Mammalian DNA Topoisomerase I Activity and Poisoning by Camptothecin Are Inhibited by Simian Virus 40 Large T Antigen†

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Received August 20, 1997; Revised Manuscript Received December 17, 1997

ABSTRACT: DNA topoisomerase I (top1) is a ubiquitous enzyme that forms reversible DNA single-strand breaks (cleavage complexes) and plays a role in transcription, DNA replication, and repair. Top1 is the target of camptothecins which selectively trap top1 cleavage complexes and represent a novel class of anticancer drugs active against human solid tumors,. The present study demonstrates that recombinant large T antigen (T-Ag), a virus encoded helicase with strong affinity for tumor suppressors and cell cycle- and replication-related proteins, suppresses top1 cleavage complexes and top1 catalytic activity. This top1 suppressive activity is probably not due to T-Ag binding to DNA, as a T-Ag truncation mutant containing only the first 246 amino acids and deficient in DNA binding also inhibited top1, and the inhibition was independent of ATP. T-Ag also antagonized and reversed the trapping of top1 cleavage complexes by camptothecin. These results demonstrate a functional interaction between T-Ag and top1: they also suggest the importance of top1−protein interactions for the regulation of DNA replication and modulation of camptothecin activity.

DNA topoisomerase I (top1)[1] is a ubiquitous enzyme required for development both in Drosophila (*26*) and mouse (*34*). By forming transient DNA single-strand breaks (commonly referred to as "cleavage [or cleavable] complexes") and acting as DNA strand transferase, top1 plays key roles in DNA replication, transcription, and recombination (for review, see refs *5, 17,* and *51*). Camptothecin and its derivatives are selective top1 inhibitors (for review see refs *6* and *37*), and are currently among the most promising anticancer agents. Camptothecin can also be used as a probe to monitor top1 activity, as it traps the top1 cleavage complexes that can then be easily detected as protein-associated DNA single-strand breaks (*9*). The top1 cleavage complexes are characterized by the covalent linkage of an enzyme tyrosine to the 3′-DNA terminus, while the other DNA terminus is a 5′-hydroxyl (*5, 17, 51*).

DNA helicases catalyze the separation of the two strands of duplex DNA to expose the DNA primary sequence to DNA and RNA polymerase as well as to other DNA binding proteins during DNA transactions. Melting of the DNA duplex by helicases produces positive supercoiling in the DNA template upstream and negative supercoiling downstream from the melted DNA region (*27*). As topoisomerases are specialized in relaxing DNA supercoiling, it appears logical that topoisomerases and helicases should be functionally associated (*8, 15*).

Simian virus 40 (SV40) T antigen (T-Ag) is an initiator protein for virus DNA replication. In the presence of ATP, T-Ag forms a double hexamer that binds to the GAGGC recognition pentanucleotides at the viral DNA origin (*30*). Subsequently, T-Ag functions as a DNA helicase to unwind the origin (*16, 53, 56*). This reaction requires a cellular DNA topoisomerase (*10, 12*) and a single-strand-specific DNA binding protein (RPA) (*23, 48, 55*). In the next step, the cell DNA polymerase α-primase (*7, 13, 14, 31, 35*) binds to unwound origin complexes through specific protein−protein contacts with T-Ag and perhaps with RPA, and short RNA−DNA primers are synthesized (*4, 11, 36, 50*).

We have recently reported (*46*) that T-Ag binds to top1, but the functional significance of this interaction is not known. In the present study, we tested the effect of the simian virus 40 (SV40) large T antigen (T-Ag) on mammalian top1 activity. We report for the first time that direct interaction of T-Ag with top1 can inhibit top1 activity and that T-Ag can antagonize and reverse the trapping of top1 cleavage complexes by camptothecin.

EXPERIMENTAL PROCEDURES

Chemicals and Enzymes. [α-^{32}P]Cordycepin 5′-triphosphate, [γ-^{32}P]ATP, and [α-^{32}P]dCTP were purchased from Dupont NEN Research Products (Boston, MA). Polyacrylamide was purchased from Bio-Rad, Inc. (Richmond, CA). Camptothecin (CPT) was provided by Drs. Wani and Wall (Research Triangle Institute, Research Triangle Park, NC) or the Drug Synthesis and Chemistry Branch (National Cancer Institute, Bethesda, MD). CPT (10 mM aliquots)

† D.T.S. was supported by PHS Grant CA36118.

