Simian Virus 40 Large T Antigen Binds to Topoisomerase I

ARTICLE *in* VIROLOGY · SEPTEMBER 1996

Impact Factor: 3.32 · DOI: 10.1006/viro.1996.0433 · Source: PubMed

VIROLOGY 222, 365–374 (1996)
ARTICLE NO. 0433

Simian Virus 40 Large T Antigen Binds to Topoisomerase I

DANIEL T. SIMMONS,*[,1] THOMAS MELENDY,†[,2] DAVID USHER,* and BRUCE STILLMAN†

*Department of Biological Sciences, University of Delaware, Newark, Delaware 19716-2590; and
†Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724-2206

Received April 22, 1996; accepted June 6, 1996

Binding of simian virus 40 (SV40) large T antigen to human and calf thymus topoisomerase I (topo I) was readily detected by using modified enzyme-linked immunosorbent assays and immunoblots. In addition to WT T antigen, binding could also be readily demonstrated with T antigen fragments from the amino-terminal region as well as with fragments missing this region, but much less so with small t antigen or with human p53. Antibody-blocking experiments showed that a monoclonal antibody that binds to the N-terminal region and several antibodies that recognize the central region of T antigen interfere with the binding to topo I. Our data are consistent with the existence of two separate topo I-binding regions in T antigen, one mapping within residues 82 to 246 and an apparently weaker one present after residue 246. By comparing the binding of T antigen to topo I with that of T antigen to DNA polymerase α or RPA, a single-stranded DNA-binding protein, it was determined that the T antigen–topo I interaction is much stronger and that the binding sites for topo I and DNA polymerase overlap, whereas the one for RPA differs. Several unwinding-defective mutants of T antigen were partially defective in their binding to topo I, suggesting that the binding to topo I is required for unwinding circular DNA. Finally, immunoprecipitation experiments demonstrated that T antigen can interact with DNA-bound topo I, indicating that such an interaction may take place during SV40 DNA replication.

INTRODUCTION

The simian virus 40 (SV40) large tumor (T) antigen is required for the initiation of SV40 DNA replication in infected monkey cells (Tegtmeyer, 1972). Various T antigen activities are required for this reaction to proceed. First, T antigen binds to the pentanucleotide sequences at the SV40 origin (Myers and Tjian, 1980; Shortle and Nathans, 1979a,b). In the presence of ATP, T antigen forms a double hexamer (Mastrangelo *et al.,* 1989) that covers a 64-bp region known as the core origin of DNA replication (Deb *et al.,* 1987). The protein then induces a structural change in the DNA. The imperfect palindrome on one (early) side of the pentanucleotides is partially melted (Borowiec and Hurwitz, 1988; Borowiec *et al.,* 1991; Roberts, 1989) and the AT-rich tract on the other (late) side is untwisted (Borowiec and Hurwitz, 1988; Dean and Hurwitz, 1991). At this point, it is believed that cellular proteins are recruited to the origin to help unwind the DNA and to begin the replication process. RPA, a single-stranded DNA-binding protein, and DNA polymerase α-primase are attracted to the origin, forming a primosome (Collins and Kelly, 1991; Melendy and Stillman, 1993). T antigen binds to DNA polymerase α directly and this association appears to be required for the initiation of DNA replication (Dornreiter *et al.,* 1992, 1993; Collins *et al.,* 1993). Likewise, the interactions between RPA and T antigen (Dornreiter *et al.,* 1992; Melendy and Stillman, 1993) and RPA and the p48 subunit of DNA polymerase α-primase complex (Dornreiter *et al.,* 1992; Nasheuer *et al.,* 1992) may be important for the stimulation of SV40 DNA unwinding and subsequent replication (Kenny *et al.,* 1990; Stillman *et al.,* 1992). Perhaps using its intrinsic helicase activity (Wessel *et al.,* 1992), T antigen then unwinds the origin in association with these cellular proteins (Goetz *et al.,* 1988; Wold *et al.,* 1987; Wiekowski *et al.,* 1988). The primase initiates replication by synthesizing short RNA primers and DNA pol α extends the primers with DNA (Bullock *et al.,* 1991; Denis and Bullock, 1993; Waga and Stillman, 1994; Kamakaka *et al.,* 1994). There is a polymerase switching event where DNA polymerase δ takes over to synthesize the body of the new DNA (Tsurimoto and Stillman, 1991a; Tsurimoto *et al.,* 1990; Waga and Stillman, 1994).

Subsequent steps in viral DNA replication are less well understood. In addition to RPA, DNA pol α-primase, and DNA polymerase δ, other cellular proteins including PCNA, RFC, DNA helicases, DNA topoisomerases (Tsurimoto and Stillman, 1991b; Ishimi *et al.,* 1991; Fien and Stillman, 1992), and possibly DNA polymerase ϵ (Melendy and Stillman, 1991; Zahradka, 1992) are required for efficient DNA replication. Many of these proteins, including DNA ligase I are found together in a functional complex that can support SV40 DNA replication *in vitro* (Malkas *et al.,* 1990; Li *et al.,* 1993; Wu *et al.,* 1994).

[1] To whom correspondence and reprint requests should be addressed. Fax: (302) 831-2281; E-mail: dsimmons@brahms.udel.edu.
[2] Present address: Department of Microbiology, School of Medicine, SUNY at Buffalo, Buffalo, NY 14214.

0042-6822/96 $18.00

T antigen's role in the elongation reaction is not clear, although it may be present at replication forks functioning as a helicase (Scheffner *et al.,* 1989). It has also been suggested that complexes of T antigen and DNA polymerase α-primase are required for lagging strand synthesis throughout the genome (Melendy and Stillman, 1991; Collins and Kelly, 1991; Denis and Bullock, 1993).

In this communication, we report that T antigen interacts specifically with bovine and human topoisomerase I. We provide evidence that two separate regions of T antigen have the ability to bind to topo I and show that the binding of these two proteins can take place on DNA. Furthermore, we report that mutants unable to unwind circular DNA are partially defective in their association with topo I, suggesting that this interaction may be important for the proper unwinding of viral DNA at replication forks.

