The Binding of Topoisomerase I to T Antigen Enhances the Synthesis of RNA−DNA Primers during Simian Virus 40 DNA Replication

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ABSTRACT: Topoisomerase I (topo I) is required for the proper initiation of simian virus 40 (SV40) DNA replication. This enzyme binds to SV40 large T antigen at two places, close to the N-terminal end and near the C-terminal end of the helicase domain. We have recently demonstrated that the binding of topo I to the C-terminal site is necessary for the stimulation of DNA synthesis by topo I and for the formation of normal amounts of completed daughter molecules. In this study, we investigated the mechanism by which this stimulation occurs. Contrary to our expectation that the binding of topo I to this region of T antigen provides the proper unwound DNA substrate for initiation to occur, we demonstrate that binding of topo I stimulates polymerase alpha/primase (pol/prim) to synthesize larger amounts of primers consisting of short RNA and about 30 nucleotides of DNA. Topo I binding also stimulates the production of large molecular weight DNA by pol/prim. Mutant T antigens that fail to bind topo I normally do not participate in the synthesis of expected amounts of primers or large molecular weight DNAs indicating that the association of topo I with the C-terminal binding site on T antigen is required for these activities. It is also shown that topo I has the ability to bind to human RPA directly, suggesting that the stimulation of pol/prim activity may be mediated in part through RPA in the DNA synthesis initiation complex.

The replication of SV40[1] DNA has been studied by a number of investigators over more than 20 years (*1–3*). We know which proteins are involved in this complex process and generally what they do in the replication reaction. However, in spite of major advances in our knowledge, we still have a poor conceptual understanding of the composition, workings and dynamics of the replication machinery. The structure and activity of the T antigen helicase is understood fairly well (*4, 5*). We know for instance how it recognizes the origin of replication (*6*−*8*) and how it unwinds DNA (*9, 10*) although the joining of these two events is not entirely clear.

Initiation of DNA synthesis in eukaryotes is believed to rely primarily on the activity of polymerase α/primase (pol/prim). This enzyme lays down the RNA primer followed by about 30 nucleotides of DNA (*11, 12*). This RNA-DNA primer is then extended with DNA by polymerase δ for both leading and lagging DNA synthesis (*13*). In the case of SV40 and other polyomaviruses as well as papillomaviruses, the synthesis of this primer is dependent on replication protein A (RPA) (*14*), a three subunit SS DNA binding protein (*15*). RPA, in association with T antigen and pol/prim participates in the initiation of new chains (*16–18*). The associations of RPA with T antigen (*19*) and with SS DNA (*20*) are necessary for priming and elongation. Furthermore, the T antigen-pol/prim interaction is necessary for correct initiation (*21*). Together, these findings strongly indicate that T antigen, RPA and pol/prim work together to initiate new chains.

Our laboratory has been involved in sorting out the details of the reactions that lead to initiation of DNA synthesis from the viral origin. In addition to T antigen, RPA, and pol/prim, we have determined that topoisomerase I (topo I) is also involved in this process. We previously demonstrated that topo I enhances the synthesis of SV40 DNA in crude replication assays (*22, 23*). The major effects of topo I are to promote the synthesis of replicative intermediates and completed molecules. Halmer et al. (*24*) made similar conclusions about the role of topo I in DNA replication from SV40 chromatin. Topo I is a component of the initiation complex (*25*) and can associate with T antigen at both the N-terminal (residues 83−160) and C-terminal (residues 602−708) ends (*26*). The enzyme preferentially binds to double hexamers of T antigen associated with origin DNA (*27*), and it appears that two molecules of topo I are bound per double hexamer (*25*). It is needed from the start of replication and we therefore proposed that it is required for efficient initiation of DNA synthesis (*23*) as did Halmer et al. (*24*).

A number of our studies have focused on the association of topo I with the replication complex (*25, 27, 28*) and we have begun to understand how topo I is recruited to the initiation machinery. Although little is known about the function of the N-terminal topo I binding site in T antigen, we showed in a recent publication (*28*), that the binding of topo I to the C-terminal binding site is necessary for normal levels of DNA replication in vitro. We identified the specific

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[1] Abbreviations used: SV40, simian virus 40; T antigen, large tumor antigen; topo I, topoisomerase I; RPA, replication protein A; pol/prim, polymerase alpha/primase; SSB, single-stranded DNA binding protein; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GST, glutathione S transferase; nu, nucleotides; HRP, horseradish peroxidase.