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[1] Abbreviations: CPT, camptothecin; SDS, sodium dodecyl sulfate; T-Ag, SV40 large T antigen; top1, topoisomerase I; top2, topoisomerase II.

S0006-2960(97)02067-9 CCC: $15.00
Published on Web 02/25/1998

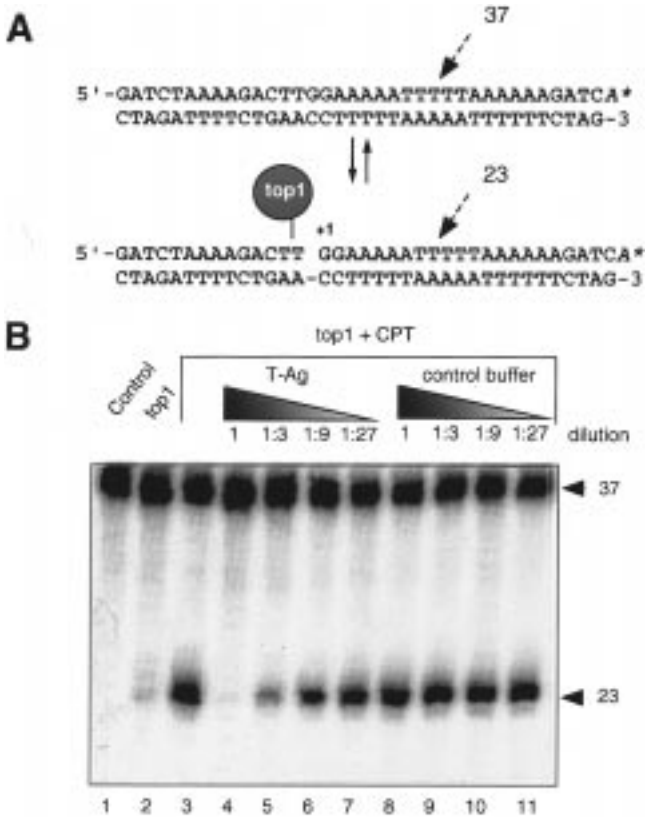

FIGURE 1: Inhibition of top1 cleavage complexes by T-Ag. (A) Oligonucleotide used. The asterisk indicates the ^{32}P label incorporated at the 3′-end of the scissile strand with cordycepin (*A*). (B) Image of a typical experiment. Lane 1, DNA alone; lane 2, + top1; lanes 3−11, + top1 + 10 μM camptothecin; lanes 4−7, serial dilutions (as indicated) of T-Ag were added to the top1−camptothecin reactions; lanes 8−11, control mock-T-Ag purified sample. An amount of 174 ng of T-Ag (2 pmol) was used in lane 4. Three picomoles of top1 was used per reaction in lanes 2−11.

was stored at −20 °C, thawed, and diluted to 1 mM in dimethyl sulfoxide (DMSO) just before use. Teniposide (VM-26) was obtained from Bristol-Myers Squibb (Wallingford, CT) and was diluted at 10 mM in DMSO immediately before use. Further dilutions were made in distilled water.

Wild-type T-Ag was purified by immunoaffinity chromatography as described (*46*) using PAb101 antibodies. Deletion mutant 1−246 was purified in the same way using PAb419 antibodies. Calf thymus top1 was purchased from Gibco-BRL (Grand Island, NY). Human top1 either was a kind gift of Drs. Lance Stewart and James Champoux (University of Washington, Seattle, WA) or was purified from Sf9 cells using a baculovirus construct containing the top1 full-length cDNA (*54*, *57*). The baculovirus construct for the N-terminus truncated human top1 was a kind gift from Dr. Jean-François Riou (RPR, Vitry sur Seine, France). DNA topoisomerase II was purified from mouse leukemia L1210 cell nuclei as described (*33*).