MATERIALS AND METHODS

Recombinant baculoviruses

A recombinant baculovirus expressing wild-type T antigen was obtained from R. E. Lanford (1988). Baculoviruses expressing T antigen mutants with single amino acid substitutions have been previously described (Simmons *et al.,* 1993; Wun-Kim *et al.,* 1993). Recombinants expressing truncated T antigen proteins were generated by first amplifying the appropriate region of SV40 T antigen cDNA by PCR. The amplified DNA was cloned into p941 or p1393 (Stratagene) and checked for proper orientation. The resulting plasmid was transfected into insect Sf9 cells along with linearized BaculoGold DNA (Stratagene) according to the manufacturer's directions. After 5–7 days, virus in the medium was subjected to a plaque assay; individual plaques were picked and screened for T antigen expression by immunofluorescence. A recombinant expressing wild-type human topoisomerase I was generated by Stewart *et al.* (1996) by cloning the cDNA (D'Arpa *et al.,* 1988) into pBluebac transfer vector from Invitrogen Corporation. The resulting plasmid was transfected into Sf9 cells along with linearized baculovirus DNA and, after several days, the medium was subjected to a plaque assay in the presence of X-gal (5-bromo-4-chloro-3-indoyl-D-galactoside). Blue plaques were selected and recombinants were subjected to several rounds of plaque purification until occlusion body-positive cells were no longer visible. Expression of full-length topo I was determined by SDS gel electrophoresis followed by immunoblotting.

Purification of T antigen

Wild-type and mutant T antigens were purified by immunoaffinity chromatography using appropriate monoclonal antibodies according to the procedure described by Simanis and Lane (1985). Wild-type T antigen and all single point mutants were purified using antibody PAb419 (Harlow *et al.,* 1981) or PAb101 (Gurney *et al.,* 1980). Deletion mutants 1-627 and 1-246 were purified using PAb419, whereas 131-708 and 246-708 were purified using PAb101. In all cases, the protein was eluted from immunoaffinity beads with triethylamine buffer at a pH of 10.6, quickly neutralized with 0.5 *M* PIPES, pH 6.7 (Simanis and Lane, 1985), and dialyzed against a solution containing 0.01 *M* Tris (pH 8.0), 0.1 *M* NaCl, 0.001 *M* EDTA, 0.001 *M* dithiothreitol, and 50% glycerol and stored at −20°. Protein concentrations were estimated by $AgNO_3$ staining of acrylamide gels using phosphorylase B as a standard.

Purification of calf thymus and human topoisomerase I

Calf thymus topoisomerase I was purified from whole calf thymuses according to Melendy and Stillman (1993). Briefly, crude nuclei were extracted with high salt in the presence of 0.01% Nonidet-P40. The topo I was then purified by ammonium sulfate fractionation, followed by chromatography on Affigel Blue (Bio-Rad), hydroxylapatite, and finally phosphocellulose columns. The protein was dialyzed in 0.02 *M* Tris–HCl, pH 7.5, 0.0001 *M* EDTA, 50% glycerol, 0.01% Nonidet-P40, 0.0001 *M* phenylmethylsulfonyl fluoride, 0.001 *M* dithiothreitol, and 0.025 *M* NaCl and stored at −20°.

Human topoisomerase I was a kind gift from Lance Stewart and James Champoux (University of Washington, Seattle). It was purified from Sf9 cells infected with recombinant topo I-expressing baculovirus as described (Stewart *et al.,* 1996). Briefly, isolated cell nuclei were lysed in 1 *M* NaCl and the solution was slowly adjusted to a polyethylene glycol 8000 concentration of 6%. After centrifugation, the proteins remaining in solution were dialyzed against a KCl solution and subjected to chromatography on phosphocellulose (Whatmann P-11). The topo I eluate was then sequentially purified on phenyl Sepharose, Mono S, and Mono Q (all from Pharmacia) columns, in that order. The Mono Q flowthrough, which contained the topo I, was dialyzed against 0.01 *M* Tris (pH 7.5), 0.001 *M* EDTA, 0.001 *M* dithiothreitol, and 50% glycerol and stored at −20°. Protein concentrations were determined with Bio-Rad protein assay reagents using BSA as a standard.

Purification of RPA and DNA polymerase

RPA was purified according to the method described by Brill and Stillman (1989). Fraction I (Prelich *et al.,* 1987) was used as the source of RPA in this work. DNA polymerase α/primase complex was purified according to Murakami *et al.* (1986) as modified by Tsurimoto and Stillman (1989). These proteins were at least 90% pure as determined by silver staining of acrylamide gels.

Purification of small t antigen

SV40 small t antigen was purified from expressing *Escherichia coli* cells as described (Goswanmi *et al.,* 1992). The small t antigen was a kind gift from Dr. Kathleen Rundell.

Purification of p53

Human p53 was purified by immunoaffinity chromatography as previously described (Oberosler *et al.,* 1993), using antibody PAb421 (Harlow *et al.,* 1981).

Purification of monoclonal antibodies

About 200 ml of hybridoma supernatant was passed over a 1.5-ml column of protein A agarose (Pharmacia) overnight at 4° with recirculation. The beads were washed with PBS until the A_{280} went to 0. IgG_{2a} was eluted with 0.1 *M* citric acid, pH 4.5. IgG_{2b} was eluted with the same buffer at pH 3.0. The eluates were collected in tubes that contained 1/20 volume of 2 *M* Tris-base (pH was not adjusted) to neutralize the samples. The IgG peak was determined by absorbance at 280 nm and fractions were pooled and dialyzed for several days versus PBS at 4°. Protein concentrations were determined by absorbance at 280 nm (1.5 OD is 1 mg/ml).

Biotinylation of IgG

One milligram of purified IgG in 1.79 ml of PBS was mixed with 358 μl of 1 *M* $Na_2CO_3/NaHCO_3$ buffer, pH 9.5. With continuous mixing, 117 μl of biotin X-NHS (Sigma) (at 5 mg/ml in dimethylformamide) was slowly added and the solution incubated overnight at 4° with constant agitation. The biotinylated IgG was dialyzed against six changes of Tris-buffered saline at 4°.

