 = 38 bp (1 bp duplication), #3 = 37 bp (wild-type) and #4 = 35 bp (2 bp deletion), were sequenced. DNA bands were cut out from the gel, eluted in 1× TBE buffer overnight, phenol-extracted and ethanol-precipitated prior to purine sequencing. Middle lane (C) corresponds to a 5′-end-labeled full-length minus strand oligonucleotide that was sequenced under similar conditions as a control. (**B**) DNA sequence across DR1 and 'r' in which the recombination sites at the 3′ end of the minus strand are indicated by arrows.

the terminal G residue (nt 2537) was required for linkage to the 3′ top1 cleaved minus strand end in our simulated circularization reaction using synthetic oligonucleotides.

Another feature in common between our work and that of Yang and Summers (36) is the production of recombination joints with short 1–3 bp duplications and 2 bp deletions. In fact, each of the top1-generated recombination joints that we observed corresponds exactly to one of the *in vivo* generated recombinants reported by Yang and Summers (figure 10 in ref. 36). Certainly, structural and sequence differences exist between the DSL DHBV molecules in the Yang and Summers experiments. However, the production of structurally identical junctions suggests that our *in vitro* model may be relevant to the *in vivo* processing of DHBV DNAs.

Plus strand cleavage

The plus strand cleavage activity of top1 at the 3′ side of nt 2528 has the potential to act as a double-edged sword. For example, top1 cleavage at this site in OC DHBV DNA, before circularization of the minus strand, can lead to linearization of the molecule and could eliminate the virion DNA from the DHBV replication cycle. On the other hand, after CCC DHBV DNA is produced, transcription of the essential pregenome RNA from the minus strand is initiated at nt 2529 opposite the 2528 top1 cleavage site in the plus strand. Since top1 has also been linked to recruitment of components of the Pol II initiation complex to transcription initiation sites (37–39), this cleavage activity could have regulatory functions in transcription of CCC DHBV DNAs.

DHBV DNA integration

When the *in situ* priming mechanism for synthesis of DSL DHBV molecules was described, the authors speculated that DSL DHBV DNAs might serve as integration substrates (40). This hypothesis has been supported by recent data showing that an LMH cell line that replicates only DSL DHBV acquires DHBV integrations at a much higher frequency than comparable cells replicating WT DHBV (41). The data in this report demonstrate that it is also possible to produce linear DHBV DNAs as a result of top1-mediated DNA cleavage. For example, double cleavage of DHBV virion DNAs at nt 2528 of the plus strand and at positions 2537 and 2536 of the minus strand would produce a linear molecule with reactive top1 molecules at each end. These molecules could also serve as integration substrates. In fact, in *in vitro* reactions, top1 was previously shown to link WHV virion DNAs with cellular acceptor sites (25). While some data support a possible role for top1 in DHBV integration, additional nucleic acid enzymes most likely also participate in the integration mechanism (42).

ACKNOWLEDGEMENTS

The authors would like to thank Dr W. S. Mason for providing the LMH-D2 cell line, and Dr O. Westergaard for providing purified human top1 and for helpful discussion and suggestions. This work was supported by United States Public Health Service Grants RO1CA37232 from the National Cancer Institute (to C.E.R.), and Grants DK-17702 from Digestive Disease Center Grant Program, Cancer Center Grant P30CA13330, and NIH training grant CA09060 (S.S.G.). The costs of publication of this article were defrayed in part by 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.

REFERENCES

1 Ganem,D. (1996) In Fields,B.N., Knipe,D.M., Howley,P.M. *et al.* (eds), *Fields Virology*, 3rd ed. Lippincott-Raven, Philadelphia, PA, pp. 2703–2737.
2 Seeger,C., Summers,J. and Mason,W.S. (1991) *Curr. Top. Microbiol. Immunol.*, **168**, 41–60.
3 Summers,J. and Mason,W.S. (1982) *Cell*, **29**, 403–415.
4 Tuttleman,J.S., Pourcel,C. and Summers,J. (1986) *Cell*, **47**, 451–460.
5 Tuttleman,J.S., Pugh,J.C. and Summers,J.W. (1986) *J. Virol.*, **58**, 17–25.
6 Bullock,P., Champoux,J.J. and Botchan,M. (1985) *Science*, **230**, 954–958.
7 Cheng,C., Kussie,P., Pavletich,N. and Shuman,S. (1998) *Cell*, **92**, 841–850.
8 Pommier,Y., Pourquier,P., Fan,Y. and Strumberg,D. (1998) *Biochim. Biophys. Acta*, **1400**, 83–105.
9 Wang,J.C. (1996) *Annu. Rev. Biochem.*, **65**, 635–692.

