Autonomous Parvovirus DNA Replication Requires Topoisomerase I and Its Activity Is Increased during Infection

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Received 17 August 1990/Accepted 7 December 1990

Topoisomerases I and II (topo I and topo II) are nuclear enzymes functioning to resolve DNA topological problems during replication, transcription, and other DNA processes. We tested the effects of camptothecin and VP16, specific inhibitors of topo I and II, respectively, on the DNA replication of parvoviruses LuIII and H-1 and found that viral DNA synthesis was suppressed by camptothecin but not by VP16. Transcription of H-1 virus was measured by a nuclear runoff assay and showed no inhibition by camptothecin. Interestingly, topo I in the LuIII virus-infected cell nuclear extract appears to have more activity for covalently binding to viral DNA than that in mock-infected cell nuclear extracts. Our data suggested that this activity was not due to an increased transcription of the topo I gene or to greater amounts of topo I.

In eukaryotic cells two major topoisomerases have been identified, topoisomerases I and II (topo I and II), which have demonstrated roles in DNA replication, transcription, DNA segregation, and other processes, such as DNA recombination and mutagenesis (8, 9, 13, 16, 18, 29, 30). During its reaction with DNA, topo I cleaves one of the DNA strands and forms a DNA-protein intermediate by attaching to the phosphate at the 3' end of the DNA at the site of the nick, leaving a free 5' hydroxyl. It catalyzes a reaction topologically equivalent to the passage of an intact single strand through the break, thereby effecting DNA relaxation. The cleaved DNA strand is restored by resealing of the broken DNA, and the whole reaction is independent of either divalent cations or ATP (16, 19). In contrast, topo II transiently cleaves both strands of a DNA helix and covalently binds to the 5' end of the DNA, catalyzing the passage of a duplex DNA segment through the break. Thus, topo II can relax supercoiled DNA and catenate and decatenate DNA circles. The reaction by topo II requires the presence of a divalent cation and hydrolysis of ATP to complete DNA cleavage and religation (19, 29).

The study of the requirements for topoisomerases in DNA replication and RNA transcription was made feasible by the use of a topo I inhibitor, camptothecin, and many topo II inhibitors, such as VP16 and MV26 (9). These inhibitors trap topoisomerases in the intermediate steps of the DNA breakage-reunion cycle, in which the enzymes are covalently attached to the DNA; therefore, they interfere with the replication or transcription of the template. In the cases of simian virus 40, adenovirus, and herpesvirus, the evidence indicates that DNA replication is inhibited by the formation of the camptothecin-induced intermediate (cleavable complex) (1, 2, 4, 24, 26, 27, 31, 32). The transcription of adenovirus is also inhibited by camptothecin (23, 31).

To study whether autonomous parvoviruses, which have single-stranded linear DNA genomes of about 5,000 nucleotides (3), have the topological constraints that require topoisomerases during viral DNA replication and transcription, we examined the effects of camptothecin and VP16 on the replication of parvoviruses LuIII and H-1.

Effects of drugs on viral replication and transcription.

HeLa G cells were seeded at 5×10^5 cells per 60-mm dish in Eagle minimal essential medium containing 5% fetal bovine serum and were infected with LuIII virus. Camptothecin or VP16, at a final concentration of 20 or 5 μ M, respectively, was added to the medium at 16 h postinfection for 30 min. The medium was then removed, and 1 ml of fresh medium containing 10 μ Ci of [*methyl*- 3 H]thymidine and the same concentration of camptothecin or VP16 was added. The cells were incubated for another 30 min and washed twice with phosphate-buffered saline, and viral DNA was collected by the Hirt extraction method (15). The viral DNA was analyzed on 1% agarose gels in TAE buffer (40 mM Tris-acetate [pH 8.0], 1 mM EDTA). After electrophoresis, the gel was prepared for fluorography (17). The synthesis of LuIII virus DNA (Fig. 1) was greatly inhibited by camptothecin, suggesting that even though LuIII virus has a small linear genome, the unwinding of its DNA during viral replication would generate topological constraints which required topo I to resolve.