Antiserum

Immunoaffinity-purified polyclonal anti-topo I IgG was a king gift from Lance Stewart and James Champoux. It was raised in a rabbit that had been injected with purified recombinant topo I. The antibody was purified on Affi-10 agarose beads (Bio-Rad) to which topo I had been conjugated. The affinity beads were washed extensively with 0.1 *M* Tris, pH 8.0, followed by 0.01 *M* Tris, pH 8.0, and the IgG was eluted with 0.1 *M* glycine, pH 3.0. The eluate was neutralized with the addition of one-tenth volume of 1 *M* Tris, pH 8.0, and dialyzed against 50% glycerol in phosphate-buffered saline and then stored at −20°.

Enzyme-Linked Immunosorbent Assays (ELISAs)

All steps were carried out at room temperature with constant shaking on a rotary shaker. The general strategy was to allow one protein to bind to the surface of microtiter wells, incubate it with a second protein, and then measure the binding of the second protein by reactivity with a specific antibody. Purified proteins to be bound to microtiter wells were diluted directly from stock solutions containing 10–50% glycerol into 50 μl of filtered PBS (important: diluted stock solutions were not made first). After 2 hr, wells were washed with PBS and then blocked with 300 μl of PBS containing 1% gelatin for 1 hr. Wells were washed again with PBS and 50 μl of a solution containing various amounts of the second protein diluted in buffer 1 (PBS containing 0.05% Tween 20 and 0.1% BSA) was added and incubation was continued for 2 hr. After washing with PBS containing 0.05% Tween 20 (PBS–Tween), 100 μl of a biotin-conjugated antibody to the second protein (300 ng in buffer 1) was added for 2 hr. The wells were washed with PBS–Tween, and 50 μl of streptavidin-conjugated horseradish peroxidase (Sigma), diluted 1:5000 in PBS containing 3% BSA, was added for 1 hr. After another wash, 100 μl of a substrate solution [0.4 mg/ml OPD (ortho-phenylenediamine, Sigma) in 0.05 *M* citric acid, 0.1 *M* Na_2HPO_4, pH 5.3, containing 1 μl of H_2O_2/mg of OPD] was added and the reaction was allowed to proceed for 10 to 20 min. In some cases, optical density was immediately read at 450 nm in a Dynatech plate reader, but in most cases, absorbance was measured at 490 nm after the reaction was stopped by the addition of 50 μl of 2.5 *M* H_2SO_4.

In some experiments, T antigen, as the second protein, was first incubated for 2 hr with various amounts of purified monoclonal antibodies or topo I in a solution of buffer 1, before being transferred to microtiter wells containing bound topo I. In other experiments, T antigen was adsorbed to the wells first and various amounts of topo I (in buffer 1) were added. Binding of topo I to T antigen was measured by incubation with immunoaffinity-purified rabbit anti-topo I IgG (4 ng in 100 μl of buffer 1) followed with goat anti-rabbit IgG conjugated with horseradish peroxidase (Sigma). Enzyme reactions were carried out as described above.

Immunoblotting

One microgram of purified topo I was subjected to electrophoresis through a 10% acrylamide gel and electrophoretically transferred to a nitrocellulose membrane (Bio-Rad). Purified T antigen or T antigen fragments (1 μg/ml) were incubated with membrane strips and their binding was detected by incubation with a biotinylated anti-T antibody (6 μg/ml) followed with streptavidin-conjugated horseradish peroxidase. All steps followed the conditions recommended by the manufacturer of the ECL Western detection kit (Amersham).

Binding of T antigen to topo I on DNA

Purified topo I (200 ng) was incubated with 3 ng of a ^{32}P-end-labeled 160-bp *Hin*dIII to *Pvu*II fragment of pSK(−) (Stratagene) in the presence of 50 μl SV40 DNA replication buffer (Simmons *et al.,* 1993). After 30 min at

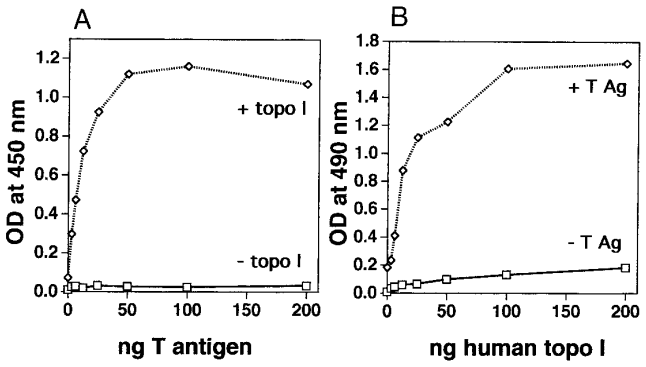

FIG. 1. ELISA of the binding of WT T antigen and human topoisomerase I. (A) Binding of T antigen to human topo I. 150 ng of purified human topo I was adsorbed to the wells of a microtiter plate. After washing and blocking the wells, increasing amounts of WT T antigen were added. Complexes were detected with the use of a biotinylated anti-T monoclonal antibody (PAb419), a streptavidin-conjugated horseradish peroxidase, and OPD peroxidase substrate. (◇) Plate coated with topo I, (□) plate not coated. (B) Binding of topo I to T Ag. 200 ng of WT T antigen was bound to microtiter wells and increasing amounts of human topo I were added. Complexes were detected with a rabbit anti-topo IgG followed with goat anti-rabbit IgG conjugated with horseradish peroxidase. (◇) Plate coated with T antigen, (□) plate not coated. Absorbance was detected with a Dynatech plate reader.

37°, 3 μg of unlabeled calf thymus DNA was added, followed by various amounts of T antigen. After 30 min at 37°, PAb419 was added for 1 hr at room temperature, followed by a suspension of *Staphylococcus aureus.* After 30 min at 0°, the bacteria were washed with DNA wash buffer (Simmons *et al.,* 1993) and the radioactivity in each tube was determined by scintillation counting.