10 Chen,A.Y. and Liu,L.F. (1994) *Annu. Rev. Pharmacol. Toxicol.*, **34**, 191–218.
11 Champoux,J. (1990) In Wang,J.C. and Cozarelli,N.R. (eds), *DNA Topology and its Biological Effects*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 217–242.
12 Halligan,B.D., Davis,J.L., Edwards,K.A. and Liu,L.F. (1982) *J. Biol. Chem.*, **257**, 3995–4000.
13 Pommier,Y., Jenkins,J., Kohlhagen,G. and Leteurtre,F. (1995) *Mutat. Res.*, **337**, 135–145.
14 Henningfeld,K.A. and Hecht,S.M. (1995) *Biochemistry*, **34**, 6120–6129.
15 Christiansen,K., Svejstrup,B.D., Andersen,A.H. and Westergaard,O. (1993) *J. Biol. Chem.*, **268**, 9690–9701.
16 Pourquier,P., Pilon,A.A., Kohlhagen,G., Mazumder,A., Sharma,A. and Pommier,Y. (1997) *J. Biol. Chem.*, 26441–26447.
17 Shuman,S. (1992) *J. Biol. Chem.*, **267**, 16755–16758.
18 Shuman,S. (1992) *J. Biol. Chem.*, **267**, 8620–8627.
19 Christiansen,K. and Westergaard,O. (1994) *J. Biol. Chem.*, **269**, 721–729.
20 Kjeldsen,E., Svejstrup,J.Q., Gromova,I., Alsner,J. and Westergaard,O. (1992) *J. Mol. Biol.*, **228**, 1025–1030.
21 Bonven,B.J., Gocke,E. and Westergaard,O. (1985) *Cell*, **41**, 541–551.
22 Jaxel,C., Capranico,G., Kerrigan,D., Kohn,K.W. and Pommier,Y. (1991) *J. Biol. Chem.*, **266**, 20418–20423.
23 Porter,S.E. and Champoux,J.J. (1989) *Mol. Cell Biol.*, **9**, 541–550.
24 Tanizawa,A., Kohn,K.W., Kohlhagen,G., Leteurtre,F. and Pommier,Y. (1995) *Biochemistry*, **43**, 7200–7206.
25 Wang,H.P. and Rogler,C.E. (1991) *J. Virol.*, **65**, 2381–2392.
26 Condreay,L.D., Aldrich,C.E., Coates,L., Mason,W.S. and Wu,T.T. (1990) *J. Virol.*, **64**, 3249–3258.
27 Pugh,J.C., Yaginuma,K., Koike,K. and Summers,J. (1988) *J. Virol.*, **62**, 3513–3516.
28 Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Sping Harbor, NY.
29 Seeger,S. and Mason,W.S. (1996) In DePamphilis,M.L. (ed.), *DNA Replication in Eukaryotic Cells*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 815–831.
30 Hsiang,Y.H., Hertzberg,R., Hecht,S. and Liu,L.F. (1985) *J. Biol. Chem.*, **260**, 14873–14878.
31 Svejstrup,J.Q., Christiansen,K., Gromova,I.I., Andersen,A.H. and Westergaard,O. (1991) *J. Mol. Biol.*, **222**, 669–678.
32 Pommier,Y. (1996) *Semin. Oncol.*, **23**, 3–10.
33 Fan,Y., Weistein,J.N., Kohn,K.W., Shi,L.M. and Pommier,Y. (1998) *J. Med. Chem.*, **41**, 2216–2226.
34 Stewart,L., Redinbo,M.R., Qiu,X., Hol,W.G. and Champoux,J.J. (1998) *Science*, **279**, 1534–1541.
35 Redinbo,M.R., Stewart,L., Kuhn,P., Champoux,J.J. and Hol,W.G. (1998) *Science*, **279**, 1504–1513.
36 Yang,W. and Summers,J. (1995) *J. Virol.*, **69**, 4029–4036.
37 Merino,A., Madden,K.R., Lane,W.S., Champoux,J.J. and Reinberg,D. (1993) *Nature*, **365**, 227–232.
38 Shykind,B.M., Kim,J., Stewart,L., Champoux,J.J. and Sharp,P.A. (1997) *Genes Dev.*, **11**, 397–407.
39 Kretzschmar,M., Meisterernst,M. and Roeder,R.G. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 11508–11512.
40 Staprans,S., Loeb,D.D. and Ganem,D. (1991) *J. Virol.*, **65**, 1255–1262.
41 Gong,S.S., Jensen,A.D., Chang,C.J. and Rogler,C.E. (1999) *J. Virol.*, **73**, 1492–1502.
42 Schirmacher,P., Wang,H., Stahnke,G.H.W. and Rogler,C.E. (1995) *J. Hepatol.*, **22**, 21–33.