The Activity of Topoisomerase I Is Modulated by Large T Antigen during Unwinding of the SV40 Origin*

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When simian virus 40 (SV40) large T antigen binds to the virus origin of replication, it forms a double hexamer that functions as a helicase to unwind the DNA bidirectionally. We demonstrate in this report that T antigen can unwind and release an origin DNA single strand of less than full length in the presence of purified human topoisomerase I. The sites nicked by topoisomerase I in the strands released by T antigen during DNA unwinding were localized primarily to the “late” side of the origin, and the template for lagging strand synthesis was preferred significantly over the one for leading strand synthesis. Importantly, these sites were, for the most part, different from the sites nicked by topoisomerase I in the absence of T antigen. These data indicate that T antigen activates topoisomerase I nicking at discrete sites and releases these nicked strands during unwinding. We hypothesize that a single molecule of topoisomerase I can form a functional complex with a double hexamer of T antigen to simultaneously relax and unwind double-stranded origin-containing DNA.

Our understanding of mammalian DNA replication has originated mostly from work on simian virus 40 (SV40). The virus DNA has a single origin of replication that has been extensively characterized. This origin is a tripartite stretch of DNA consisting of a central pentanucleotide repeat that serves as the binding sites for T antigen (the virus initiator protein) (1, 2), an early palindrome from where melting originally takes place (3, 4), and an AT-rich track that is structurally distorted by T antigen (5–7). All three regions are required for DNA replication (1, 2, 8) and for origin unwinding (9). Neighboring sequences improve the efficiency of replication but are not absolutely required (1, 10). These auxiliary sequences are located on both sides of the origin and may facilitate the unwinding reaction (11).

A great deal of effort has gone into trying to understand the composition of the initiation complex at the origin. In the presence of ATP, T antigen forms a double hexamer that completely protects the core origin from accessibility to Dnase (12–15). This double hexamer functions as an efficient helicase (16–18) that unwinds the DNA in both directions (19). At least three cellular proteins have been shown to interact with T antigen and are believed to be recruited to the origin to form a functional initiation complex. These include DNA polymerase α-primase (20–23), replication protein A (RPA) (24–26), and topoisomerase I (27). The order in which these three proteins bind is not known; nor do we know if all proteins are present simultaneously.

Recently, the work in our lab has concentrated on the interaction between T antigen and topoisomerase I. A complex between these two proteins readily forms in vitro (28), but it has been difficult to demonstrate binding in vivo.1 Nevertheless, there are a number of reasons for thinking that an interaction between T antigen and topoisomerase I has functional significance during SV40 DNA replication. First, topoisomerase I is required for DNA replication (29–32) as shown in in vitro DNA replication reactions. Its most obvious function is to relax the torsionally twisted DNA during replication. Second, topoisomerase I inhibits the ability of T antigen to unwind DNA at sites other than a complete origin that includes binding site I, one of the auxiliary sequences. This suggests that it is present in a protein complex that functions during initiation (27). Third, topoisomerase I is part of a large protein complex that can support SV40 DNA replication in vitro (33). Fourth, T antigen and topoisomerase I can form a complex in the presence of DNA (34).2 Finally, in collaboration with Yves Pommer, we (34) have recently shown that T antigen can reverse the nicking of DNA by topoisomerase I in the presence of the drug camptothecin, suggesting that T antigen can influence the activity of topoisomerase I.

When circular DNA is unwound by T antigen, topoisomerase I must relax the unreplicated, supercoiled portions of the DNA ahead of the replication forks. Intuitively, a careful balance must exist between unwinding and nicking activities, and the two must be tightly linked to one another. If the unwinding activity is much higher than the nicking activity, the helicase will have to slow down because of torsional strain in the molecule. On the other hand, if topoisomerase activity is too high, the DNA will be nicked at too many places and broken strands may be released by the helicase.

In this study, we investigated the effects of topoisomerase I on origin DNA unwinding. Using linear DNA fragments, we observed that T antigen can unwind and release broken single strands in the presence of topoisomerase I. The DNA breaks were mapped to discrete sites and differed almost completely from the ones recognized by topoisomerase I in the absence of T antigen. These results are discussed in terms of a model whereby a complex of T antigen and topoisomerase I simultaneously unwinds and relaxes double-stranded DNA.

