Topoisomerase I Associates Specifically with Simian Virus 40 Large-T-Antigen Double Hexamer–Origin Complexes

DAHAI GAI, RUPA ROY, CHUNXIAO WU, AND DANIEL T. SIMMONS*

Department of Biological Sciences, University of Delaware, Newark, Delaware 19716-2590

Received 15 September 1999/Accepted 14 March 2000

Topoisomerase I (topo I) is required for releasing torsional stress during simian virus 40 (SV40) DNA replication. Recently, it has been demonstrated that topo I participates in initiation of replication as well as in elongation. Although T antigen and topo I can bind to one another in vitro, there is no direct evidence that topo I is a component of the replication initiation complex. We demonstrate in this report that topo I associates with T-antigen double hexamers bound to SV40 origin DNA (T_{DH}) but not to single hexamers. This association has the same nucleotide and DNA requirements as those for the formation of double hexamers on DNA. Interestingly, topo I prefers to bind to fully formed T_{DH} complexes over other oligomerized forms of T antigen associated with the origin. High ratios of topo I to origin DNA destabilize T_{DH} . The partial unwinding of a small-circular-DNA substrate is dependent on the presence of both T antigen and topo I but is inhibited at high topo I concentrations. Competition experiments with a topo I-binding fragment of T antigen indicate that an interaction between T antigen and topo I occurs during the unwinding reaction. We propose that topo I is recruited to the initiation complex after the assembly of T_{DH} and before unwinding to facilitate DNA replication.

The mechanism of initiation of eukaryotic DNA replication is not yet clearly understood. To study this process, currently the best model systems are those of simian virus 40 (SV40) and other small DNA tumor viruses. SV40 DNA replication initiates from a well-defined single origin. The core of the origin consists of three parts, a central region known as site II (which consists of four GAGC pentanucleotide repeats), an AT-rich track, and an early palindrome (EP) region (14). This 64-bp-long core is sufficient for SV40 DNA replication (15), but the efficiency of replication is enhanced by auxiliary regions on both sides of the core, especially in vivo (23).

The large tumor (T) antigen is the only viral protein essential for SV40 DNA replication, while the host cells provide all other required factors (33, 34, 56, 62). The initiation of SV40 DNA replication is a multistep event. In the presence of ATP, T antigen specifically interacts with the core of the origin and assembles into a double-hexamer structure (T_{DH}) (12, 30, 36, 61). This causes partial melting of the EP region and untwisting at the AT track of the origin (3, 4, 5, 7, 13, 45, 47). This T_{DH} complex appears to be the basic frame around which the replication initiation complex forms, and T_{DH} is the functional helicase during elongation (53, 54, 61).

At least 10 cellular proteins have been identified to be essential for complete replication of SV40 DNA (33, 34, 56, 62). Among them, DNA polymerase α /primase, replication protein A (RPA), and topoisomerase I (topo I) are believed to participate in DNA replication at a very early stage (19, 21, 37, 40, 41, 51, 57, 59, 63, 64, 65, 67). Topo I is a critical enzyme needed to release the topological stress created by DNA unwinding. RPA is required to stabilize regions of single-stranded DNA (22, 62) and to promote the synthesis of RNA primers (9, 29, 39). DNA polymerase α /primase lays down the RNA primer and extends it with a short stretch of DNA (20, 44).

Recent work in our lab (50) and by others (26) demonstrated a direct interaction between topo I and T antigen; two regions

of topo I bind to two regions on T antigen. By using in vitro replication assays, we (50, 57) and others (25) have shown that topo I stimulates T-antigen-mediated DNA replication and that it must be present from the beginning of the reaction to promote initiation. Topo I has no effect if it is introduced during the elongation stage (57). Also, topo I nicks origin DNA at specific and unique sites during T-antigen-mediated DNA unwinding, indicating that the interaction between T antigen and topo I is functionally significant (51). Furthermore, topo I enhances the fidelity of origin unwinding by T antigen (52). These results are consistent with the hypothesis that topo I and the T-antigen helicase are components of a replication initiation complex, but direct evidence is lacking. At least two critical questions remain to be answered: at what stage does topo I join the replication complex and how is topo I recruited to the complex?

In order to start answering these questions, we used Western blotting to detect an association between topo I and T_{DH} under replication buffer conditions. We found that topo I preferentially associates with fully formed T_{DH} complexes over intermediates in assembly and that topo I is recruited to the initiation complex prior to the beginning of unwinding.