Top1-Mediated DNA Cleavage Assays. Oligonucleotides (see Figure 1A) were purchased from The Midland Certified Reagent Company (Midland, TX). 3′-Labeling and preparation of the oligonucleotide used in Figures 1 and 5 were performed using terminal deoxynucleotidyl transferase (Stratagene, La Jolla, CA) with [α-^{32}P]cordycepin as described previously (*41*). In some of our cleavage assays (Figure 2), we used a *Pvu*II−*Hin*dIII 161 bp fragment from pBluescript (Stratagene). The fragment (200 ng) was labeled at the *Hin*dIII site by filling in the 3′-recessed end with [α-^{32}P]-dCTP and cold 0.5 mM dATP, dGTP, and dTTP in 50 mM Tris-HCl, pH 8.0, 100 mM $MgCl_2$, and 50 mM NaCl containing 0.5 unit of DNA polymerase I (Klenow fragment). At the end of the labeling reaction, the DNA was phenol/chloroform extracted, ethanol precipitated, and resuspended in water.

DNA substrates (approximately 50 fmol/reaction) were incubated with 5 units (≈3 pmol) of calf thymus or human recombinant top1 for 15 min at 25 °C with or without camptothecin or T-Ag in top reaction buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 μg/mL bovine serum albumin). Reactions were stopped by adding 0.5% sodium dodecyl sulfate (SDS), ethanol precipitated, resuspended in loading buffer (80% formamide, 10 mM sodium hydroxide, 1 mM sodium EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue, pH 8.0), and separated in a denaturing gel (16% polyacrylamide, 7 M urea in TBE) run at 51 °C. Gels were visualized with a Phosphorimager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Top2-Mediated DNA Cleavage Assays. Simian virus 40 (SV40) DNA (Gibco-BRL, Long Island, NY) was 5′-end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase as described (*39*). A second digestion with *Hpa*II was used to generate a single-end-labeled fragment (see Figure 3). Top2 reactions were performed in top buffer supplemented with 1 mM ATP for 30 min at 25 °C. Reactions were stopped by adding 0.5% SDS and electrophoresed as for the top1 assays, except that 7% acrylamide gels were used.

T-Ag Binding Reactions and Supershift Assays. Immunoaffinity purified T-Ag was incubated for 1 h at 37 °C with 40 ng of 5′-^{32}P-end-labeled (*) double-stranded DNA oligonucleotide

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5'*GGACTTGCCTGGCCTTGCCTACAA 3'
   TGTTCCTGAACGGACCGGAACGGA
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in a total volume of 30 μL replication buffer (0.03 M *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.5, 0.007 M $MgCl_2$, 0.04 M ATP, 0.001 M dithiothreitol, 20 μg/mL creatine phosphokinase, 0.1 mg/mL bovine serum albumin) in the presence or absence of top1. The protein−DNA complexes were cross-linked with glutaraldehyde (0.1%) and subjected to electrophoresis in a 4% acrylamide gel in TBE at 25 mA for 3 h at 3 °C.

Top1-Mediated DNA Relaxation Assays. Reaction mixtures (10 μL) contained 0.3 μg of supercoiled SV40 DNA in top reaction buffer and 5 units of purified calf thymus top1. Reactions performed were at 37 °C for 30 min and were terminated by the addition of 0.5% SDS. An amount of 1.1 μL of 10× loading buffer (20% Ficoll 400, 0.1 M Na_2EDTA, pH 8, 1.0% SDS, 0.25% bromophenol blue) was then added, and reactions were loaded onto a 1% agarose gel made in 1× TBE buffer. After electrophoresis, the gel was stained in 1× TBE buffer containing 10 μg/mL ethidium bromide and visualized by transillumination with UV light (300 nm).