EXPERIMENTAL PROCEDURES

Plasmids and DNA Substrates Used for Unwinding—pSKori was generated by introducing the TAg1–KpnI origin-containing fragment of SV40 DNA into the large KpnI–ClaI fragment of pSK(−) (Stratagene). The DNA substrates used in the unwinding reactions described in this

1 D. T. Simmons, R. Roy, L. Chen, D. Gai, and P. W. Trowbridge, unpublished results.
2 C. Wu, unpublished results.
study were generated by PCR amplification of various regions of the SV40 DNA insert of pSori. An “up” primer was used in combination with a “down” primer to amplify a certain region of the DNA. The up and down primers used in this study were as follows: up primers, 4800, 5'-GCT TCA TCC TCA GAG AGC-3'; 4919, 5'-CAC TCC TCC TCC TTT ATT T-3'; 5019, 5'-ATC TCC TCC TCC TTT ATT T-3'; 5171, 5'-AAG CTT TTT TGT AAA GCC-3'; down primers, 42, 5'-CTG CTG CAT CCG ATT TTT-3'; 276, 5'-CTT ATC ATT ATT GCA AGA GAA GGA GTA GTC-3'; 176, 5'-TGT GAA AAG ACC CCA GCC-3'.

The number given for each primer refers to the nucleotide number of the 5'-end of the primer in SV40 DNA. The PCR-generated DNA was purified by gel electrophoresis and 5'-end-labeled with [γ-32P]ATP in the presence of T4 polynucleotide kinase and subsequently purified on a “spin” column of Sephadex (Amersham Pharmacia Biotech) G-50. In some experiments, the DNA was cleaved with a restriction endonuclease, and a smaller fragment was purified by agarose gel electrophoresis. This permitted us to generate a DNA fragment labeled only at one end.

**Results**

**T Antigen Releases Nicked DNA During Unwinding in the Presence of Topoisomerase I**—We have previously demonstrated that when SV40 large T antigen unwinds an origin-containing linear DNA fragment under DNA replication conditions, added topoisomerase I has relatively little effect except at high concentrations, where it inhibits unwinding (27, 28). These unwinding assays were performed with a 112-bp DNA fragment containing the minimal SV40 origin of replication and T antigen binding site I. However, when the DNA contains sequences from the late side of the origin as well, the DNA becomes nicked by the added topoisomerase I, and the nicked strands become released by T antigen during unwinding (Fig. 1). A single major band (actually a doublet; see below) of less than full-length single-stranded DNA was observed (Fig. 1, lanes 11–14). The pattern of labeled single strands produced under these latter conditions was different from the one obtained from single strands released by T antigen in the presence of topoisomerase I. Topoisomerase I alone nicked the DNA at multiple sites, whereas the single strands released by T antigen were incised at relatively few sites (see below).

**Fig. 1. T antigen unwinding of SV40 DNA in the presence of topoisomerase I (Topo I).** An origin-containing fragment of DNA was generated by PCR from pSori using the up 5171 and down 176 primers and end-labeled. The DNA was incubated with or without purified T antigen (400 ng) and various amounts of topoisomerase I as shown. The reaction was terminated with stop buffer, and some of the samples were boiled for 5 min to denature the DNA, as indicated. The DNA was applied to a 4% acrylamide gel in TBE buffer. The positions of the nicked DNA (Nicked), native DNA (Native), and boiled DNA (Boiled) are indicated. The star indicates the position of the major nicked and released single-stranded DNA.

**Fig. 2. T antigen releases strands nicked at select sites.** A fragment from pSori was generated by PCR using primers up 5171 and down 276. The end-labeled fragment was subjected to an unwinding reaction with or without 400 ng of T antigen and various amounts of topoisomerase I (Topo I), as indicated. Some reactions contained camptothecin. Some samples were boiled to denature the DNA. The double-stranded (DS), full-length single strands (SS1 and SS2) (SS on the left side), and less than full-length single strands (nicked) are shown.
Nick DNA during Unwinding—

To eliminate the possibility that an enzyme other than topoisomerase I was nicking the DNA released by T antigen, we used a single point substitution mutant of topoisomerase I (Y723P). This mutant is catalytically inactive due to a mutation at the catalytic tyrosine but retains its ability to bind DNA (41). It has recently been used as a source of protein for determining the three-dimensional structure of this enzyme (42, 43). Fig. 3 demonstrates that this mutant topoisomerase I is totally inactive in generating the nicked forms of DNA. The mutant protein can still interact with T antigen as determined by enzyme-linked immunosorbent assays (data not shown) and inhibits, at higher concentrations, the unwinding of DNA by T antigen (Fig. 3A, lanes 7–10), just like WT topoisomerase I (Fig. 3A, lanes 3–6).