RESULTS

Binding of large T antigen and topo I

To determine if SV40 large T antigen and topoisomerase I have the ability to interact with one another, we set up an ELISA with purified human topo I and large T antigen isolated from insect cells infected with recombinant baculoviruses. Both of these protein preparations were judged to be at least 90% pure by silver staining of acrylamide gels (not shown). A fixed amount of topo I (Fig. 1A) or T antigen (Fig. 1B) was first adsorbed on the surface of a microtiter plate and incubated with increasing amounts (0 to 200 ng) of T antigen or topo I, respectively. When topo I was first attached to the wells, complexes were detected by incubation with a biotinylated anti-T monoclonal antibody (PAb419 or PAb101), followed with streptavidin-conjugated horseradish peroxidase and finally with peroxidase substrate. When T antigen was first bound to the wells, complex formation was detected with rabbit anti-topo I IgG followed with peroxidase-conjugated mouse anti-rabbit IgG and then substrate. The association of these two proteins can readily be detected even at very small (2–5 ng) levels of T antigen (Fig. 1A) or topo I (Fig. 1B) and binding is saturated at about 50 to 100 ng of the second protein. The slope of the curve shown in Fig. 1A, which measures the association of T antigen to bound topo I, was similar to what we observe for antibody–antigen binding (not shown). Similar results were obtained with purified calf thymus topo I (Fig. 2A), and no apparent differences in binding were noticed between human and calf thymus topo I. Control reactions with all components except for topo I (−topo I curve in Fig. 1A) or without T antigen (0 point of +topo I curve in Fig. 1A) showed that the background is low in this system. In all subsequent graphs, the background is subtracted in order to show only the binding signal.

Binding of large T antigen fragments to topo I

In order to map the site on T antigen responsible for binding to topo I, we generated various deletion mutants of large T antigen and cloned them into recombinant baculoviruses. A fragment missing the C-terminal 81 amino acids (1–627) bound calf thymus topo I at close to WT levels (Fig. 2A). A much smaller fragment consisting only of the N-terminal 246 amino acids also bound to topo I normally (Fig. 2B). Interestingly, SV40 small t antigen, which shares the first 82 amino acids with large T antigen, did not bind well, except at high concentrations (Fig. 2B). However, a low level of functionally important interaction between small t antigen and topoisomerase I cannot be excluded. An unrelated protein (human p53) also did not bind (Fig. 2B).

To test the effect of N-terminal deletions on the ability of T antigen to bind to topo I, we constructed mutants missing the first 130 or 245 amino acids. Both of these proteins bound well (Fig. 2C), although the binding of the 246-708 fragment was reduced slightly. These data point to the existence of two binding regions, one located in the first 246 amino acids, the other located in the remainder of the molecule.

Blocking T antigen:topo I binding with monoclonal antibodies

In order to obtain additional mapping information about the T antigen sites that bind to topo I, various monoclonal antibodies were used as specific inhibitors of the binding reaction. Topo I was first adsorbed onto the surface of microtiter plates and then reacted with WT T antigen that had been previously incubated with increasing amounts of various purified monoclonal antibodies. The binding epitopes of these antibodies and their effects on the T:topo I binding reaction are shown in Table 1. An antibody (PAb419) that reacts with an epitope within the first 82 amino acids of large T antigen does not inhibit the binding reaction (Fig. 3A), in agreement with the observation that small t antigen does not bind well. Two antibodies that recognize different determinants (Table 1) in the central region of the molecule (PAb114 and PAb204) also do not inhibit (Fig. 3A). However, an antibody (PAb416) that binds to residues 82 to 115 and several other antibodies (PAb413, PAb402, PAb420, and PAb205) that react with a segment between

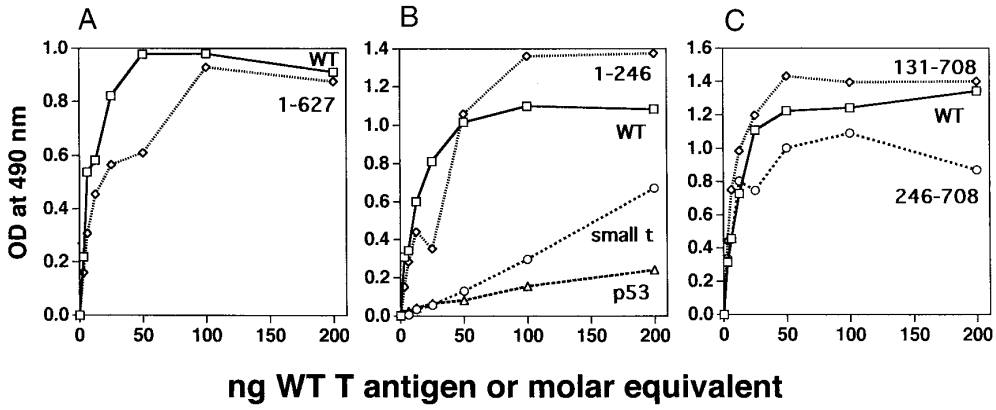

FIG. 2. Binding of WT and deletion mutants (A) 1-627, (B) 1-246, and (C) 131-708 and 246-708 of large T antigen to topo I. 100 ng of purified calf thymus topo I (A) or 200 ng of human topo I (B and C) was adsorbed to microtiter wells and reacted with 0 to 200 ng of WT T antigen (□) or the molar equivalent of fragment 1-627 (◇ in A), fragment 1-246 (◇ in B), small t antigen (○ in B), human p53 (△ in B), fragment 131-708 (◇ in C), or fragment 246-708 (○ in C). Complexes were detected with biotinylated PAb419 (WT, 1-627, 1-246, and small t antigen in A and B), PAb421 (p53 in B), or PAb101 (C) as described in Fig. 1. Background due to the nonspecific binding of the proteins to the wells was subtracted in this and all subsequent graphs.

residues 333 and 451 inhibit the binding reaction to various extents (Fig. 3B). These data are therefore consistent with the existence of two separate regions on large T antigen that can independently bind to topo I. One region is located in the NH_2-terminal 246 amino acids, but probably excludes the first 82 amino acids; the second lies somewhere between residues 246 and 708 and probably includes a sequence within residues 333–451.

Immunoblotting

As another demonstration of the binding between T antigen and topo I, we performed an immunoblotting experiment. Topo I was subjected to electrophoresis on an acrylamide gel and blotted onto nitrocellulose sheets. Strips were incubated with WT T antigen or various T antigen fragments (Fig. 4) and bound T antigen was detected by incubation with biotinylated PAb101 or PAb419. As observed in the ELISA reactions, both an N-terminal fragment containing the first 246 amino acids (Fig. 4, lane 3) and C-terminal fragments consisting of residues 131–708 or 246–708 (Fig. 4, lanes 6 and 7) clearly bound to topo I. As detected in ELISAs (Fig. 2C), the binding of 246-708 (Fig. 4, lane 7) was somewhat reduced compared to WT (Fig. 4, lane 5). Control reactions with human p53 and SV40 small t antigen showed very little if any activity (not shown).