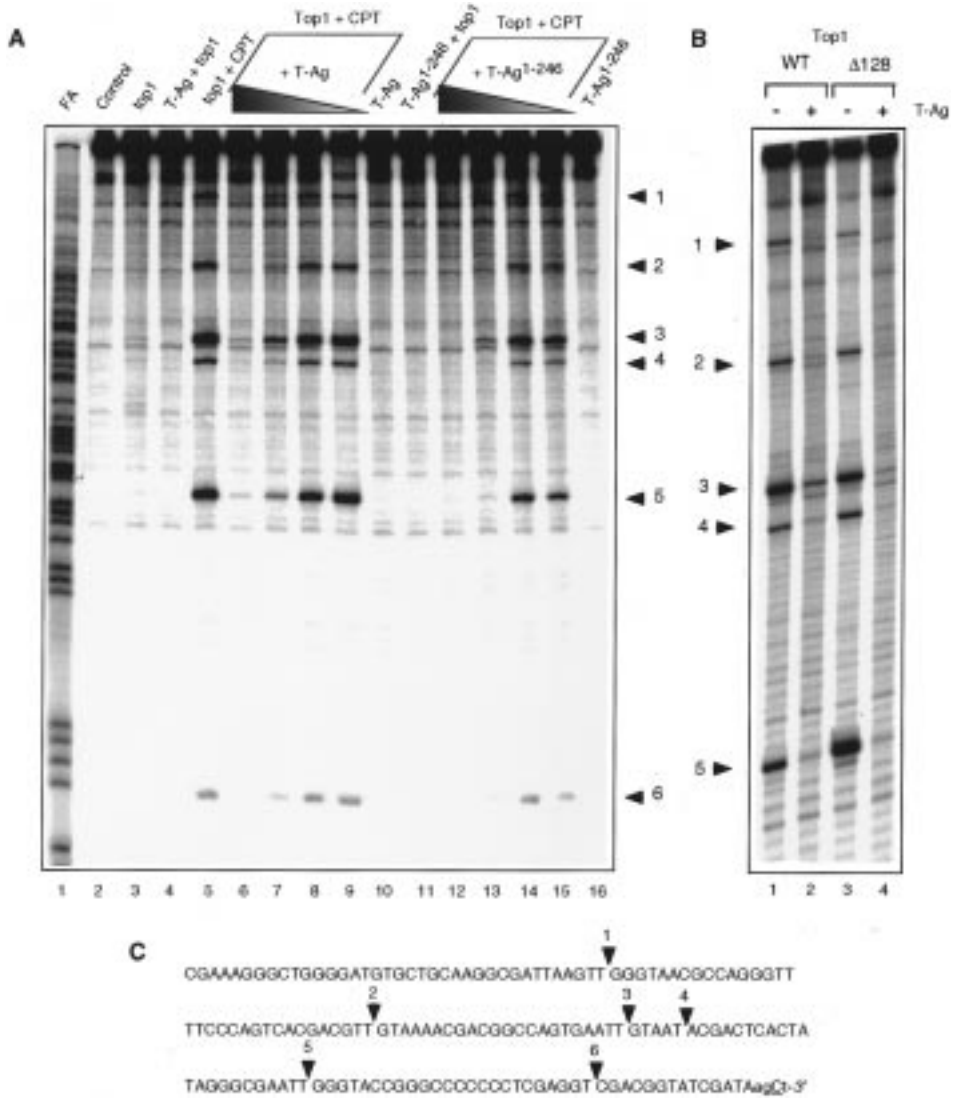

FIGURE 2: Effects of truncated mutants of T-Ag and top1. (A) Global inhibition of top1 cleavage complexes by T-Ag and a truncation mutant of T-Ag containing the first 246 amino acids (T-Ag$^{1-246}$). Lane 1, purine ladder after formic acid reaction; lane 2, DNA alone; lane 3, + top1; lane 4, + top1 + T-Ag; lane 5, + top1 + 10 μM camptothecin (CPT); lanes 6−9, same but in the presence of 1:3 serial dilutions of T-Ag; lane 10, T-Ag without top1; lane 11, T-Ag$^{1-246}$ + top1; lane 12−15, + top1 + CPT in the presence of 1:3 serial dilutions of T-Ag$^{1-246}$; lane 16, T-Ag$^{1-246}$ without top1. (B) Inhibition by T-Ag of a truncated mutant of top1 missing the first 128 amino acids. Lanes 1 and 2, DNA + wild-type human top1 + 10 μM camptothecin; lanes 3 and 4, DNA + truncated top1 + 10 μM camptothecin; lanes 4, 6, 10−12, and 16 in panel A and lanes 2 and 4 in panel B contained 174 ng (2 pmol) of T-Ag or T-Ag$^{1-246}$ (6 pmol) per reaction. Lanes 3−9 and 12−15 in panel A and lanes 1−4 in panel B contained ≈3 pmol of top1. The cleavage sites (arrows and numbers) are indicated between panels A and B. (C) Sequence and position of the cleavage sites in the 161 bp pBluescript fragment that was 3′-end-labeled at a *Hin*dIII site (underlined C).