**FIG. 3.** A catalytically inactive mutant of topoisomerase I (Topo I) does not nick DNA during unwinding. T antigen (400 ng) and various amounts of WT and Y723P mutant topoisomerase I were used to program an unwinding reaction of an end-labeled DNA fragment containing sequences 5171–276. The positions of the double-stranded (DS), full-length single-stranded (SS), and nicked single-stranded DNAs (star) are indicated. A and B represent different exposures of the same gel.

Fig. 2 demonstrates that if the DNA from a reaction containing T antigen and topoisomerase I was denatured by boiling prior to electrophoresis (lanes 7 and 8), the prominent released strands were seen in a background of less than full-length single strands. These other strands presumably represent DNA nicked by the topoisomerase I present in the reaction and not resealed. However, the strands nicked at these sites were not released during DNA unwinding by T antigen (compare lanes 3 and 7). Importantly, a large proportion of the DNA nicked at the major sites (star in Fig. 2) was released by T antigen during unwinding.

Since topoisomerase I alone nicks the DNA at multiple sites (Fig. 1), we asked whether it does so in the presence of T antigen during an unwinding reaction. To assay for this, we compared the pattern of single-stranded DNA generated by topoisomerase I in the presence of the inhibitor camptothecin and in the absence of T antigen with the one produced under the same conditions but in the presence of T antigen. Camptothecin acts as a topoisomerase I poison by inhibiting the second step in catalysis. It permits strand breakage and the formation of a covalent intermediate between the 3’-phosphate of the nicked strand with a tyrosine residue (Tyr723) in the protein but prevents ligation of the broken strand (38–40). We incubated origin-containing DNA with topoisomerase I and camptothecin in the presence or absence of T antigen under DNA-unwinding conditions, and the DNA was analyzed by electrophoresis on an acrylamide gel after denaturation (Fig. 2, lanes 9–13). In the absence of T antigen (lanes 12–13), multiple nicked strands were detected. When T antigen was present, this same pattern was observed except that it also displayed the strands released by T antigen (compare lanes 10 and 12, for instance). Therefore, during DNA unwinding, topoisomerase I is able to carry out the first step of its reaction at multiple sites on the DNA, but T antigen releases strands nicked only at very discrete and, for the most part, different sites.

**FIG. 4.** Deletion mutant 110–708 has enhanced nicked strand-releasing activity. An unwinding reaction was carried out as described for Fig. 1 using either no T antigen (no T) or molar equivalents of WT T (400 ng) or deletion mutant 110–708 (338 ng) in the presence or absence of topoisomerase I (Topo I), as shown.

**FIG. 5.** Deletion mutants catalyze the release of much larger amounts of nicked single strands—Deletion mutants of T antigen missing sequences from the N-terminal end are still able to support DNA replication in vitro (44, 45). One such deletion mutant missing the first 109 amino acids (designated 110–708) may be lacking part of a topoisomerase I-binding region (residues 83–246) (28). We were interested in determining, therefore, whether this mutant was able to promote the release of nicked single strands. With equal molar amounts of protein, the deletion mutant had slightly more unwinding activity than WT in the absence of topoisomerase I (Fig. 4, compare lanes 11 and 6), but it possessed substantially more nicked strand-releasing activity than WT T antigen (Fig. 4, compare lanes 12–15 with lanes 7–10). Also, whereas WT T antigen’s release of nicked DNA was inhibited at higher concentrations of topoisomerase I (Fig. 4, lanes 7–10, and Figs. 1 and 3), the ability of mutant 110–708 to release nicked strands was not affected at all concentrations tested (Fig. 4, lanes 12–15). Our interpretation of these results is that a region was removed in the deletion mutant that down-regulates nicked strand-releasing activity. Importantly, the pattern of released strands was not altered with the deletion mutant, indicating that the DNA was nicked at the same sites. However, the higher nicked strand-releasing activity of the mutant along with its altered response to different concentrations of topoisomerase I suggest that its interaction with topoisomerase I is different in some important way compared with WT T antigen.