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binding site within the C-terminal end of the helicase domain of T antigen and generated a model of the complex between T antigen, topo I and DNA that might begin to explain how the initiation complex assembles. In this model, one topo I molecule binds to one monomer T antigen on the "back edge" of the helicase domain in the double hexamer structure with origin DNA (*28*). We proposed that the DNA becomes threaded into topo I and then fed into the "back end" of the helicase where it becomes unwound. However, the exact biochemical function of topo I in this complex is still unclear. Since topo I is primarily an enzyme that relaxes supercoiled DNA, it makes sense to assume that the interaction of topo I with the C-terminal end of T antigen is required primarily for the proper unwinding and relaxation of the DNA; that is, the complex might function as a swivelase, carefully controlling the relative activities of the helicase (T antigen) with that of the relaxation enzyme (topo I). The association of topo I with the "back edges" of the double hexamer (*26, 28*) would make sense if that were the case because the DNA could be relaxed by topo I just before it is presented to the helicase for melting. The assumption was that this concerted reaction would provide the proper template for efficient synthesis of DNA.

In this manuscript, we demonstrate, however, that the binding of topo I to the C-terminal end of the helicase domain of T antigen is not to permit unwinding of closed circular DNA, but rather to promote initiation of RNA-DNA primers by pol/prim. Our finding is that topo I bound to that site stimulates pol/prim activity within this replication machine to initiate the synthesis of new chains and that unwinding activity must be provided by other topo I molecules bound to the DNA. These results fortify our model that this enzyme is an integral component of the DNA replication initiation machinery.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification. All proteins were purified exactly as described in previous publications. WT and mutant full length T antigens were purified by immunoaffinity chromatography from infected High 5 insect cells using PAb101 antibody-coupled Sepharose 4B beads as described by Mastrangelo et al. (*29*) and Khopde and Simmons (*28*). WT topoisomerase I (topo I), the catalytic mutant Y723F, and GST-topo 70 were all purified from infected Hi5 insect cells as previously described (*30*). Human RPA was expressed by IPTG induction of p11d-tRNA transformed *Escherichia coli* strain BL21 (Novagen) and purified by column chromatography according to Henricksen et al. (*31*). Pol/prim was purified as previously described (*25*) using the IMPACT-CN system (New England Biolabs) system where one of the subunits was tagged with a modified form of intein. The enzyme was purified in two steps by using chitin affinity chromatography followed by chromatography on hydroxyapatite. All purified proteins were loaded on SDS-acrylamide gels to determine their concentration and integrity.

Minicircle Unwinding Assays. A circular DNA unwinding substrate was made as previously described (*27*) by ligating a 32-P end-labeled *Hin*dIII-*Kpn*I origin-containing fragment from pSKori (*32*) with the following double-stranded oligonucleotide having the same restriction enzyme overhangs:

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 HindIII                                  KpnI
5' AGCTTGGTCGACCCACGCCATGGTAC 3'
3'       ACCAGCTGGGTGCGGTAC       5'
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Circular DNA (388 bp) was separated and purified from all other ligation products by electrophoresis on a 2% agarose gel in Tris borate EDTA where it migrated faster than linear unligated fragment. DNA unwinding assays were performed as previously described (*22, 33*). One ng of gel purified 32-P labeled circular DNA substrate was incubated in DNA replication buffer containing 20 μg/mL creatine phosphokinase, 80 ng of *E. coli* SSB (Pharmacia), 400 ng of immnoaffinity purified T antigen with or without 40 ng of topo I in a total reaction volume of 20 μL. After 1 h at 37 °C, the reactions were terminated by the addition of 5 μL of stop buffer (2% SDS, 0.1 M EDTA, 1 mg/mL proteinase K), incubated at 37 °C for 30 min and at 65 °C for 5 min. Samples were applied to composite gels containing 2.5% acrylamide and 0.6% agarose in Tris borate EDTA buffer and subjected to electrophoresis for 550 V-h at 3 °C. The gels were dried and exposed to a PhosphorImager screen.

SV40 DNA Monopolymerase Synthesis Assays. The reaction mixture (40 μL) contained 200 ng of CsCl purified pSKori, 400 ng T antigen, 50 ng pol/prim, 300 ng of RPA with or without 150 ng of topo I in a complete replication buffer (30 mM HEPES-KOH [pH 8.0], 7 mM $MgCl_2$, 40 mM creatine phosphate, 4 mM ATP, 0.2 mM each CTP, GTP and UTP, 0.1 mM each dATP, dGTP and dTTP, 20 μM dCTP, 4 μCi of [α-^{32}P]dCTP, 25 μg/mL creatine phosphokinase, 0.5 mM dithiothreitol, 50 μg/mL BSA). After incubation for 2 h at 37 °C, the reactions were terminated by the addition of 9 μL of stop buffer (2% SDS, 0.1 M EDTA, 1 mg/mL proteinase K), incubated at 37 °C for 30 min followed by passage through Centri-Spin 20 columns (Separation Science) to remove unincorporated [α-^{32}P]dCTP. The DNA was denatured by incubation with 5 μL of 10× denaturing buffer (50 mM NaOH, 10 mM EDTA) for 15 min at 37 °C. Two microliters of ficoll-bromophenol blue were added followed by electrophoresis on 1.5% alkaline agarose gels in 1× denaturing buffer for 7.5 h at 42 V. The gels were soaked in 250 mL of 5% acetic acid for 30 min with rocking, dried and exposed to phosphorImager screens.