RESULTS

Inhibition of Top1-Induced Cleavage Complexes by T Antigen. We first used an oligonucleotide containing a single top1 cleavage site (*41*) (Figure 1A) to determine the effects of T-Ag on top1 cleavage complexes. Camptothecin (CPT) markedly enhances top1-mediated cleavage (*6*, *37*) and can be used to monitor top1 DNA nicking activity. Figure 1B represents a typical experiment showing that T-Ag inhibited top1 DNA nicking activity (lanes 4−7). As a control, we used a sample purified by immunoaffinity from cells infected with a non-T-Ag expressing baculovirus (lanes 8−11). These data demonstrate that T-Ag inhibits top1 cleavage complexes at approximately stoichiometric concentrations relative to top1. This inhibition appears independent from T-Ag binding to DNA as the oligonucleotide used does not contain a T-Ag binding site, and T-Ag was purified and assayed under conditions that result in low DNA binding activity.

A Truncated T Antigen Mutant Defective in DNA Binding and Helicase Activity Also Inhibits Top1 DNA Nicking Activity. We then used a 161 bp fragment from pBluescript DNA to further study the inhibition of top1 by T-Ag (Figure 2). Top1 induced several cleavage sites in the presence of camptothecin in this DNA fragment (Figure 2). The DNA sequence of these sites is indicated in panel C. All these cleavage sites were linked to top1 by a thymidine at the 3′-DNA terminus, and four out of six sites had a guanine at the 5′-terminus. This base preference is consistent with our previous results that top1 cleaves DNA preferentially between T and G in the presence of camptothecin (*21*, *47*). All these cleavage sites were inhibited by T-Ag, indicating a global effect of T-Ag on top1 activity. The truncated T-Ag