MATERIALS AND METHODS

Cells. Sf9 insect cells were routinely maintained in spinner flasks, transferred to T150 flasks, and infected with recombinant baculoviruses using standard protocols (PharMingen).

Protein purification. Human topo I was purified by column chromatography as described by Stewart et al. (55) and estimated to be about 90% pure.

Wild-type (WT) T antigen and T antigen harboring residues 1 to 246 (T antigen 1–246) were immunoaffinity purified from baculovirus-infected Sf9 cells with monoclonal antibody pAb101 (24) for WT T antigen and pAb419 (24) for T antigen 1–246. The antibody was covalently cross-linked to CNBr-activated Sepharose 4B beads (Pharmacia) according to the manufacturer's procedure. T antigens were eluted with ethylene glycol elution buffer (36), dialyzed against storage buffer (10 mM Tris [pH 8], 1 mM EDTA, 100 mM NaCl, 1 mM dithiothreitol, 50% [vol/vol] glycerol), and stored at -20°C . Silver staining of 10% Laemmli gels allowed for the estimation of protein concentrations relative to that of a phosphorylase B standard.

Plasmids. pSKori contains the small *TaqI*-to-*KpnI* fragment of SV40 DNA inserted into pSK(–) (Stratagene) (51). pSKORI and pSKIR/AT are equivalent plasmids missing T-antigen binding site I and sites I and II, respectively. They

* Corresponding author. Mailing address: University of Delaware, Department of Biological Sciences, Newark, DE 19716-2590. Phone: (302) 831-8547. Fax: (302) 831-2281. E-mail: dsimmons@udel.edu.

FIG. 1. (A) Topo I binds to T_{DH} in the presence of ATP. Different combinations of an origin-containing 362-bp *HindIII-KpnI* DNA fragment (5 ng), T antigen (400 ng), ATP (4 mM), topo I (50 ng), and ethidium bromide (50 ng), as shown, were incubated in replication buffer for 30 min. Samples were subjected to electrophoresis on a nondenaturing acrylamide-agarose composite gel, and protein complexes were transferred to a nitrocellulose membrane. Topo I and T antigen were detected with the monoclonal antibodies 8G6 and pAb101 as described in Materials and Methods. The positions of T_{DH} s and T_H s are shown. (B) Binding reactions contained T antigen (400 ng), ATP (4 mM), and equal molar amounts of a 64-bp core origin fragment (0.9 ng), a 112-bp *HindIII-NcoI* WT-origin-containing fragment (1.6 ng), or a 362-bp origin-containing fragment (5 ng). Samples were subjected to electrophoresis on a nondenaturing 4 to 20% acrylamide gradient gel. The positions of various T-antigen oligomers are shown.

were generated by replacing the WT-origin-containing *HindIII*-to-*NcoI* fragment of pSKori with the equivalent fragments from pORI (18) and pIR/AT (45), respectively.

Linear origin-containing DNA fragments. pSKori was cleaved with *HindIII* and *KpnI* to generate a 362-bp WT-origin-containing DNA fragment. Similar fragments missing site I (341 bp) or sites I and II (317 bp) were made from pSKORI or pSKIR/AT, respectively. A 112-bp *HindIII-NcoI* WT-origin-containing fragment was also generated from pSKori. These fragments were purified from 2% agarose gels with a Bio 101 GeneClean II kit according to the manufacturer's recommendations.

Oligonucleotides. 5' TAATTTTTTTTATTTATGCAGAGGCCGAGGCCGCTCGGCCTCTGAGCTATTCCAGAAGTAGTG 3' and 5' CACTACTTCTGGAATAGCTCAGAGGCCGAGGCCGCTCGGCCTCTGCATAAATAA AAAAAATTA 3' were annealed to one another, and the 64-bp product representing the SV40 core origin of replication was purified by gel electrophoresis.

Circular-DNA substrate. A circular-DNA-unwinding substrate was made by ligating an end-labeled *HindIII-KpnI* origin-containing fragment from pSKori with the following double-stranded oligonucleotide:

The circular DNA (388 bp) was purified by electrophoresis on a 2% agarose gel in Tris-borate-EDTA (TBE), where it migrated faster than the linear unligated fragment. The circular DNA was electroeluted out of the gel and purified further by phenol and chloroform extractions and ethanol precipitation.