Primer RNA-DNA Synthesis Assays. These assays were carried out essentially as described by Murakami et al. (*34*). Briefly, the monopolymerase reactions were modified by the omission of all four dNTPS, the inclusion of 10 units of RNasin (Sigma) and twice the concentrations of pSKori, T antigen, RPA and pol/prim in the presence or absence of 200 ng of topo I. After 1 h at 37 °C, newly synthesized RNA-DNA primers were pulse labeled for 1 min at 37 °C with 10 μCi [α-^{32}P]dCTP, in the presence of 100 μM dATP, dTTP and dGTP. The purified DNA was incubated with 15 μL of 95% formamide, 10 mM EDTA, 0.01% bromophenol blue and 0.01% xylene cyanol at 90 °C for 3 min and subjected to electrophoresis for 3 h at 1200 V on 12% polyacrylamide-urea sequencing gels in Tris borate EDTA buffer.

ELISAs. ELISAs were performed essentially as detailed by Simmons et al. and Roy et al. (*26, 35*) with 100 ng of WT topo I as the protein bound to the wells. Various amounts of WT RPA were added followed by incubation with 300 ng of anti-RPA antibody 1 (Oncogene Research). After washing each well, horseradish-conjugated antimouse antibody (Sigma) was added, followed by substrate. The reaction

was terminated with one-half volume of 2.5 M H_2SO_4, and read at 490 nm on a DYNEX MRX plate reader.

Immunoblot Assays. Various amounts of WT topo I or control protein were spotted on a nitrocellulose membrane. After blocking with milk buffer (7.5% milk powder in Tris buffered saline containing 0.3% Tween 80), the membrane was incubated with 1 μg/mL WT RPA in milk buffer for 3−4 h at RT. After washing in Tris buffered saline containing 0.3% Tween 80, the membrane was incubated with 1 μg/mL anti RPA 70 kDa (7G9) (a generous gift from Marc Wold) followed by incubation with HRP conjugated goat anti mouse IgG (Sigma) and Alpha Innotech Chemiglow substrate. Chemiluminescence was detected with an Alpha Innotech FluorChem 8800.

GST Pull down Assays. Glutathione S transferase (GST) protein binding assays were performed by a modification of Herbig et al. (*36*). 1.25 μg of GST-tagged topo 70 attached to glutathione Sepharose 4B beads or control GST beads were incubated with 1 μg of WT RPA in 150 μL of binding buffer (30 mM HEPES-KOH pH 7.8, 10 mM KCl, 7 mM $MgCl_2$, 2% non fat dry milk) for 3 h at RT in an end-over-end shaker. The beads were washed 4 times with 0.5 mL of wash buffer I (0.05 M Tris-HCl pH 7.4, 0.5 M NaCl, 0.001 M EDTA, 10% glycerol, 1.0% NP40), and 4 times with the same buffer without the NP40. Bound RPA was eluted with electrophoresis sample buffer and detected by Western blotting using antibodies 1 and 2 (Oncogene Research Products) directed against the 70 kDa and 32 kDa subunits, respectively.

RESULTS

Circular DNA Unwinding by Mutant T Antigens. In a recent publication (*28*), we described the isolation of mutant T antigens defective in binding to topo I and showed that these mutants are incapable of supporting proper levels of DNA replication. We concluded from these studies that the association of these two enzymes with one another greatly augments the production of completed molecules, consistent with earlier reports (*22–24*). In this study, we relied on the same mutants to first investigate if the association between these two enzymes is required for partial unwinding of closed circular DNA. To address this, we used a previously described assay that converts relaxed mini circular DNA containing the origin of replication to underwound forms (*27, 37*). In this assay, both T antigen and topo I are absolutely needed to underwind the DNA [refs *27, 37 and Figure 1*]. In most respects, this assay resembles the form U unwinding assay that utilizes a circular plasmid DNA (*38*). Contrary to our expectations and as shown in Figure 1, all the topo I-binding defective T antigen mutants (shown in bold in Table 1) were fully functional in the formation of underwound minicircular DNA showing that the binding of topo I to the C-terminal end of T antigen is not required for unwinding circular DNA containing the origin. These assays were performed with different concentrations of T antigen and for different times of incubation and no significant differences were found (data not shown). Although the catalytic activity of topo I is required in this assay [ref *27* and unpublished data], the association of topo I with the C-terminal end of T antigen is not needed. DNA unwinding must be promoted by other topo I molecules, either free or bound to the N-terminal site on T antigen.