We found that cellular DNA synthesis in uninfected cells was inhibited 70 to 80% with the 10 μ M concentration of camptothecin (data not shown). However, this effect cannot account for the inhibition of viral DNA synthesis because the virus infection has already inhibited cell DNA synthesis (7). Similar inhibition of viral DNA synthesis was observed in H-1 virus-infected cells (data not shown). However, VP16 did not inhibit parvovirus DNA synthesis, unlike the previously observed inhibition of adenovirus DNA replication (24, 31), though a 25% inhibition of cellular DNA synthesis in uninfected cells was detected with the same concentration of VP16 (data not shown). Because the genomes of parvoviruses are linear, parvovirus genomes may not require topo II to decatenate the daughter DNAs after replication. In contrast, simian virus 40, which has a circular genome, requires topo II to separate its progeny DNA from the parental template (28).

Since camptothecin traps topo I in the cleavable complex, it could interfere with DNA replication indirectly if the enzyme was required for transcription and if DNA replication occurred on templates that were also being transcribed. To examine whether topo I was required for parvovirus transcription, we used camptothecin in nuclear runoff assays. Two plasmids, pUP4HE and pUP38HE, were used as probes to detect both P4 and P38 parvovirus transcripts (21).

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FIG. 1. Incorporation of [methyl-³H]thymidine into LuIII virus DNA after treatment with camptothecin (CPT), VP16, or dimethyl sulfoxide (DMSO) (drug solvent). Lanes 2, 3, and 4 were Hirt extracts from LuIII virus-infected HeLa G cells; lanes 1, 5, and 6 were extracts from mock-infected cells. The arrow indicates the position of LuIII virus monomer replicative-form DNA.

pUP4HE and pUP38HE were made by cloning the 1.1- and the 2.5-kb fragments which were cut with *Eco*RI and *Hind*III from the H-1 virus genome in the plasmid pSR1 (22) into pUC19. pUP4HE contains H-1 virus sequences from nucleotides 1 to 1,088, and pUP38HE contains H-1 virus sequences from nucleotide 2,655 to the right end. Both 1- and 0.1- μ g amounts of each plasmid DNA were denatured and blotted to Nytran in duplicate. Two 60-mm dishes of HeLa G cells were infected with H-1 virus for 16 h and treated with camptothecin or dimethyl sulfoxide control solution for 30 min at a final concentration as described before. Cell nuclei were prepared and labeled (14) in the presence of the same concentration of the drug. RNA was extracted and DNA-RNA hybridization was carried out at 65°C overnight. The membrane was washed with 2 $\times$ SSPE (1 $\times$ SSPE is 0.18 mM NaCl, 10 mM sodium phosphate, and 1 mM EDTA [pH 7.7]) and 0.1% sodium dodecyl sulfate (SDS) twice and with 0.2 $\times$ SSPE and 1% SDS at 65°C once and exposed to film.

The labeling of the transcripts from both the P4 and the P4 plus p38 promoters was not decreased by camptothecin (Fig. 2), indicating that topo I activity was not required to relax DNA structural tension during transcription. Thus, parvoviruses are unlike adenovirus, for which Wong and Hsu (31) and Schaack et al. (24) observed that viral transcription was inhibited by camptothecin. It was suggested that the transcription template of adenovirus was folded into loop domains that were topologically constrained. In another experiment (data not shown), viral transcription was inhibited by treatment of the infected culture with alpha amanitin, and the effect of camptothecin on the viral DNA synthesis was tested as in Fig. 1. The inhibitor of RNA synthesis did not reduce the inhibition of DNA synthesis by camptothecin.

FIG. 2. Nuclear runoff assays of viral transcription. The amounts of plasmid probes blotted to the membrane are indicated above the figure, and the plasmids used are marked at the bottom. The rows are labeled RNA from a mock-infected culture (MI), H-1 virus-infected culture (H-1), and camptothecin-treated H-1 virus-infected culture (H-1, CPT).