Western blotting. Unless otherwise indicated, 400 ng of WT T antigen; 4 mM ATP, ADP, or γ -S-ATP (Sigma); 5 ng of a 362-bp *HindIII-KpnI* origin DNA fragment; and 50 ng of topo I were incubated in a total volume of 20 μ l in replication buffer (30 mM HEPES-KOH [pH 7.5], 7 mM $MgCl_2$, 1 mM dithiothreitol, 40 mM creatine phosphate, 0.1 mg of bovine serum albumin per ml) at 37°C for 25 min. Glutaraldehyde was added to the reaction mixture to a final concentration of 0.1%, followed by incubation at 37°C for 10 min. The samples were applied to nondenaturing composite gels containing 2.5% acrylamide and 0.6% agarose in TBE buffer or to gradient gel (4 to 20% acrylamide in 0.05 M Tris [pH 8.8], 0.05 M glycine). Composite and gradient gels were subjected to electrophoresis at 70 V for 2 h in TBE buffer and 100 V for 12 h in 0.05 M Tris (pH 8.8)–0.05 M glycine, respectively. Gels were then transferred at 4°C to nitrocellulose membranes (Amersham) for 2 h at 200 V, followed by 6 h at 80 V

in transfer buffer (0.025 M Tris [pH 8.5], 0.192 M glycine, 20% [vol/vol] methanol). Membranes were screened for topo I using monoclonal antibody 8G6 (57) and ECL reagents (Amersham) according to the manufacturer's recommendations. The same membranes were stripped with stripping buffer (ECL kit; Amersham) and then probed for WT T antigen using the biotin-labeled monoclonal antibody pAb101 (24) and streptavidin-conjugated horseradish peroxidase (Sigma).

DNA unwinding. The conditions for DNA unwinding were previously described (52, 66). One nanogram of the gel-purified-circular-DNA substrate was incubated in DNA replication buffer containing 20 μ g of creatine phosphokinase per ml, 80 ng of *Escherichia coli* single-strand DNA binding protein (SSB; Pharmacia), 400 ng of immunoaffinity-purified T antigen, and/or various amounts of topo I and T antigen 1–246 in a total reaction volume of 20 μ l. After 1 h at 37°C, the reactions were terminated by the addition of 5 μ l of stop buffer (2% sodium dodecyl sulfate, 0.1 M EDTA, 1 mg of proteinase K per ml) and the mixtures were 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 TBE and subjected to electrophoresis for 550 V $\cdot$ h at 3°C. The gels were dried and exposed to X-ray film.

RESULTS

Topo I binds to a complex of SV40 T-antigen double hexamers and origin DNA (T_{DH}) in the presence of ATP. It has long been known that topo I is required for SV40 DNA replication in vivo (32) and in vitro (59, 63, 67). Recently, our lab demonstrated that topo I promotes initiation of SV40 DNA replication in vitro (57), and we (50) and others (26) have shown that topo I can interact directly with T antigen. However, it has not been shown that topo I is actually a component of the initiation complex. To investigate this, we incubated T antigen, a 362-bp origin-containing DNA, topo I, and nucleotides in various combinations under replication buffer conditions. T-antigen single hexamers (T_H s) and T_{DH} s were separated from one another by nondenaturing gel electrophoresis.

Protein complexes were then transferred to nitrocellulose, and the presence or absence of T antigen and topo I was determined by reaction with specific monoclonal antibodies.

As expected (36), in the absence of origin-containing DNA, ATP directed the formation of T_{DH} s (Fig. 1A, anti T lane 1). When both ATP and origin DNA were incubated with T antigen, T_{DH} s also formed (Fig. 1A, anti T lane 3). The T_{DH} complex consists of T antigen, ATP, and origin DNA (36). When topo I was introduced in the reaction mixture, it could be found at the same position as that of T_{DH} complexes but not at the position of T_H (Fig. 1A, anti topo I lane 7). Association of topo I was dependent on the presence of T antigen, origin DNA, and ATP (Fig. 1A, anti topo I lanes 4 to 7). When ethidium bromide was also present in the reaction mixture, no double hexamers or topo I-containing complexes could be detected (Fig. 1A, lane 8). Since ethidium bromide disrupts protein-DNA interactions (31), this implies that the formation of the initiation complex is DNA dependent.