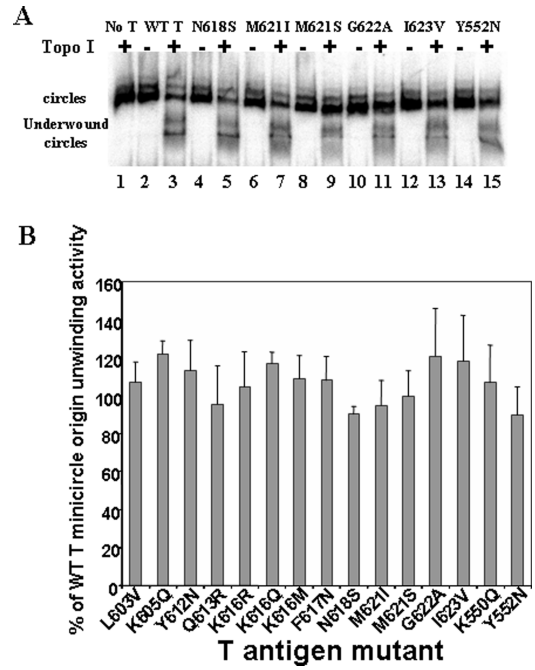

FIGURE 1: Minicircle unwinding assay of T antigen mutants. P-32 labeled 388 bp minicircles containing the SV40 origin were incubated with WT or mutant T antigen in the absence or presence of topo I. The reaction was deproteinized and the DNA analyzed on a native agarose-acrylamide composite gel followed by scanning with a PhosphorImager. (A) A representative gel result with WT T antigen and several mutants. The positions of the labeled circular substrate and various underwound forms of the DNA are shown. The reaction in lane 1 contained topo I but no T antigen. (B) Bar graph of the origin unwinding activity of all studied T antigen mutants. Activity is expressed as a percentage of WT T antigen's ability to generate underwound forms. Error bars represent standard deviations obtained from three data sets.

Effect of Topo I on the Synthesis of DNA by pol/prim. Because topo I is needed for efficient initiation of SV40 DNA synthesis and topo I is associated with the initiation machinery, we next investigated whether topo I directly stimulates the initiation of SV40 DNA synthesis. This was probed by performing DNA synthesis reactions with polymerase alpha/primase, RPA and topo I in the so-called monopolymerase system. The reaction is completely dependent on the presence of T antigen (Figure 2A) and synthesizes, in the presence of topo I, short (about 200−500 nu) and long (about 2100 nu) molecular weight DNAs (Figure 2A, lanes 4 and 6) (*39*). These long products have been previously described to represent leading strand synthesis (*39*). As shown in Figure 2, in this assay, topo I stimulates overall DNA synthesis by about 2.5 fold and the synthesis of large molecular weight DNA (about 2100 nucleotides) by about 4 fold. This second number is undoubtedly higher because, in the absence of topo I, there is a background of variously sized DNAs (Figure 2A, lanes 1 and 3) partially obscuring the 2100 nu DNA that appears to be made only in the presence of topo I (Figure 2A). Mutant T antigens (e.g., Y612N, K616R, K616Q, etc.) defective in binding topo I at their C-terminal ends (*28*) were not stimulated by topo I to promote the synthesis of either large molecular weight DNA nor shorter DNAs (Figure 2B,D and

Table 1: Summary of Properties of T Antigen Mutants

T antigen	defective in DNA replication[a,b]	defective in topo I binding[a]	defective in origin DNA unwinding	defective in forming large molecular weight DNA[a]	defective in synthesis of primer-DNA[a]
WT T	N	N	N	N	N
L603V *Leu* → *Val*	N	N	N	N	N
K605Q *Lys* → *Gln*	N	N	N	N	N
Y612N *Tyr* → *Asn*	Y	Y	N	Y	Y
Q613R *Gln* → *Arg*	N	N	N	N	N
K616R *Lys* → *Arg*	Y	Y	N	Y	Y
K616Q *Lys* → *Gln*	Y	Y	N	Y	Y
K616M *Lys* → *Met*	Y	Y	N	Y	Y
F617N *Phe* → *Asn*	Y	Y	N	Y	Y
N618S *Asn* → *Ser*	N	N	N	N	N
M621I *Met* → *Ile*	Y	Y	N	N	Y
M621S *Met* → *Ser*	Y	Y	N	Y	Y
G622A *Gly* → *Ala*	N	N	N	N	N
I623V *Ile* → *Val*	N	N	N	N	N
K550Q *Lys* → *Gln*	Y	Y	N	Y	Y
Y552N *Tyr* → *Asn*	Y	Y	N	Y	Y

[a] Mutants that possessed 60% or less of the WT activity were considered to be defective. [b] Summary of DNA replication activity of mutants in a crude extract system (*28*).