TABLE 1

Binding Epitopes of Anti-T Monoclonal Antibodies

Antibody	Binding epitope	Reference	Inhibit topo binding?
PAb419	1–82	(Harlow *et al.,* 1981)	–
PAb416	83–115	(Harlow *et al.,* 1981)	+
PAb114	269–522	(Gurney *et al.,* 1986)	–
PAb402	333–451	(Harlow *et al.,* 1981; Lane and Gannon, 1986)	+
PAb413	333–451	(Harlow *et al.,* 1981; Lane and Gannon, 1986)	+
PAb420	333–451	(Harlow *et al.,* 1981; Lane and Gannon, 1986)	+
PAb205	333–451	(Mole and Lane, 1985)	+
PAb204	448–509	(Mole and Lane, 1985)	–

Comparison of the binding of T antigen to topo I with that of T antigen to DNA polymerase α and RPA

Several reports have demonstrated an interaction between SV40 large T antigen and DNA polymerase α

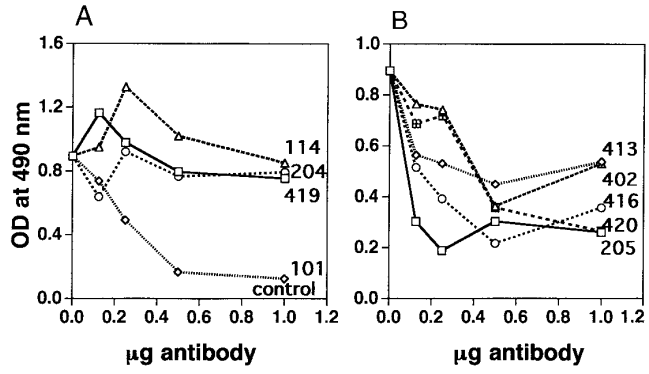

FIG. 3. Effects of various monoclonal antibodies on the binding of WT T antigen to topo I. 100 ng of calf thymus topo I was adsorbed to microtiter wells. To each well was added 100 ng of WT T antigen that had been preincubated with 0 to 1 μg of various purified monoclonal antibodies to T antigen (in A, PAb114 △, PAb204 ○, PAb419 □, and in B, PAb413 ◇, PAb402 △, PAb416 ○, PAb420 ⊞, PAb205 □). Complexes were detected with biotinylated PAb101. The effects of blocking (B) and nonblocking (A) antibodies are shown. As a positive control, PAb101 (◇ in A) was included to show that this antibody prevents the detection of complexes with biotinylated PAb101.

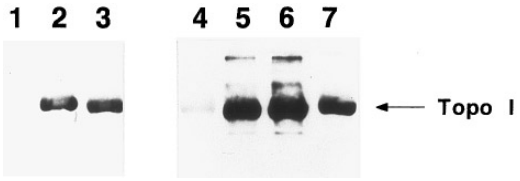

FIG. 4. Immunoblotting. Human topo I was applied to a denaturing acrylamide gel, transferred to a nitrocellulose membrane, and incubated with buffer alone (lanes 1 and 4), WT T antigen (lanes 2 and 5), 1-246 (lane 3), 131-708 (lane 6), or 246-708 (lane 7). Binding was detected with biotinylated PAb419 (lanes 1–3) or PAb101 (lanes 4–7) followed with streptavidin-conjugated horseradish peroxidase and ECL (Amersham) substrate. The membrane was exposed to X-ray film for 15 sec.

(Smale and Tjian, 1986; Gough *et al.,* 1988; Dornreiter *et al.,* 1992; Collins *et al.,* 1993) and T antigen and RPA (Dornreiter *et al.,* 1992; Collins *et al.,* 1993; Melendy and Stillman, 1993). It was of interest to determine the relationship between the interaction of T antigen to topo I and those between T antigen and the other two cellular proteins. ELISAs were performed to compare the binding of T antigen to adsorbed topo I with that of T antigen to adsorbed DNA polymerase (Fig. 5A) or RPA (Fig. 5B). At equal molar amounts of these various highly purified cellular proteins attached to the plates, the binding of T antigen to topo I appeared to be much stronger than its binding to DNA polymerase or RPA. To determine if the binding sites for these proteins on T antigen were distinct, or if they overlapped, a competition experiment was performed (Fig. 5C). Adsorbed RPA or DNA polymerase was reacted with large T antigen that had been previously incubated with increasing amounts of purified topo I. Topo I was a very effective competitor of the binding of T antigen to DNA polymerase (Fig. 5C) but a rather poor competitor of T antigen binding to RPA. This suggests that the binding sites on T antigen for topo I and DNA polymerase are identical or that they overlap, whereas the binding site for RPA is mostly different.

Binding of T antigen mutants to topo I

We have previously generated a large collection of SV40 T antigen mutants with single-point substitution mutations in the DNA-binding domain (Simmons *et al.,* 1990a,b). Many of these mutants are unable to support virus replication in monkey cells. The replication-defective mutants fall into six distinct classes based on their ability to bind the SV40 origin of replication, to bind nonspecifically to double-stranded DNA, to oligomerize into hexamers in the presence of ATP, to unwind origin-containing DNA, and to support virus DNA replication *in vitro* and *in vivo* (Wun-Kim *et al.,* 1993; Simmons *et al.,* 1993). The mutants belonging to class 1 fail to bind specifically to the origin, but retain the ability to bind nonspecifically to DNA (Simmons *et al.,* 1993). These mutants are normal in binding to topo I (not shown). The mutants in class 4 (e.g., 213QH, 215LV, and 216CS) retain the ability to oligomerize, to bind origin and non-origin DNA, and to unwind an origin-containing DNA fragment, but are unable to unwind an origin-containing circular DNA plasmid in the presence of RPA and topoisomerase I (form U unwinding assay) (Wun-Kim *et al.,* 1993). Since T antigen binds to RPA (Dornreiter *et al.,* 1992; Melendy and Stillman, 1993) and is now known to bind to topoisomerase I (this report), one explanation for their inability to carry out the form U unwinding assay is a failure to properly bind to one of these cellular proteins. The binding of these mutants to RPA was tested and found to be normal (data not shown). However, a defect was detected in the binding to topo I (Fig. 6). At saturation, the topo I bound significantly less mutant T antigens than WT. This experiment suggests that the interaction between these two proteins is needed for unwinding circular DNA and that these mutants are defective in this activity.