To confirm that we have correctly identified the hexamers and double hexamers on the composite gels, we separated reaction products on 4 to 20% gradient gels in order to detect lower-molecular-weight oligomers as well. Three different DNA fragments were used: a 64-bp synthetic DNA representing the core origin of replication (15, 28), a 112-bp *HindIII*-*NcoI* origin fragment containing the core and T-antigen binding site I, and the 362-bp *HindIII*-*KpnI* origin fragment used in the experiment described above. In the absence of DNA, T-antigen dimers, trimers, tetramers, and hexamers could be detected (Fig. 1B, lane 1). Monomers were present but were not very reactive with pAb101 (data not shown). In the presence of origin DNA (Fig. 1B, lanes 2 to 4), the amounts of lower-molecular-weight oligomers were reduced as expected (46) and a new slowly migrating band, most likely a DNA-protein complex, was detected in each case. The complex migrated to slightly different positions in variation with the size of the DNA (Fig. 1B). The electrophoretic behavior of these DNA-protein complexes is consistent with the idea that they are double hexamers associated with origin DNA (T_{DH}), and this interpretation is in agreement with published results (46). By comparison of Fig. 1B, lane 4, with Fig. 1A, lane 3, it is apparent that the two bands in Fig. 1A correspond to T_H and T_{DH} .

Nucleotides and origin DNA constructs that support the formation of double hexamers also support topo I binding. Many nucleotides other than ATP can participate in the formation of double hexamers (36). To investigate the nucleotide requirements of topo I association, ADP and the nonhydrolyzable ATP analog γ -S-ATP were also tested (Fig. 2A). Both of them supported the formation of T_{DH} complexes (Fig. 2A, lanes 2 and 4). Topo I also bound to these complexes in the presence of all three nucleotides (Fig. 2A, lanes 6 to 8). AMP, which does not support the formation of T_{DH} (5, 12), did not permit topo I binding (data not shown). Therefore, the association of topo I with T antigen depends on a T_{DH} structure but not on ATP hydrolysis.

T-antigen binding site II, which is at the center of the origin, is required for double hexamer-origin DNA complexing (28, 46), but site I, another T-antigen binding site, is needed for optimal DNA replication (23). We asked whether only site II or both sites were required for the formation of topo I-containing complexes (Fig. 2B). When site I was deleted from the origin, T_{DH} formation was close to normal by comparison with the whole origin (Fig. 2B, lanes 2 and 3). The association of topo I with this structure was also normal (Fig. 2B, lanes 6 and 7). However, when both sites I and II were deleted, the remaining DNA could not permit formation of T_{DH} or T_{DH} -topo

FIG. 2. Nucleotide and DNA requirements for the association of topo I with T_{DH} . (A) Binding reactions were carried out as described for Fig. 1 using T antigen (400 ng), the 362-bp origin DNA fragment (5 ng), topo I (50 ng), and 4 mM ATP, ADP, or nonhydrolyzable γ -S-ATP. (B) Binding reaction mixtures contained T antigen (400 ng), topo I (50 ng), ATP (4 mM), and DNA (5 ng) containing the complete origin (362 bp), a deletion of site I (Δ site I) (341 bp), or a deletion of both sites I and II (Δ site I & II) (317 bp).

I (Fig. 2B, lanes 4 and 8). The correspondence between T_{DH} formation and topo I association further demonstrates that the conditions that allow the assembly of T_{DH} also permit the association of the complex with topo I.

Topo I exclusively associates with a fully formed T_{DH} structure. There is a substantial amount of evidence that double hexamers assemble on origin DNA from individual monomers (11, 27) and that one hexamer forms first over pentanucleotides 1 and 3 in site II, followed by the cooperative assembly of the second hexamer over pentanucleotides 2 and 4 (28, 30). To determine if the association of topo I with this T_{DH} structure is dependent on the formation of a complete double hexamer or can take place with intermediates in the assembly process, we performed binding experiments with various amounts of origin-containing DNA (Fig. 3). When the ratio of DNA to T antigen was increased, faster-migrating complexes formed (Fig. 3, anti T lanes 2 to 7). These complexes probably represent intermediates that contain single hexamers and various

FIG. 3. Topo I preferentially associates with fully formed T_{DH} . Binding reaction mixtures contained T antigen (400 ng), topo I (50 ng), ATP (4 mM), and increasing amounts of the complete origin-containing 362-bp DNA as shown.

numbers of additional monomers associated with DNA but are not fully assembled because of limiting amounts of T antigen. Interestingly, topo I associated primarily with the fully formed double hexamers and bound poorly to intermediate forms (Fig. 3, anti topo I lanes 5 to 7). These results indicate that topo I is added to the T-antigen–origin complex after T_{DH} is completely formed.