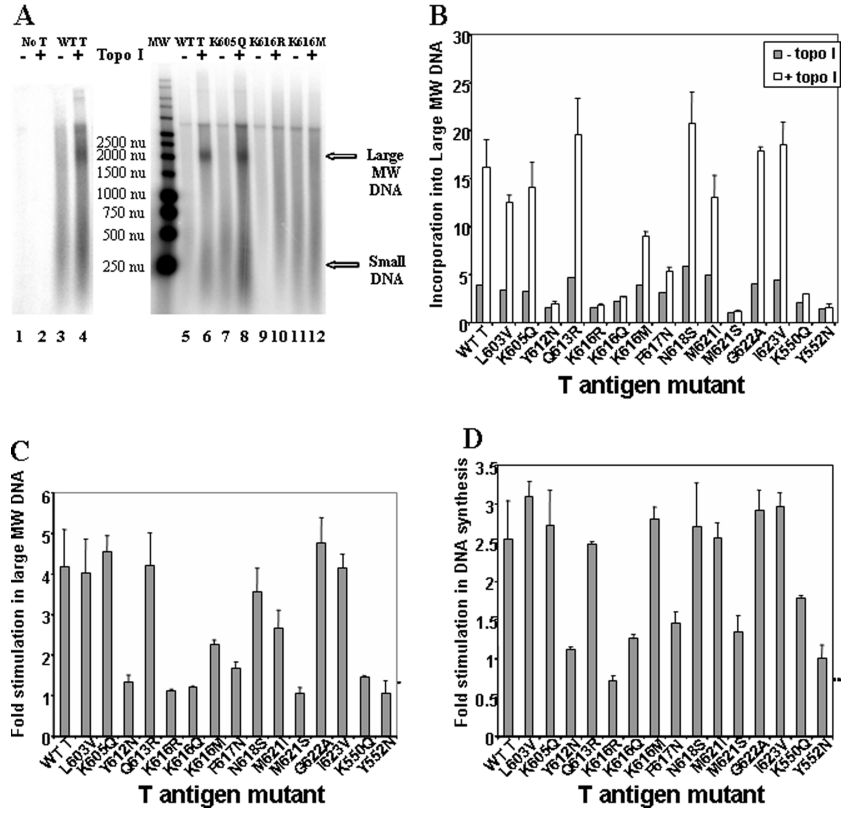

FIGURE 2: Monopolymerase reactions with T antigen mutants. pSKori was incubated without T antigen as a control or with WT or mutant T antigens in the presence of RPA, pol/prim, and ^{32}P-dCTP with or without topo I. The reactions were deproteinized and the labeled DNA analyzed on a denaturing agarose gel followed by scanning with a PhosphorImager. (A) Sample gels of reactions with and without T antigen (left) and with various mutant T antigens (right). The lane marked "MW" contains end-labeled 1 kb ladder (Sigma) and the size of each marker band is shown between the two panels. The positions of the large and small molecular weight DNAs are indicated. (B) Quantitation of the radioactivity incorporated into the large molecular weight DNA in the absence and presence of topo I for reactions with WT T antigen and all mutants (arbitrary units). (C) Fold stimulation of incorporation into the large molecular weight DNA by topo I for WT T antigen and each mutant. A value of 1 corresponds to no stimulation. (D) Fold stimulation of total DNA synthesis by topo I for WT T antigen and each mutant. In each graph, error bars represent standard deviations from three data sets.

Table 1) whereas other mutants that are normal in topo I binding (e.g., Q613R, N618S, G622A, etc.) supported normal DNA synthesis. The one exception to this correlation was M621I, a mutant that is only slightly defective in topo I binding (*28*) and is close to normal in supporting the synthesis of large molecular weight DNA (Figure 2 and Table 1). The differences observed for the two classes of mutants cannot be explained by differences in protein stability because that all of these mutant proteins were stable and expressed in normal amounts (*28*). The monopolymerase