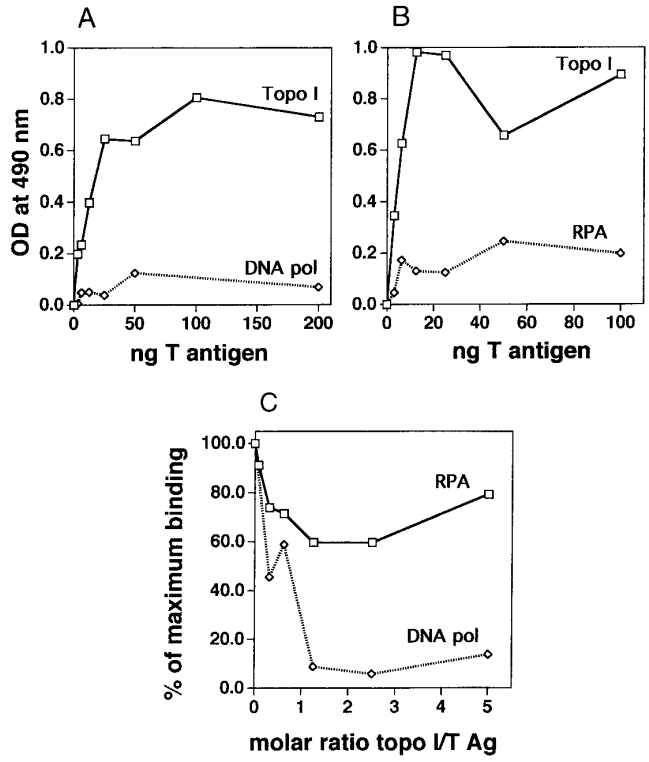

FIG. 5. Comparative binding of WT T antigen to (A) topo I and DNA pol α and (B) topo I and human RPA. (A and B) Microtiter wells received either 100 ng of calf thymus topo I (□) or the molar equivalent of purified DNA polymerase α/primase complex (358 ng, ◇ in A) or human RPA (116 ng, ◇ in B). Various amounts of WT T antigen were added, as shown, and complexes were detected with biotinylated PAb419 (A) or PAb101 (B). (C) Competition of T Ag binding to RPA and DNA pol α by topo I. 1 μg of either RPA (□) or DNA polymerase α/primase complex (◇) was adsorbed to microtiter wells. Each well was then incubated with 100 ng of WT T antigen preincubated with 0- to 5-molar excess of calf thymus topo I. T antigen:RPA or T antigen:DNA polymerase complexes were detected with biotinylated PAb101.

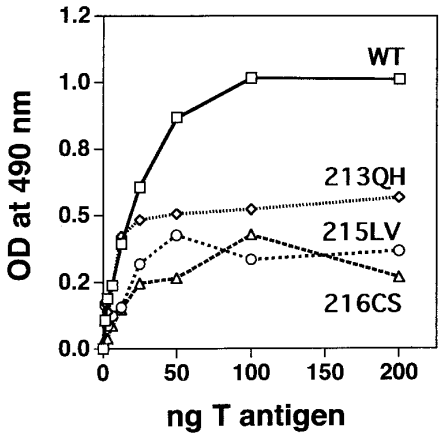

FIG. 6. Binding of substitution mutants of T antigen to topo I. 100 ng of human topo I was bound to microtiter wells. Various amounts, as shown, of purified WT or mutant T antigens were added. Complexes were detected with biotinylated PAb419. WT (□) or class 4 mutants (213QH, ◇; 215LV, ○; 216CS, △).

Interaction of T antigen with topo I bound to DNA

If T antigen and topo I interact with each other at replication forks, they should be able to bind together when one of them is bound to DNA. To test this, purified topo I was first incubated with a labeled plasmid DNA fragment under the buffer conditions used for SV40 DNA replication (Simmons *et al.,* 1993). A 1000-fold mass excess of unlabeled calf thymus DNA was then added, followed by a second incubation in the presence of increasing amounts of WT T antigen. Complexes containing labeled DNA were then immunoprecipitated with an anti-T antibody, and the amount of labeled DNA precipitated was determined by scintillation counting (Fig. 7). Plasmid DNA was used in this experiment, instead of origin-containing DNA, to minimize the binding of T antigen to the labeled DNA. This binding was further inhibited by adding unlabeled calf thymus DNA as a competitor in the second incubation. In the absence of topo I, T antigen did not bind appreciable amounts of labeled DNA in the presence of the competitor DNA (Fig. 7), indicating that, under these conditions, there is very little direct binding of T antigen to the labeled plasmid DNA fragment. When topo I was first bound to DNA, much larger amounts of labeled DNA were precipitated after WT T antigen was added. This amount corresponded to approximately 75% of the total amount of labeled DNA precipitable with anti-topo I antibodies, demonstrating that most topo I–DNA complexes were also associated with T antigen. The precipitation of the labeled DNA in the presence of topo I and T antigen appears, therefore, to be due to a direct interaction between these two proteins. The possibility that topo I stimulates T antigen binding to DNA was eliminated by experiments (not shown) where T antigen and topo I were added simultaneously to labeled DNA (no stimulation was observed). These experiments have also been duplicated by using a gel-shift assay to detect DNA–protein complexes (C. Wu and D. T. Simmons, unpublished). Our data, therefore, demonstrate that T antigen and topo I can interact on DNA.