These results suggest that the interaction of viral DNA with topo I is not the result of transcription.

Examination of topo I activity in mock- and LuIII virus-infected HeLa S3 cell nuclear extracts. To test whether there was any change in topo I activity upon parvovirus LuIII infection, HeLa S3 suspension cells were grown to final concentrations of about 5×10^5 cells per ml in Eagle minimal essential medium with 5% fetal bovine serum and infected with LuIII virus for 16 h. The mock-infected and the virus-infected cell nuclear extracts were made by Shapiro's method (25). A 500-bp DNA fragment representing the right end of the LuIII virus genome was restricted from the plasmid pGlu883 (11) with *Xba*I and labeled with [α -³²P]dCTP by treatment with the Klenow fragment. This labeled DNA was then incubated with the same amount of extract either in the presence or in the absence of camptothecin for 2 h at 30°C (1 $\times$ reaction buffer containing 10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid] [pH 7.9], 10% glycerol, 50 mM KCl, 0.1 mM EDTA, and 1 mM dithiothreitol). SDS was then added to a final concentration of 0.2%, and the samples were heated for 10 min at 65°C. The samples were analyzed in a 1.4% agarose gel (10 mM NaH₂PO₄, Na₂HPO₄ [pH 6.8], and 0.1% SDS). After electrophoresis, the gel was dried and exposed to film.

In the absence of camptothecin, the covalently bound DNA-protein complex formed in a mock-infected cell nuclear extract was hardly visible, but it was readily apparent in the virus-infected cell nuclear extract (Fig. 3). This implied that either the amount of topo I in the infected extract was increased or the activity of topo I was modified. When camptothecin was added to the reaction, multiple DNA-protein complexes were found in both extracts, but the amounts were increased in the infected extract. Topo I in mock-infected extracts mainly formed complex I and a small amount of complex II (probably two topo I molecules bound to the DNA); however, topo I in the virus-infected extract formed complexes II and III (probably three topo I molecules binding to the DNA). To exclude the possibility that topo II would form the gel-shift bands during this reaction, unhydrolyzable τ , β -methylene ATP was added to a final concentration of 10 mM. The results showed that the formation of the DNA-protein complexes was not ATP dependent.

To verify that topo I activity was increased in infected

FIG. 3. Gel-shift analysis of topo I DNA complexes formed by mock-infected and LuIII virus-infected nuclear extracts. Mock-infected nuclear extracts (MOCK INF. EXT) were used in lanes 2 through 5; LuIII virus-infected extracts (LuIII INF. EXT) were used in lanes 6 through 9. The amounts of nuclear extracts used were 5 μ l, with a concentration of 12 μ g of protein per ml. Camptothecin (CPT) and γ - β -methylene ATP (γ - β -MeATP) were included in the reaction as indicated. The SDS-stable protein complexes are indicated as complexes I, II, and III.