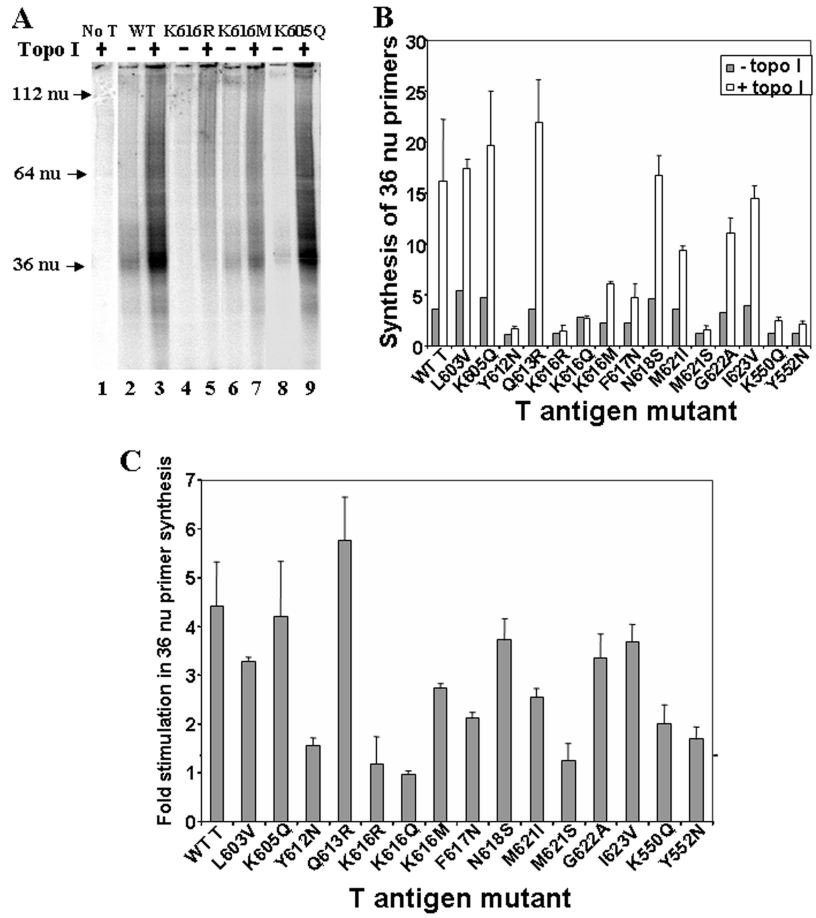

FIGURE 3: Primer RNA-DNA synthesis induced by T antigen mutants. Primer RNA-DNA synthesis was measured by incorporation of ^{32}P-dCTP in a 1 min labeling reaction. After deproteination, the DNA was denatured with formamide and subjected to electrophoresis on a 12% acrylamide sequencing gel followed by detection of the labeled DNA with a PhosphorImager. (A) Sample gel of several mutants. Molecular weights of single stranded DNA standards are shown on the left. (B) Quantitation of incorporation into 36 nucleotide RNA-DNA primers in the presence of WT T antigen and all mutants with and without topo I. (C) Fold stimulation of 36 nucleotide primer synthesis by topo I for WT T antigen and all T antigen mutants.

system, therefore, comes very close to mimicking the complete replication assay using cell extracts when considering the effects of topo I on DNA synthesis (Table 1).

Effect of Topo I on the Synthesis of RNA-DNA Primers by pol/prim. To investigate whether the stimulation of DNA synthesis by topo I occurs because topo I enhances the synthesis of primers containing RNA and short DNA strands, the monopolymerase system was again utilized. Newly synthesized RNA-DNA primers were identified by labeling them in the presence of ^{32}P-dCTP for one minute in a complete monopolymerase reaction (Figure 3), as previously described (*34*). Under these conditions, an RNA-DNA primer of approximately 36 nucleotides is synthesized and readily detected (Figure 3A). With WT T antigen, topo I greatly augments the synthesis of this product (Figure 3A, lanes 2 and 3, and Figure 3B,C). This demonstrates that one function of topo I during DNA synthesis is to stimulate the initiation reaction with polymerase alpha/primase. Importantly, mutant T antigens defective in binding topo I were not able to support the synthesis of significant amounts of this product whereas other mutants normal in binding topo I did (Figure 3B,C and Table 1). This correlation appeared to be perfect for every mutant examined. Thus, the binding of topo I to the C-terminal site on T antigen is needed for stimulating the initiation reaction.

The stimulation of primer RNA-DNA synthesis by topo I in the presence of WT T antigen was examined in more detail (Figure 4). By varying the amount of topo I in the reaction, it was determined that topo I can stimulate the synthesis of the 36 nucleotide RNA-DNA primer by 5 fold or more (Figure 4A,C). The production of larger RNA-DNA chains was also stimulated up to 5 fold (Figure 4C). In addition, we showed (Figure 4B) that the nicking/relaxation activity of topo I is important for this stimulation. Previously, Murakami et al. (*34*) showed that the synthesis of the RNA primer itself appeared to be stimulated by the inclusion of topo I in a similar reaction. This effect was not studied in more detail but the data are consistent with the interpretation that topo I stimulates the activities of pol/prim to both synthesize the RNA primer and to extend this primer with DNA.