Order of assembly of the T_{DH} -topo I complex. How SV40 DNA initiation complexes assemble is essential to understanding early events of SV40 DNA replication. Topo I preferentially binds to fully formed double hexamers (Fig. 3), strongly indicating that topo I associates with the initiation complex after the T-antigen double hexamer forms around origin DNA. To obtain additional evidence for this, each component of the binding reaction was individually omitted during the first 12 min of incubation and then included during a second 12 min of incubation. When ATP was added at 12 min (Fig. 4, lane 3), very small amounts of T_{DH} formed and topo I was not detected in the complex. This observation indicates that an interaction between topo I and T antigen in the absence of ATP interferes with subsequent oligomerization of T antigen in the presence of ATP. When the DNA or topo I was added at 12 min, T_{DH} and T_{DH} -topo I formed readily (Fig. 4, lanes 5 and 7). However, when T antigen was added at 12 min, T_{DH} assembled normally (Fig. 4, anti T, compare lanes 9 and 1) whereas topo I associated very poorly with T_{DH} (Fig. 4, anti topo I lane 9). These results demonstrate that T antigen must be present from the beginning for T_{DH} -topo I complexes to form and indicate that the order of assembly is indeed T antigen and then topo I.

High concentrations of topo I destabilize the T_{DH} complex. We then tested the effect of various amounts of topo I on the formation of T_{DH} complexes by adding increasing amounts of topo I to binding reaction mixtures (Fig. 5A). As the amount of topo I increased from 12 to 100 ng, there was a significant reduction in the total amount of T_{DH} (Fig. 5A, lanes 3 to 6), with no detectable signal in the presence of 100 ng of topo I (Fig. 5A, lane 6). The amount of topo I bound to the T_{DH}

FIG. 4. Order of assembly of the T_{DH} -topo I complex. For lane 1, T antigen (400 ng), ATP (4 mM), the 362-bp origin DNA (5 ng), and topo I (50 ng) were incubated for 30 min as a positive control. For lanes 2, 4, 6, and 8, the reaction mixtures were missing one component as shown and incubated for 24 min. For lanes 3, 5, 7, and 9, the reaction mixtures were missing one component for 12 min, as shown, the missing component was then added to the reaction mixture, and incubation was continued for another 12 min.

complex increased as the amount added to the reaction mixture increased, with a maximum signal at 50 ng of topo I, and decreased to undetectable levels at 100 ng (Fig. 5A, lanes 7 to 12).

There are at least two possible reasons why T_{DH} was not detectable in the presence of excess topo I. One is that high concentrations of topo I prevent the interaction between origin DNA and T antigen. The other is that excess topo I renders the T_{DH} structure unstable. To distinguish between these two possibilities, 50 ng of topo I was incubated with T antigen, the 362-bp origin DNA, and ATP for 12 min, allowing T_{DH} -topo I complexes to form. The binding reaction is essentially over after 8 min of incubation, and further incubation does not alter the amounts of complexes formed (data not shown). At 12 min, another 50 ng of topo I was added to the reaction mixture and incubation continued for another 12 min. Under these conditions, no T_{DH} was observed (Fig. 5B, lane 2), and consequently, very little topo I was present in the complex (Fig. 5B, lane 5). Our interpretation of this result is that high concentrations of topo I destabilize preformed T_{DH} complexes.

The ratio between topo I and DNA is critical for T_{DH} -topo I complex formation. To investigate the mechanism by which high concentrations of topo I destabilize T_{DH} s, different ratios of topo I to origin DNA, topo I to T antigen, and T antigen to origin DNA were used (Fig. 6). In all cases where the mass ratio between topo I and origin DNA was 10 (e.g., 50 ng of topo I to 5 ng of origin DNA, which is equal to a molar ratio of 30), T_{DH} -topo I complexes formed (Fig. 6, anti topo I lanes

FIG. 5. High concentrations of topo I destabilize the T_{DH} structure. Binding reaction mixtures contained T antigen (400 ng), ATP (4 mM), and the complete 362-bp origin DNA (5 ng). (A) Increasing amounts of topo I were included as shown. (B) Fifty or 100 ng of topo I was added at 0 min. For lanes 2 and 5, another 50 ng of topo I was added at 12 min and incubation continued for another 12 min.

2, 4, and 5). Higher ratios of topo I to DNA inhibited T_{DH} formation and topo I association (Fig. 6, lanes 1, 3, 6, and 7). On the other hand, the ratios of T antigen to DNA and T antigen to topo I were not as critical (Fig. 6).