DISCUSSION

The binding between SV40 large T antigen and human or calf thymus topo I appears to be very strong since it was easily detected with small amounts of protein in ELISAs and immunoblot assays. Binding was normal or nearly normal with various N-terminal and C-terminal deletion mutants of large T antigen but occurred much less readily with small t antigen or with p53. These data lead us to conclude that topo I binds to two discrete regions of large T antigen. Antibody blocking experiments supported this conclusion because an N-terminal monoclonal antibody (PAb416) as well as several antibodies that recognize a central region (333–451) of T antigen interfered with the formation of WT T–topo I complexes (Table 1). This second region appears to be a weaker binding site for topo I based on ELISA (Fig. 2C) and immunoblotting (Fig. 4) results with fragment 246-708.

By comparing the binding of T antigen to topo I with the binding to RPA and DNA polymerase α, it was determined that the binding to topo I is significantly stronger. Competition experiments with RPA and DNA polymerase allowed us to deduce that the binding sites on T antigen for topo I and DNA polymerase overlap or interfere with one another, whereas the binding site for RPA is different. This suggests that any one molecule of T antigen might be able to interact simultaneously with RPA and topo I or RPA and DNA polymerase but not with topo I and DNA polymerase.

The observation that two different regions of T antigen

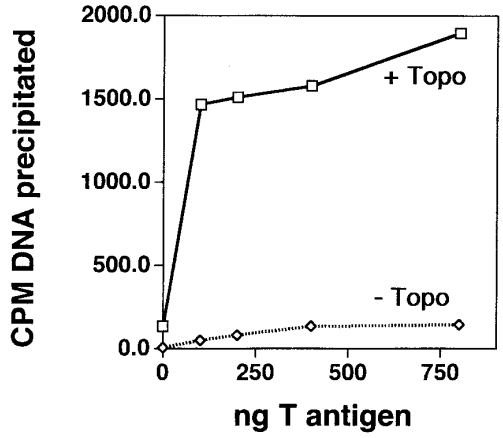

FIG. 7. T:topo I binding on DNA. Human topo I was incubated with an end-labeled 160-bp plasmid DNA fragment under SV40 DNA replication conditions. 1000-fold excess of unlabeled calf thymus DNA was added to block the binding of T antigen to labeled DNA. WT T antigen (□) was added in various amounts as shown, followed by PAb419 and *S. aureus.* The labeled DNA in the bacterial precipitate was quantitated by scintillation counting. Control reactions without topo I are shown (◇).

have similar activities is apparently not limited to topo binding. The interaction of T antigen to DNA polymerase α may be very similar to the situation with topo I, in that both an NH_2-terminal fragment of T antigen (Dornreiter *et al.,* 1990, 1992) and a more central region of the protein (Gannon and Lane, 1987) bind independently. Second, both the amino-terminal 147 amino acids of T antigen (Sompayrac and Danna, 1991; Cavender *et al.,* 1995) and a fragment containing the remainder of the molecule (Cavender *et al.,* 1995) can independently immortalize cells in the presence of activated ras oncogene. Third, although the transactivation domain of T antigen has been mapped to the NH_2-terminal end (Srinivasan *et al.,* 1989; Zhu *et al.,* 1991), deletion mutants lacking that region still exhibit the ability to activate transcription (Gruda *et al.,* 1993). There seems to be, therefore, a duplication of activities in T antigen. One group of functions is localized to the amino-terminal region, another is present in the middle of the molecule. It will be vital to the understanding of the biochemistry of this complex protein to determine whether both or only one activity is functional in the intact protein.

There is some evidence that both T antigen (Scheffner *et al.,* 1989) and topo I (Avemann *et al.,* 1988) are present at replication forks. Since T antigen can bind strongly to topo I, one obvious possibility is that this association promotes the proper unwinding and relaxation of the DNA at replication forks. We showed, in support of this model, that an interaction between the two proteins can take place on DNA (Fig. 7). RPA is also required for DNA unwinding (Dean *et al.,* 1987) indicating that RPA and topoisomerase I may cooperate with T antigen to unwind SV40 DNA at replication forks. This concept is consistent with our observation that topo I and RPA do not interfere with one another for binding to T antigen (Fig. 5C).

The mutants in class 4 are partially defective in their association with topo I (Fig. 6). Since these mutants are unable to unwind circular DNA but can unwind an origin-containing DNA fragment (Wun-Kim *et al.,* 1993), their defect may be due to a failure to associate with a protein complex that is needed to properly unwind circular DNA. Such a complex is likely to contain topoisomerase I.

Another, but less likely, explanation for the interaction between T antigen and topo I is that it is involved in the transcriptional activation property of T antigen. The mechanism by which T antigen activates the late viral gene region is not known but is believed to occur, in part, by binding and presumably activating several cellular transcription factors. Topo I has been implicated in the activation of transcription (Kretzschmar *et al.,* 1993; Merino *et al.,* 1993), therefore, it is possible that the function of this association is related to T antigen's role in viral transcription.

Several investigators have reported that purified large T antigen contains topoisomerase activity (Marton *et al.,* 1993; Mann, 1993). This activity appears to be different from cellular topo I in that it responds differently to salt concentrations. The relationship between this activity and any cellular topo I that may remain bound to T antigen after purification is not known.

In summary, an association between T antigen and topo I was detected by ELISAs and immunoblots. Two discrete regions of T antigen have the ability to bind separately to topo I. This association can take place on DNA and may be important during the unwinding of circular DNA at replication forks.

ACKNOWLEDGMENTS

This work was supported by PHS Grant CA36118 from the National Cancer Institute to D.T.S. and by CA13106 to B.S. We thank Dr. Kathleen Rundell for purified small t antigen and Drs. Lance Stewart and Jim Champoux (University of Washington) for purified human topo I and for immunoaffinity-purified anti-topo I.

REFERENCES

Avemann, K., Knippers, R., Koller, T., and Sogo, J. M. (1988). Camptothecin, a specific inhibitor of type I DNA topoisomerase, induces DNA breakage at replication forks. *Mol. Cell. Biol.* 8, 3026–3034.

Borowiec, J., and Hurwitz, J. (1988). Localized melting and structural changes in the SV40 origin of replication induced by T-antigen. *EMBO J.* 7, 3149–3158.

Borowiec, J. A., Dean, F. B., and Hurwitz, J. (1991). Differential induction of structural changes in the simian virus 40 origin of replication by T antigen. *J. Virol.* 65, 1228–1235.