RPA and Topo I Can Bind to One Another. There are at least three different ways that topo I could enhance the activity of pol/prim. One way is by directly binding to one or more component of pol/prim and the other two ways are by binding to RPA or T antigen which then in turn activates pol/prim. We have tried, but failed, to identify an interaction of topo I with pol/prim (data not shown), although it is possible that we did not have the proper binding conditions. We did find that topo I can bind directly to RPA, however.

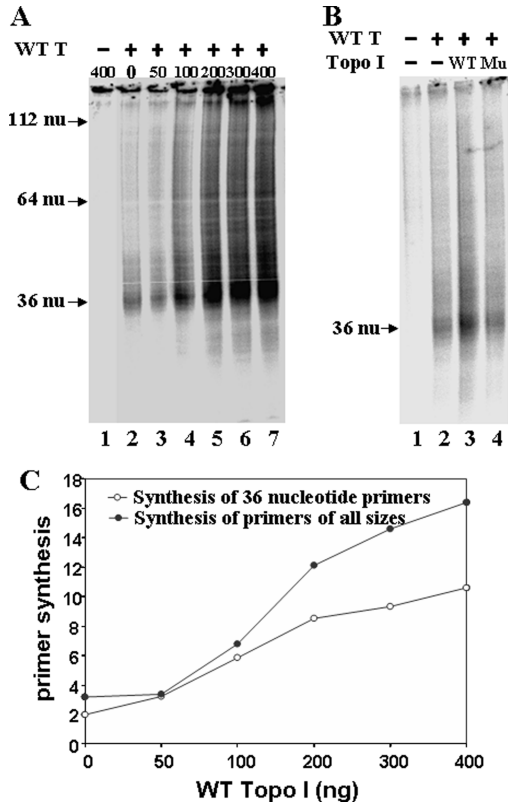

FIGURE 4: Effect of topo I concentrations and catalysis on the production of RNA-DNA primers by pol/prim. (A) Primer synthesis reactions were carried out as described in Figure 3 in the presence of WT T antigen and various amounts of topo I. The sequencing gel was scanned with a PhosphorImager. The positions of various single stranded DNA markers are shown. (B) Reactions were similarly carried out without T or topo I (lane 1), with T but without topo I (lane 2), with 100 ng of WT topo I (lane 3) or with 100 ng of Y723F catalytic mutant of topo I (lane 4). (C) Quantitation of incorporation into the 36 nucleotide RNA-DNA primers and into primers of all sizes in the presence of increasing amounts of WT topo I.

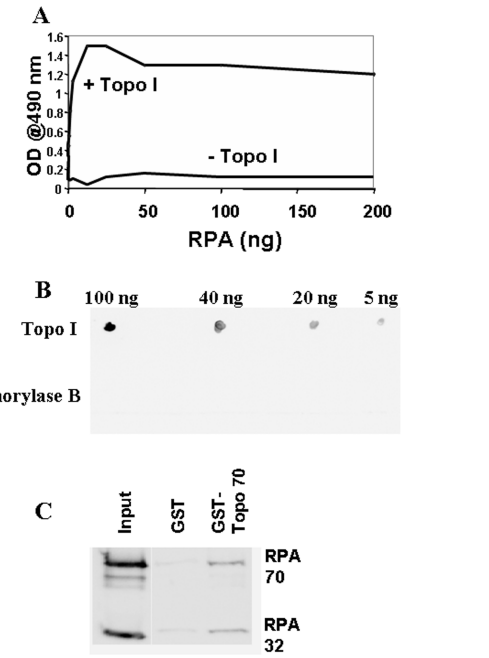

FIGURE 5: Binding of topo I and RPA. (A) ELISA. 100 ng of WT topo I (or no topo I as controls) was bound to wells of a microtiter plate and incubated with increasing amount of WT RPA. After washing the wells, anti RPA 70 Kda was added followed by anti mouse HRP (Sigma) and then substrate. The reactions were stopped and absorbance read at 490 nm using a DYNEX MRX plate reader. (B) Immunoblot. Various amounts of WT topo I or phosphorylase B (as controls) were spotted on a nitrocellulose membrane as shown. Subsequently, the membrane was incubated with WT RPA and then with anti RPA 70 Kda followed by incubation with HRP-conjugated goat anti mouse IgG and Chemiglow substrate. Chemiluminescence was detected with a FluorChem 8800. (C) GST-pull down assay. Purified WT RPA was incubated with beads containing either GST alone or a GST-topo 70 fusion protein. After thorough washing, bound proteins were released with SDS and analyzed by a Western blot using anti-RPA monoclonal antibodies against the 70 kDa and 32 kDa subunits. A sample of the total reaction (input) was applied to the first lane.