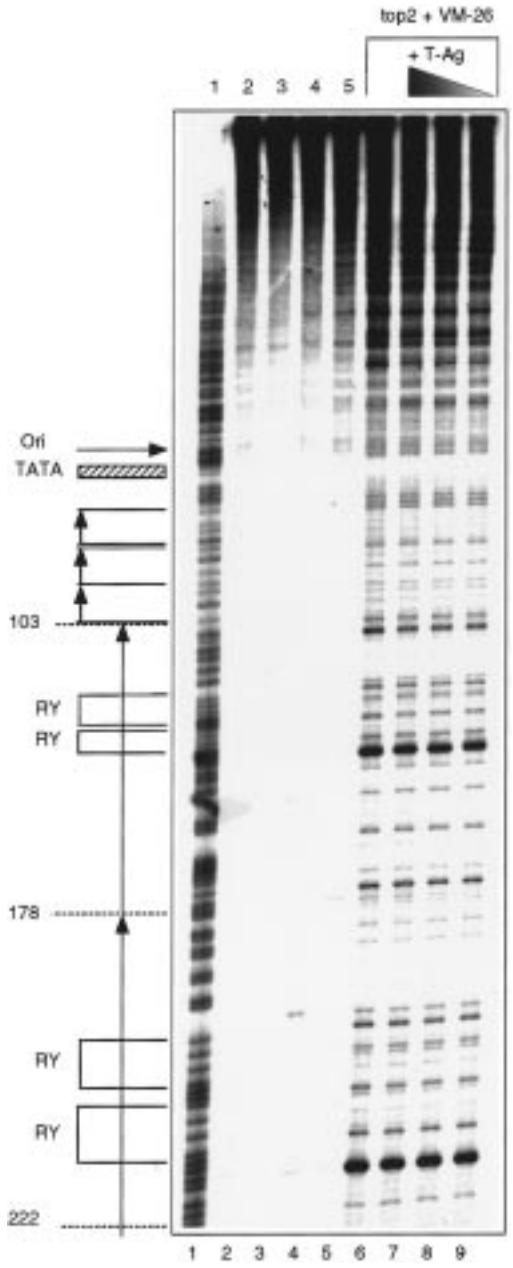

FIGURE 3: T-Ag does not inhibit top2 cleavage complexes. The long *Ban*I−*Hpa*II fragment of SV40 DNA was 5′-end-labeled and reacted as follows: lane 1, purine ladder after formic acid reaction; lane 2, DNA alone; lane 3, + T-Ag; lane 4, + top2; lane 5, + top2 + T-Ag; lanes 6−9, + top2 + 10 μM teniposide (VM-26); lanes 7−9, + 1:3 serial dilutions of T-Ag (174 ng was in reactions corresponding to lanes 5 and 7). Ori and TATA correspond to the SV40 replication origin and TATA box, respectively. The three short arrows below correspond to the 21 bp repeats. The two long arrows correspond to the 72 bp repeats, and the RY boxes correspond to the alternating purine−pyrimidines repeats.

containing amino acids 1−246 inhibited top1 equally well. Since this mutant lacks DNA binding activity (*46*), it appears that top1 inhibition is not due to the interaction of T-Ag with DNA but rather to direct protein interaction (*46*).

We also tested a top1 truncation mutant missing the first 128 amino acids. This mutant (top1 Δ128) retains top1 nicking activity and sensitivity to camptothecin (Figure 2B). T-Ag also inhibited top1 Δ128 (Figure 2B), indicating that the first 128 amino acids at the N-terminus of top1 are not required for T-Ag contact.

DNA Supershift Assay with T Antigen and Top1. To ask if T-Ag and top1 can form a trimeric complex with DNA, purified top1 was incubated with an end-labeled 21 bp double-stranded oligonucleotide in the presence of increasing amounts of T-Ag. DNA−protein complexes were analyzed on nondenaturing acrylamide gels. T-Ag by itself bound to the DNA inefficiently because the protein was purified under high pH conditions which partially inactivate DNA binding activity [but not top1 binding activity (*46*)]. Top1 by itself generated a DNA−protein complex that migrated faster than the T-Ag−DNA complex. When both proteins were present, a slower migrating complex appeared (T-Ag−top1−DNA) (data not shown). These results suggest that T-Ag and top1 can associate together with DNA.

T Antigen Does Not Affect DNA Topoisomerase II Activity. Another major topoisomerase in human cells is topoisomerase II (top2) (*51*). We next studied the effect of T-Ag on top2. Top2 cleavage complexes can be trapped specifically by the epipodophyllotoxin derivative and anticancer agent VM-26 (teniposide) (*38*). For these experiments, we used a 5′-end-labeled fragment corresponding to the SV40 enhancer region and replication origin (Figure 3) (*39*). As expected, top2 produced a series of cleavage sites in this DNA (Figure 3). Neither the wild-type nor the T-Ag$^{1-246}$ truncated mutant (not shown) affected top2−DNA cleavage activity. These results indicate that the inhibition of top1 DNA nicking activity by T-Ag is specific for top1.

T Antigen Inhibits Top1 DNA Relaxing Activity. Top1 catalytic activity can be monitored by DNA relaxation assays. Figure 4 demonstrates that both the full-size and the T-Ag$^{1-246}$ truncation mutant inhibited top1-mediated DNA relaxation. By contrast to previous reports (*28, 29*), under the conditions used here, T-Ag by itself has no detectable top1 activity (Figure 4, lanes 7, 8 and 13, 14).

T Antigen Reverses Top1 Nicking of DNA in the Presence of Camptothecin. Trapping of top1 cleavage complexes is the primary mechanism of camptothecin cytotoxicity (*6, 17, 37*). The data presented so far (Figures 1 and 2) demonstrated that T-Ag added immediately before top1 prevented camptothecin-induced top1 cleavage complexes. We next wished to determine the effects of T-Ag on preexisting camptothecin-trapped cleavage complexes. Top1 was first incubated with the double-stranded oligonucleotide shown in Figure 1A in the presence of camptothecin. T-Ag was added, and at various times the reaction was stopped and products analyzed by gel electrophoresis (Figure 5). The results demonstrate that T-Ag can efficiently reverse camptothecin-stabilized nicked intermediates and regenerate an intact DNA strand.