The topo I-to-DNA ratio is critical for T-antigen-mediated unwinding of circular origin-containing DNA. The effect of high topo I-to-DNA ratios on the formation of T_{DH}s led us to ask if high topo I-to-DNA ratios inhibit T antigen in a functional assay. We generated a labeled, origin-containing, circular, 388-bp DNA (Fig. 7A) that was very similar to the linear DNA fragment used in our binding reactions. The circular DNA was incubated under DNA replication buffer conditions with T antigen and various amounts of topo I to determine the conditions under which this substrate would be unwound (Fig. 7B). Figure 7B (lane 2) demonstrates that T antigen alone has no effect on this substrate. When topo I was added in addition to T antigen, two faster-migrating bands appeared with a concomitant decrease in the amounts of starting circular DNA (Fig. 7B, lanes 4 to 6). The identities of these DNA products were determined by comparing them to DNA incubated in the presence of *E. coli* gyrase (data not shown). The faster-moving product is a topoisomer with a -5 linking order, whereas the slower-moving product has a -2 linking number, consistent with results previously obtained by Roberts (47). The amount of these underwound products increased with increasing topo I-to-DNA ratios and reached their maximum level at a mass ratio of 9 (molar ratio of about 29). At this ratio, maximum levels of topo I are bound to the origin (Fig. 5A, lane 11). Higher topo I-to-DNA ratios reduced the amounts of underwound circular products (Fig. 7B, lanes 6 and 7). This result is consistent with those of Fig. 5 showing that excess topo I inhibits T_{DH} formation.

T antigen-topo I interactions occur during the unwinding reaction. Although topo I is required for the partial unwinding of a small circular DNA substrate (Fig. 7B), it is not known if this involves an interaction between T antigen and topo I. To address this question, we tested if a topo I-binding fragment of T antigen interferes with partial unwinding. T antigen 1-246 has been previously shown to bind topo I (50), but it fails to

bind origin DNA (49) and does not interfere with DNA unwinding by itself (52). Furthermore, this fragment has no effect on topo I DNA relaxation activity, even at high concentrations (data not shown). Various amounts of this fragment were added to the circular-DNA-unwinding reaction mixture, and it was evident that it strongly inhibited unwinding (Fig. 7C). The results indicate that, under the conditions of unwinding, T antigen-topo I interactions take place and suggest that such an interaction may participate in DNA unwinding.

DISCUSSION

T antigen is the only viral protein involved in SV40 DNA replication. It has two critical functions: one is to recognize the viral origin specifically, and the second is to separate the DNA strands at the origin and at replication forks. The first function takes place through its DNA-binding domain (2, 35, 49). Upon binding of ATP (5, 8, 17), T antigen assembles on the origin to form a double-hexamer structure (11, 27, 30, 36). This double-hexamer structure is the helicase that unwinds DNA (53, 54, 61). Eventually, topo I is needed to release the torsion created by the progressing replication fork. At the same time, topoisomerase activity must be controlled according to the pace of the helicase to prevent too much or too little nicking, which might interfere with efficient replication. Although how helicase and topoisomerase activities are coordinated during DNA replication is still unclear, recent evidence (25, 26, 50, 51, 57) points to the likelihood that T antigen and topo I function together from the very beginning of replication.

In this report, we demonstrated that topo I physically associates with T_{DH} initiation complexes and that it prefers fully formed T_{DH}s over intermediates in assembly. A previous study in our lab showed that topo I binds to T antigen monomers in enzyme-linked immunosorbent assays and immunoblot assays (50), so it is interesting that in solution and under replication conditions, topo I selectively associates with the T_{DH} structure but not with hexamers or other oligomerized forms of T antigen associated with origin DNA. An explanation for this difference is that one of the binding sites on T antigen is buried

FIG. 6. The ratio between topo I and origin DNA is critical for the formation of T_{DH} and topo I association. Binding reaction mixtures contained ATP (4 mM) and different amounts of T antigen (T), topo I, and the complete 362-bp origin DNA (ori) as shown.

in the native monomeric protein but becomes exposed on partially denatured monomers during enzyme-linked immunosorbent assays and immunoblot reactions and on double hexamers bound to DNA. We further observed, in this study, that topo I readily associates with preformed T_{DH} complexes (Fig. 4), supporting the conclusion that topo I binds primarily after the double hexamer completely assembles over the origin.