To map the inhibitory region removed in deletion mutant 110–708, we generated several mutants containing smaller deletions from the N-terminal end and tested them for the ability to unwind and release nicked DNA in the presence of topoisomerase I. Fig. 5 demonstrates that deletion mutant 83–708 has a reduced origin-unwinding activity and no ability to release nicked DNA in the presence of topoisomerase I (lanes 3–6). Deletion of an additional 16 amino acids (mutant 99–708) resulted in a protein with similar unwinding and nicked strand-releasing activities as WT (Fig. 5, lanes 7–10). In contrast, deletion mutant 110–708, as shown in Fig. 4, had dramatically (about 10-fold) more nicked DNA-releasing activity (Fig. 5, lanes 12–14). The results indicate that the region from residue 99 to 109 is necessary for this inhibitory activity. This region overlaps with the retinoblastoma-binding domain of T.
Antigen (residues 103–115) (46–48) and contains one site of phosphorylation (a serine at residue 106) (49). The 110–708 portion of the molecule seems to concentrate topoisomerase I's nicking activity to a few specific sites, and another region overlapping with residues 99–109 appears to down-regulate this process.

Mapping of Sites Nicked by Topoisomerase I in the Presence or Absence of T Antigen—In order to map the sites close to the SV40 origin that are nicked by topoisomerase I in the presence or absence of T antigen, we generated four singly end-labeled DNAs (Fig. 6A), representing each strand on either side (early or late) of the origin, and incubated each one with T antigen and topoisomerase I or with topoisomerase I alone. For the purposes of mapping the sites nicked in broken unwound single strands, we used deletion mutant 110–708 because this mutant releases large amounts of nicked strands. We felt that this was appropriate, since the pattern of released strands is qualitatively identical to what is obtained with WT T antigen. Fig. 6B shows a non-denaturing gel analysis of the labeled DNA produced in this reaction from DNA representing the late side of the top strand (lagging late; Fig. 6A) and the early side of the bottom strand (lagging early). In Fig. 6C, unwinding reactions were performed with DNA representing the late side of the bottom strand (leading late) and the early side of the top strand (leading early). In the same experiment, we incubated each of the four labeled DNAs with topoisomerase I in the presence of camptothecin but in the absence of T antigen (Fig. 6, B and C, lanes 6, 7, 13, and 14) in order to map the sites nicked by topoisomerase I alone. This experiment demonstrates that T antigen releases DNA nicked on the late side of the origin (Fig. 6, B and C, lanes 3 and 4) but, for the most part, not on the early side (Fig. 6, B and C, lanes 10 and 11). A second observation was that the majority of displaced shorter strands originated from the top strand as shown in Fig. 6A (Fig. 6C was exposed about 2.5 times longer than Fig. 6B). During DNA replication, the preferred sites correspond to the template for lagging strand synthesis on the late side of the origin.

A third important observation is that, for the most part, topoisomerase I by itself incised the DNAs in the presence of camptothecin at sites completely different from the ones used to release broken strands by T antigen.

In order to map the exact nucleotides where the DNA was nicked in each case, all of these reaction products were separated on a 7.5% acrylamide sequencing gel. At the same time, we ran the products of a deoxy sequencing reaction using each of four kinased oligonucleotide primers representing the labeled end of each molecule. For instance, the DNA representing the late side of the top strand (lagging late) was labeled with $^{32}$P at nucleotide 5171 (see Fig. 6A). The sites nicked in this DNA were determined by carrying out a sequencing reaction using a primer whose 5'-end is at nucleotide 5171 (up 5171; in fact, the same primer was used to generate the DNA fragment by PCR). An example of this type of analysis is shown in Fig. 7. We looked for bands that were present in the lanes containing T antigen and topoisomerase I but that were absent from the reactions with only T antigen (lanes 1–3). The cleavage sites were then determined by comparison with the sequencing ladder (Fig. 7). Similarly, we mapped the sites nicked by topoisomerase I alone by identifying bands that appeared when topoisomerase I was added (Fig. 7, lanes 4–6).