DISCUSSION

Top1 interacts with various nuclear proteins. RNA polymerase I (*43*) and TFIID−TFIIA (*25, 32, 45*) interactions are consistent with the importance of top1 for transcription. Top1 activity is stimulated by HMG proteins (*20*) and p53 (*3*), while nucleolin which binds to the 166−120 region of top1 does not appear to alter top1 catalytic activity (*3*). Splicing proteins (U1 and snRNP) (*44*) have recently been reported to be phosphorylated by top1. Other kinase interactions involve top1 phosphorylation by casein kinase II and protein kinase C (*24, 40, 49*).

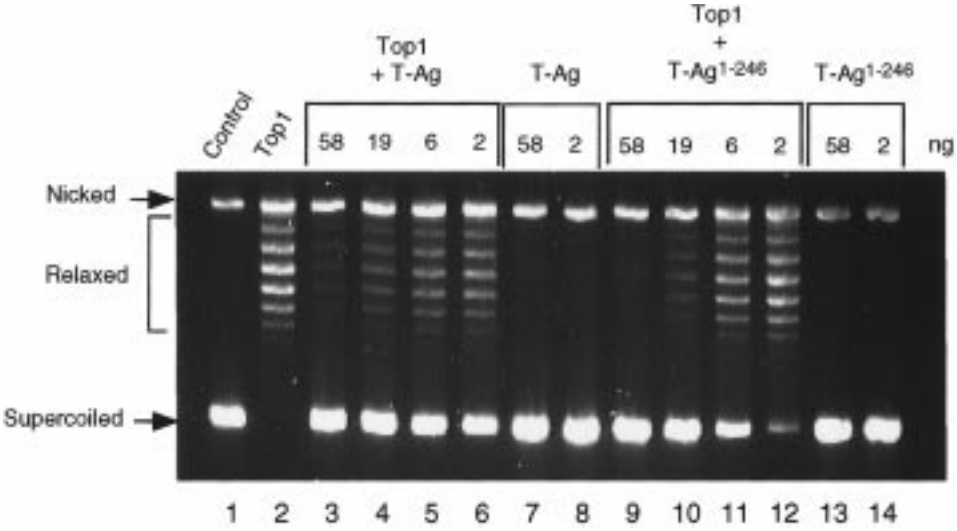

FIGURE 4: Inhibition of top1 catalytic activity by T-Ag. Native supercoiled SV40 DNA (lane 1) was reacted with top1 (5 units/reaction corresponding to approximately 3 pmol) in the absence of T-Ag (lane 2) or in the presence of wild-type T-Ag (lanes 3−6) or mutant truncated T-Ag$^{1-246}$ (lanes 9−12). Amounts of T-Ag are indicated above lanes (58 ng of T-Ag, 0.7 pmol; 58 ng of T-Ag$^{1-246}$, 2 pmol). T-Ag and mutant T-Ag$^{1-246}$ were also tested for DNA relaxing activity in the absence of top1 (lanes 7, 8 and 13, 14, respectively). A picture of a typical 1% agarose gel stained with ethidium bromide is shown.

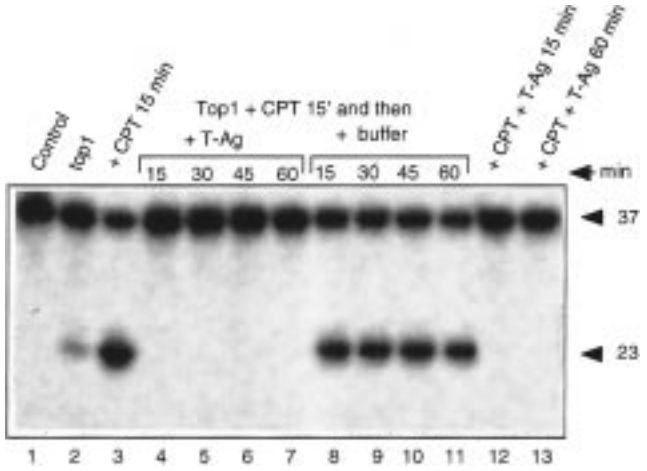

FIGURE 5: Reversal of camptothecin-trapped top1 cleavage complexes by T-Ag. The oligonucleotide shown in Figure 1A was incubated with top1 in the absence (lane 2) or presence of 10 μM camptothecin (lanes 2−13). After 15 min, an aliquot was taken (lane 3) and the reaction mixture was divided in half. T-Ag was added to one half (lanes 4−7) while only T-Ag buffer was added to the other half (lanes 5−11). Reactions were then stopped at the indicated times after T-Ag or buffer addition (15 min for lanes 4 and 8, 30 min for lanes 5 and 9, 45 min for lanes 6 and 10, and 60 min for lanes 7 and 11). Lanes 12 and 13 are control reactions with T-Ag added immediately before top1 and camptothecin, and reactions performed for 15 or 30 min, respectively. Top1 and T-Ag were 2 and 3 pmol per reaction, respectively.