One simple explanation for the selectivity of topo I binding to T_{DH} is that a stable association depends on interactions with multiple T-antigen subunits in the double hexamer. Another possibility is that binding requires a specific conformational change in T antigen or origin DNA triggered by the assembly of double hexamers. There is substantial evidence that the assembly of T antigen on the origin is accompanied by conformational changes in both T antigen and the DNA. First, formation of double hexamers is dependent on ATP binding (5, 8, 17), suggesting that an allosteric change in T antigen is required. Second, the DNA undergoes structural changes in the EP and AT regions (3, 4, 6, 7, 13, 45, 47) and EP melting is temperature sensitive, providing evidence that there are major changes in DNA conformation (7). Third, Mastrangelo et al. (36) observed that at 0°C, only 9-mers of T antigen formed and inferred that a DNA conformational change was needed to assemble a complete double hexamer. One or more of these changes may be needed for topo I binding.

The preference of topo I for a complete T_{DH} structure may have at least two biological benefits. Work by two labs (28, 46) indicated that T-antigen monomers first assemble over the origin into a hexameric structure that is used to recruit additional monomers into a second hexamer. Although topo I does not directly inhibit DNA binding or DNA structural distortion by T antigen (52), it is possible that it may interfere with double hexamer function if it binds too early. Since extensive interactions between the two T-antigen hexamers are required for DNA unwinding (53) and replication (60), it may be critical that topo I not attach to the complex too early lest it interfere

with the correct placement of each T-antigen hexamer on the DNA.

A second advantage in restricting topo I binding to double hexamers may be in preventing topo I from binding to T antigen attached to site I. This region is located adjacent to the core origin and functions in the repression of transcription from the early promoter (1, 38, 42). It has been reported that a dimer of T antigen binds to site I (16). If topo I associates with this region of the DNA, it may prevent transcriptional regulation by T antigen.

In nearly all cases, conditions that permitted T_{DH} formation also allowed topo I association. ADP and γ -S-ATP supported the formation of T_{DH} complexes and also promoted topo I binding (Fig. 2A), while AMP, which fails to support T_{DH} formation, was unable to induce detectable topo I association (data not shown). Compared to ATP and γ -S-ATP, ADP permitted smaller amounts of T_{DH} to form under these conditions, and as a consequence less topo I bound. When the T antigen-to-DNA ratio was optimal for T_{DH} formation (Fig. 3, lanes 3 and 10) or when the topo I concentration was low enough to permit stable double hexamers to form (Fig. 5A, lanes 3 to 5 and 9 to 11), topo I could be observed with the complex. These observations imply that no other factors or conditions are required for topo I binding.

Our data support the idea that topo I binds to the initiation complex before the DNA begins to unwind. First, as indicated above, ADP and γ -S-ATP, which cannot be used as an energy source during unwinding, were sufficient for topo I binding. Second, T antigen alone was incapable of partially unwinding a circular-DNA substrate and required topo I for activity (Fig. 7B). Competition experiments (Fig. 7C) with a topo I-binding T-antigen fragment (T antigen 1–246) demonstrated that T antigen-topo I interactions were taking place during the reaction, raising the possibility that such an interaction may participate in DNA unwinding. These results can be used to explain our previous observation that topo I must be present at the initiation stage to stimulate DNA replication in vitro (25, 57). Our previous results (57) also argued that topo I was stably integrated in the initiation complex and could not be exchanged with other molecules of topo I once DNA replication began. Together, these data indicate that topo I is one of the first cellular proteins to be recruited to the initiation machinery.

In an earlier report (51), we estimated that one to two molecules of topo I are present in this complex based on the optimum amounts of enzyme needed to nick origin DNA during a DNA-unwinding reaction. Although it is difficult to calculate this stoichiometry from our present data, we can estimate that there are relatively few topo I molecules bound because the presence of the enzyme had no detectable effect on the mobility of the complex on our gels. Other cellular proteins such as RPA and DNA polymerase α /primase are also known to be required for initiation of DNA replication (19, 21, 40, 41, 59, 63, 64, 65) and may be components of an initiation complex. Recent work (27) of Huang et al. showed that polymerase α associates with preformed T-antigen hexamers and that two molecules of polymerase α /primase may bind to each double hexamer. Our current working model is that each hexamer associates with one molecule of topo I and one of polymerase α /primase.