This was demonstrated in various binding assays, including ELISAs (Figure 5A), immunoblots (Figure 5B) and a GST-pull down assays (Figure 5C). In these experiments, binding of full length topo I or a deletion mutant missing the first 174 amino acids (topo 70) (*30*) to WT RPA was readily detected. So, it is possible that a topo I-RPA interaction participates in the stimulation of pol/prim activity.

DISCUSSION

In this manuscript, we demonstrate that the major role of the association of topo I with the C-terminal end of T antigen is to promote the synthesis of RNA-DNA primers by pol/prim. Surprisingly, the binding has no impact on the ability of T antigen to unwind a circular DNA template. Free topo I or topo I associated with the N-terminal binding site on T antigen must provide this function. Although we are now beginning to understand how the initiation complex forms, we still have very few clues as to exactly where and how RPA and pol/prim become engaged within this replication engine.

How does the topo I enzyme stimulate primer-DNA synthesis by pol/prim? One possibility is that topo I communicates directly with pol/prim and stimulates its ability to prime. However, we have no evidence that topo I and pol/prim can bind to one another. Furthermore, even if this were the case, an association of topo I with T antigen is needed since the mutant T antigens that are unable to bind topo I correctly (Table 1) (*28*) do not promote correct initiation (Figure 3). So, we think this is unlikely. Another possibility is that, by binding to T antigen, topo I transduces a signal that is relayed to pol/prim resulting in enhancement of activity. Since binding of topo I to T antigen is required to fully activate the polymerase (results described in this paper) and the association of T antigen with pol/prim also appears to be necessary (*21*), this model has merit. A third explanation is that topo I interacts with RPA and that RPA directly stimulates pol/prim. Here too, an interaction of topo I with T antigen would be necessary. Many investigators have shown that RPA is absolutely necessary for SV40 DNA replication (*34, 40–43*). This protein interacts with the primer strands during DNA synthesis (*18*) and stimulates pol/prim activity (*14, 17*) suggesting that it works in conjunction with the polymerase to efficiently initiate new chains. In agreement with this interpretation, recent work by Taneja et al. (*44*) indicates that the RPA-pol/prim interaction occurs prior to initiation of DNA synthesis. Our observation that topo I and RPA can bind to one another (Figure 5), suggests that this explanation is also possible. Perhaps both topo I-T

antigen and topo I-RPA interactions as well as topo I catalytic activity are important.

Topo I also stimulated the production of large molecular weight DNA by pol/prim. It is clear from the results obtained with the mutant T antigens that the association of topo I with the C-terminal end of T antigen is required for the synthesis of this product. This large molecular weight DNA is most likely made when RNA-DNA primers are extended by the polymerase activity of pol/prim during leading strand synthesis (*39*). Our overall conclusion is, therefore, that the binding of topo I to the helicase domain of T antigen significantly augments the synthesis of RNA-DNA primers and their extension by pol/prim. It should be stressed that topo I must, in addition, act to relax supercoiling created by the movements of the replication forks. Free, unbound topo I or topo I bound the N-terminal site on T antigen must participate in that reaction.

Previous reports have indicated that topo II can substitute for topo I in a DNA replication reaction (*45–47*). Our finding that topo I is specifically needed to bind to T antigen and stimulate initiation of DNA synthesis implies either that topo II also possesses the ability to bind T antigen and stimulate pol/prim or that topo II can stimulate the activity of the polymerase directly. Further investigations are required to distinguish between these possibilities.

The placement of topo I to the "back edge" of the helicase domain of each hexamer (*28*) to promote initiation of new chains by pol/prim introduces certain restrictions into how RPA and pol/prim can position themselves within this complex. RPA is known to bind to the origin binding domain of T antigen (*19*), far away from the topo I binding site at the C-terminal end. Stoichiometric analyses (*25*) showed that RPA, like topo I, is present at the origin in a 1:1 molar ratio with T antigen hexamers. Therefore, if topo I-RPA interactions are needed for DNA synthesis, extensive structural changes must occur to permit one molecule of RPA to bind both T antigen and topo I. Pol/prim is a large enzyme consisting of four subunits. It would presumably have to contact at least T antigen and RPA in addition to the SS DNA template. It will be exciting to determine how each protein component fits into the replication machine.

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