The present study further demonstrates that T-Ag can interact directly with top1 (*46*) and that this interaction results in an inhibition of both top1 cleavage complexes and DNA relaxing activity. The topoisomerase selectivity of T-Ag for top1 is suggested by the lack of effect of T-Ag on top2 cleavage complexes. Top1 inhibition by T-Ag is most likely not simply due to DNA binding of T-Ag because inhibition was observed with a T-Ag truncation mutant (T-Ag$^{1-246}$), which is deficient in DNA binding. Furthermore, top1 inhibition by T-Ag was most efficient when the two proteins were preincubated together prior to the addition of DNA (data not shown). Finally, top1 inhibition by T-Ag and T-Ag$^{1-246}$ was observed at stoichiometric concentrations of top1 and T-Ag. Thus, these observations are consistent with a direct interaction between T-Ag and top1 (*46*).

In a previous report (*46*), we demonstrated that two distinct sites on T-Ag can independently bind to top1 and that the small T antigen which shares the first 82 amino acids with the large T antigen does not bind to top1. The stronger binding site was located between amino acids 83 and 246. In the present study, the inhibition of top1 activity by a truncated mutant of T-Ag (T-Ag$^{1-246}$) indicates that the amino-terminal region of T-Ag is sufficient for top1 interaction. For top1, protein interactions have been postulated so far to involve the amino-terminus, while the carboxyl-terminus contains the catalytic residue including the tyrosine which forms the covalent catalytic intermediates of the cleavage complexes (Y723 for human top1) (*5*, *17*, *51*). For instance, nucleolin has been found to bind to the region encompassed between amino acids 166 and 210 (*2*). The top1 mutant tested in the present study (Figure 2) demonstrates that the top1 interaction does not involve the most distal amino-terminus of top1 (up to amino acid 128).

The N-terminal region of SV40 large T antigen, up to the DNA binding domain (1−256), interacts with a number of cellular proteins. Those that may be relevant to top1 binding include several transcription factors such as TFIIB, TBP, TEF-1 and SP1 (*22*), the catalytic 140 KDa subunit of RNA pol II (*22*), and DNA polymerase α (*52*). We have previously shown that T antigen binding sites for top1 and DNA polymerase α overlap extensively (*46*).

Both top1 and T-Ag are critical for DNA replication, and top1 is among the constituents of the multiprotein replication complex (*1*, *18*). Replication origins tend to be free of nucleosomes and to be underwound during replication initiation (*19*, *42*). Our results showing inhibition of top1 activity by T-Ag are consistent with the possibility that by inhibiting top1 activity and maintaining negative supercoiling at the replication origin (*19*), T-Ag may facilitate initiation. Another consequence of the T-Ag−top1 association might be that T-Ag may recruit top1 as it tethers the polymerase

α-primase complex to the SV40 replication origin (*7*). Top1 is probably required for replication elongation as the replication forks approach the nucleosomes (*19*). At this point, perhaps the two proteins no longer need to be associated, and top1 can relax DNA supercoiling adjacent to the replication forks and facilitate chromatin assembly.

Camptothecins are a novel class of anticancer agents used for the treatment of human ovarian and colon carcinomas. Camptothecins selectively trap top1 cleavage complexes and convert top1 into a cellular poison (*37*). This report is the first observation of an inhibition of the camptothecin-trapped top1 cleavage complexes by another protein. This suggests the possibility that cells could antagonize top1 poisoning by camptothecins not only by down-regulating and mutating top1 or changing top1 phosphorylation (*17*), but also by mobilizing protein modulators that would act functionally as T-Ag to suppress top1 activity while the drug is present in the cell.

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BI972067D