Our view is that topo I is also associated with the DNA-synthesizing machinery at replication forks. During bidirectional unwinding or replication, the two hexamers of T antigen remain together at replication forks and act as the functional helicase (53, 54, 61). Therefore, topo I may continue to be associated with double hexamers due to its high affinity to this

FIG. 7. (A) Construction of the circular DNA substrate. A *HindIII-KpnI* origin-containing fragment of pSKori (SV40 sequences 5171 to 294) was end labeled with [γ - 32 P]ATP and ligated to a double-stranded oligonucleotide (linker). The position of the origin in the molecules is shown. (B) Unwinding of the circular-DNA substrate with T antigen and topo I. The labeled gel-purified circular substrate was incubated with various amounts of topo I in the presence (lanes 2 to 7) or absence (lanes 9 to 13) of T antigen (T) under DNA-unwinding conditions. Products were separated from the unreacted substrate by gel electrophoresis. Lane 1 contained no T antigen or topo I, and lane 2 had T antigen only. In lanes 3 to 7 and 9 to 13, increasing amounts of topo I were used, as shown. Lane 8 contained substrate DNA that was boiled for 5 min (the circular DNA is resistant to boiling). Lane 14 contained end-labeled *HindIII-KpnI* linear double-stranded DNA (L). The positions of the circular substrate, the two products of the reaction (slow and fast), and linear *HindIII-KpnI* DNA are shown. (C) Inhibition of unwinding by T antigen 1-246. The circular substrate was incubated under DNA-unwinding conditions without proteins (except for *E. coli* single-strand DNA binding protein) (lane 1); with T antigen (lane 2); with T antigen and 10 ng of topo I (lane 3); or with T antigen, 10 ng of topo I, and various amounts of T antigen 1-246 (145, 241, 338, and 435 ng in lanes 4 to 7, respectively).

structure. In this way, its activity to relax topological stress ahead of the replication fork may be under the close control of T antigen. This interpretation agrees with previous observations that topo I preferentially associates with SV40 replication intermediates in camptothecin-treated CV-1 cells (10).

The observation that excess topo I destabilizes T_{DH} (Fig. 5B) is interesting, but the mechanism is unclear. This effect is most probably responsible for the inhibition of circular-DNA unwinding (Fig. 7B, lanes 6 to 7) at high topo I concentrations and for the inhibition of unwinding and topo I nicking of linear origin-containing DNA (51). Since the topo I-to-DNA ratio appears to be critical for this effect (Fig. 6), we surmise that double hexamers are destabilized when too many topo I molecules bind to the DNA. One possibility is that excess topo I causes structural changes in DNA that displace the bound T-antigen double hexamers.

An important question that must be answered is how topo I is recruited to the initiation complex. Since topo I binds to both T antigen and DNA, it is likely that both participate. Evidence that T antigen (in association with DNA) has a direct role in recruiting topo I comes from our observation that incompletely

assembled T antigen-DNA complexes do not bind topo I (Fig. 3). Also, the stable association of topo I with DNA is dependent on the presence of T antigen (Fig. 1A and data not shown). Since topo I-to-DNA ratios are important, we reason that topo I-DNA interactions are also involved. One possibility is that the DNA is used as the touchdown site for topo I. After binding to the DNA, topo I may then slide over until it meets preformed double hexamers and in this context may become stably associated with the complex. We found that the T_{DH} structure containing the 112-bp origin DNA fragment permitted topo I association just like the one with the 362-bp origin DNA but that the T_{DH} containing the 64-bp core DNA bound topo I very poorly, if at all (data not shown). This result may be due to the fact that all of the 64-bp DNA core is covered by a T-antigen double hexamer (5, 58) and topo I has no free DNA to bind.

According to our data, we propose a model for the assembly of the first SV40 replication initiation complex on the origin (Fig. 8). First, T antigen binds to ATP, followed by the interaction with core sequences at the origin to assemble into the T_{DH} structure. Then, a small number of topo I molecules (two?) interact with both DNA and T antigen and are recruited to the complex. Other protein factors (e.g., RPA and DNA

FIG. 8. Model of the assembly of a topo I-containing initiation complex. We propose that first T antigen assembles into a double hexamer structure over the SV40 replication origin. Topo I is then recruited to the complex and associates with T_{DH} . The binding of topo I takes place before DNA unwinding because topo I association is independent of ATP hydrolysis. During elongation, topo I may still be associated with the T-antigen double hexamer structure at replication forks.

polymerase α /primase) bind to the complex during unwinding. After replication begins, topo I continues to be associated with double hexamers during the elongation process.

ACKNOWLEDGMENTS

This work was supported by a grant from the National Cancer Institute to D.T.S. (CA36118).

We thank Pamela Trowbridge for critically reading the manuscript.

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