Results of the mapping experiment are shown in Fig. 8. The open arrows represent sites nicked on either strand by topoisomerase I alone, and the closed arrows designate sites nicked in strands unwound by T antigen. Several important features of this map should be pointed out. First, with two exceptions, these sites were completely different. Second, the major cuts in broken released strands were localized to the phosphodiester bonds between residues 121 and 122 and between residues 122 and 123. These sites correspond to the ends of the major nicked single-stranded DNA in Figs. 1–5. The second preferred sites were between nucleotides 77 and 78 and between nucleotides 78 and 79. These same sites were also recognized by topoisomerase I alone, although weakly. These two sets of sites are on the strand corresponding to the lagging strand template on the late side of the origin during DNA replication. Third, nearly all of the nicking occurred on the late side of the origin. The only site nicked on the early side of the origin was between nucleotides 5226 and 5227 (Fig. 8). It is important to recognize the fact that T antigen can unwind all four labeled DNAs in the absence of topoisomerase I (clearly seen in the lighter exposures of Fig. 6, B and C) and that topoisomerase I nicks both sides of the origin when present alone (Fig. 8). We infer from these data that the activity and specificity of topoisomerase I are altered by T antigen during DNA unwinding.

**DISCUSSION**

During DNA replication, SV40 large T antigen is believed to function as a helicase at the replication forks. T antigen initiates this reaction at the origin by first forming a double hexamer bound to the four GAGGC pentanucleotides that constitute binding site II (15) and then structurally distorting and partially melting the DNA (3–7). One consequence of the unwinding of long or circular DNA is that the DNA becomes topologically overwound. This torsional strain must be relieved by the action of a topoisomerase. Relatively little is known about how the topoisomerase reaction is coupled to unwinding at the replication forks, but intuitively, it seems that a topoisomerase must be able to nick and religate a strand before that stretch of DNA reaches the helicase at the replication fork. In this report, we demonstrated that when T antigen unwinds an origin-containing fragment of SV40 DNA in the presence of topoisomerase I, the DNA fragment is nicked at discrete sites and specific nicked single strands are released during the unwinding reaction. We have previously demonstrated that T antigen and topoisomerase I bind to one another in vitro and our interpretation of these new results is that the release of nicked forms of the DNA represents a coupled reaction of unwinding and nicking by a T antigen-topoisomerase I complex. We observed that the nicking reaction was most efficient when the molar ratio between T antigen and topoisomerase I was about 15:1. This would roughly correspond to one molecule of topoisomerase I per double hexamer of T antigen. At higher concentrations of topoisomerase I, nicking and unwinding were
inhibited (see Fig. 1, for instance). This indicates that excess topoisomerase I interferes with the activity of the double hexamer. Thus, a functional complex of a double hexamer of T antigen and a single molecule of topoisomerase I might efficiently unwind DNA at the replication forks and at the same time relax the torsional strain resulting from the unwinding reaction.

We demonstrated that topoisomerase I was carrying out the actual nicking of the DNA released by T antigen by showing that a catalytically inactive mutant form of topoisomerase I was totally incapable of participating in the release of broken strands during unwinding. Interestingly, the mutant protein was still able to inhibit unwinding to full-length single strands, indicating that the mutant interacts with T antigen in a way very similar to WT topoisomerase I.

The identification of the sites in SV40 DNA that are nicked in the coupled reaction revealed that the large majority of these sites were located on the late side of the origin. This took place although topoisomerase I by itself can nick the DNA on the other side of the origin as well. If T antigen forms a double hexamer at the origin and unwinds the DNA bidirectionally from that region, it seems likely that the bound topoisomerase I molecule is attached to the hexamer facing the late side of the origin. Fig. 9 illustrates this model of topoisomerase I-linked unwinding by T antigen. It is intriguing that, for the most part, the sites nicked by topoisomerase I in strands released by T antigen are different from those nicked by topoisomerase I alone in the presence (Fig. 2 and Fig. 8) or absence (Fig. 1) of the inhibitor camptothecin. Since topoisomerase I is apparently free to nick the DNA at its usual sites in the presence of T antigen (Fig. 2), this tells us that T antigen directs topoisomerase to inhibited sites.
T Antigen Modifies Nicking by Topoisomerase I

Model of nicking and unwinding

![Diagram](50x547 to 294x729)

In summary, the results described in this paper suggest that SV40 T antigen and topoisomerase I form a functional complex that is used to simultaneously unwind and relax torsionally strained DNA at replication forks (Fig. 9).

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