extracts rather than some other protein which might also be able to form DNA-protein complexes, we did an α - 32 P transfer experiment and immunoprecipitation with a polyclonal topo I-specific antibody (5). An H-1 virus DNA fragment was restricted from pUP38HE with *Eco*RI and *Hind*III and labeled with [α - 32 P]dCTP by a random primer extension reaction (12). Variable amounts of both nuclear extracts were incubated with 0.5 μ g of the labeled DNA for 30 min, and then 6 μ g of DNase I was added to each reaction for 1 h. Since topo I could transiently bind to the 3' end of the DNA strand via formation of a phosphodiester bond, after DNase I digestion, α - 32 P was transferred from the DNA strand to the topo I enzyme. The reaction was stopped either (i) by adding 2 $\times$ sample buffer (2% SDS, 150 mM dithiothreitol, 0.02% bromophenol blue, 10% glycerol, 0.1 M Tris [pH 6.8]) and analyzing the reaction mixture on a 6% SDS-acrylamide gel (Fig. 4A) or (ii) by adding 2 $\times$ immunoprecipitation buffer (0.2% SDS, 20 mM Tris-HCl [pH 8.5], 1% Nonidet P-40, 1% deoxycholate, 0.15 M NaCl) and immunoprecipitating with topo I polyclonal antibody and then subjecting the mixture to acrylamide gel electrophoresis (Fig. 4B). The gel was dried and exposed to film for autoradiography. The major band formed in the LuIII virus-infected extracts had a molecular mass around 100 kDa, which corresponds to the molecular mass of the eukaryotic topo I found in HeLa cells (20), and this material immunoprecipitated with the topo I antibody. This band was sensitive to protease digestion, indicating that the protein was labeled. This confirms that the protein from the infected extract that was binding to the probe DNA is topo I. In the uninfected cell nuclear extracts, much less topo I was found. These results are consistent with the results in Fig. 3 and imply that either total topo I was lower in the uninfected cell nuclear extract or the activity for forming the cleavable complex with topo I in the infected cell nuclear extracts was increased. There were also some minor bands labeled, ranging from 84 to 93 kDa. These might be the partially degraded topo I and failed to bind with topo I antibody.

FIG. 4. Assay of topo I by label transfer and immunoprecipitation of topo I. (A) Topo I in LuIII virus-infected extract (VI) or mock-infected extract (MI) was complexed with α - 32 P-labeled DNA, digested with DNase I, and analyzed by gel electrophoresis. Lanes: 1 and 2, 5 μ l of extract treated with proteinase K; 3 and 4, 5 μ l of extract; 5 and 6, 2.5 μ l of extract; and 7 and 8, 1 μ l of extract. Both extracts were adjusted to the same concentration (12 μ g/ μ l) of protein. (B) Topo I in the infected extract was labeled and digested with DNase I as described above and then treated without antibody (lane 1) or with topo I polyclonal antibody (lane 2) or control rabbit sera (lane 3). The immune complexes were precipitated with Formalin-fixed *Staphylococcus aureus* (Boehringer Mannheim Biochemicals). The topo I immune complexes were denatured before analysis by gel electrophoresis.

Rainwater and Mann have shown that the lower-mass molecules of topo I were present in preparations of TC7 cell extracts, and presumably these were the results of proteolytic degradation of topo I (20).

We tested whether the increased topo I activity in infected extracts was caused by the increased transcription of the topo I gene. The mRNA level of topo I in the cells infected with LuIII virus was measured by a Northern blot with a topo I cDNA clone for a probe (10). The results showed no increase in steady-state transcripts of topo I upon virus infection (data not shown). Also, on a Western immunoblot using a topo I-specific polyclonal antibody, the bands at 100 kDa were equivalent for infected and mock-infected nuclear extracts (data not shown). Thus, we have no evidence for an increase in the amount of topo I after infection. We have detected an increased activity for topo I for forming cleavable complexes in either the presence or absence of camptothecin in parvovirus-infected cells. Coderoni et al. (6) found that the phosphorylation of calf DNA topo I could increase its activity and that this mechanism could be significant and might play a rapid regulatory role. Also, it was reported that topo II was differentially increased in simian virus 40-infected TC7 cells, even though both topo I and topo II were required for the virus replication (20). In our case, it will be interesting to know whether such regulatory mechanisms for topo I might be present to meet the requirement of parvovirus replication.

We thank Carol Stessman for technical assistance and J. K. Vishwanatha for reviewing the manuscript. We also thank William Earnshaw for the topoisomerase I cDNA clone and Leroy Liu for topoisomerase I antibody.

This work was supported by grants from the National Institutes of Health (AI25552 [S.R.] and CA36727), grants from the American Cancer Society (ACS MV-479 and SIG-16), and funds from the Elizabeth Bruce and Parents Memorial Endowment.

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