Brill, S. J., and Stillman, B. (1989). Yeast replication factor-A functions in the unwinding of the SV40 origin of DNA replication. *Nature (London)* 342, 92–95.

Bullock, P. A., Seo, Y. S., and Hurwitz, J. (1991). Initiation of simian virus 40 DNA synthesis in vitro. *Mol. Cell. Biol.* 11, 2350–2361.

Cavender, J. F., Conn, A., Epler, M., Lacko, H., and Tevethia, M. J. (1995). Simian virus 40 large T antigen contains two independent activities that cooperate with a ras oncogene to transform rat embryo fibroblasts. *J. Virol.* 69, 923–934.

Collins, K., and Kelly, T. J. (1991). Effects of T antigen and replication protein A on the initiation of DNA synthesis by DNA polymerase α-primase. *Mol. Cell. Biol.* 11, 2108–2115.

Collins, K. L., Russo, A. A., Tseng, B. Y., and Kelly, T. J. (1993). The role of the 70 kDa subunit of human DNA polymerase alpha in DNA replication. *EMBO J.* 12, 4555–4566.

D'Arpa, P., Machlin, P. S., Ratrie, H., III, Rothfield, N. F., Cleveland, D. W., and Earnshaw, W. C. (1988). cDNA cloning of human topoisomerase I: Catalytic activity of a 67.7-kDa carboxy-terminal fragment. *Proc. Natl. Acad. Sci. USA* 85, 2543–2547.

Dean, F. B., and Hurwitz, J. (1991). Simian virus 40 large T antigen untwists DNA at the origin of DNA replication. *J. Biol. Chem.* 266, 5062–5071.

Dean, F. B., Bullock, P., Murakami, Y., Wobble, R., Weissbach, L., and Hurwitz, J. (1987). Simian virus 40 (SV40) DNA replication: SV40 large T antigen unwinds DNA containing the SV40 origin of replication. *Proc. Natl. Acad. Sci. USA* 84, 16–20.

Deb, S., Tsui, S., Koff, A., DeLucia, A. L., Parsons, R., and Tegtmeyer, P. (1987). The T-antigen binding domain of the simian virus 40 core origin of replication. *J. Virol.* 61, 2143–2149.

Denis, D., and Bullock, P. A. (1993). Primer-DNA formation during simian virus 40 DNA replication in vitro. *Mol. Cell. Biol.* 13, 2882–2890.

Dornreiter, I., Copeland, W. C., and Wang, T. S. (1993). Initiation of simian virus 40 DNA replication requires the interaction of a specific domain of human DNA polymerase alpha with large T antigen. *Mol. Cell. Biol.* 13, 809–820.

Dornreiter, I., Erdile, L. F., Gilbert, I. U., von Winkler, D., Kelly, T. J., and Fanning, E. (1992). Interaction of DNA polymerase alpha-primase with cellular replication protein A and SV40 T antigen. *EMBO J.* 11, 769–776.

Dornreiter, I., Hoss, A., Arthur, A. K., and Fanning, E. (1990). SV40 T antigen binds directly to the catalytic subunit of DNA polymerase α. *EMBO J.* 9, 3329–3336.

Fien, K., and Stillman, B. (1992). Identification of replication factor C from *Saccharomyces cerevisiae:* A component of the leading-strand DNA replication complex. *Mol. Cell. Biol.* 12, 155–163.

Gannon, J. V., and Lane, D. P. (1987). p53 and DNA polymerase α compete for binding to SV40 T antigen. *Nature (London)* 329, 456–458.

Goetz, G. S., Dean, F. B., Hurwitz, J., and Matson, S. W. (1988). The unwinding of duplex regions in DNA by the simian virus 40 large tumor antigen-associated DNA helicase activity. *J. Biol. Chem.* 263, 383–392.

Goswanmi, R., Turk, B., Enderle, K., Howe, A., and Rundell, K. (1992). Effect of zinc ions on the biochemical behavior of simian virus 40 small-t antigen expressed in bacteria. *J. Virol.* 66, 1746–1751.

Gough, G., Gannon, J. V., and Lane, D. P. (1988). Competition between DNA polymerase alpha and p53 for binding to SV40 T-antigen. *Cancer Cells* 6, 153–158.

Gruda, M. C., Zabolotny, J. M., Xiao, J. H., Davidson, I., and Alwine, J. C. (1993). Transcriptional activation by simian virus 40 large T antigen: Interactions with multiple components of the transcription complex. *Mol. Cell. Biol.* 13, 961–969.

Gurney, E. G., Harrison, R. O., and Fenno, J. (1980). Monoclonal antibodies against simian virus 40 T antigens: Evidence for distinct subclasses of large T antigen and for similarities among nonviral T antigens. *J. Virol.* 34, 752–763.

Gurney, E. G., Tamowski, S., and Deppert, W. (1986). Antigenic binding sites of monoclonal antibodies specific for simian virus 40 large T antigen. *J. Virol.* 57, 1168–1172.

Harlow, E., Crawford, L. V., Pim, D. C., and Williamson, N. M. (1981). Monoclonal antibodies specific for simian virus 40 tumor antigen. *J. Virol.* 39, 861–869.

Ishimi, Y., Sugasawa, K., Hanaoka, F., and Kikuchi, A. (1991). Replication of the simian virus 40 chromosome with purified proteins. *J. Biol. Chem.* 266, 16141–16148.

Kamakaka, R. T., Kaufman, P. D., Stillman, B., Mitsis, P. G., and Kadonaga, J. T. (1994). Simian virus 40 origin- and T-antigen-dependent DNA replication with *Drosophila* factors in vitro. *Mol. Cell. Biol.* 14, 5114–5122.

Kenny, M. K., Schlegel, U., Furneaux, H., and Hurwitz, J. (1990). The role of human single-stranded DNA binding protein and its individual subunits in simian virus 40 DNA replication. *J. Biol. Chem.* 265, 7393–7700.

Kretzschmar, M., Meisterernst, M., and Roeder, R. G. (1993). Identification of human DNA topoisomerase I as a cofactor for activator-dependent transcription by RNA polymerase II. *Proc. Natl. Acad. Sci. USA* 90, 11508–11512.

Lane, D. P., and Gannon, J. (1986). Monoclonal antibody analysis of the SV40 large T antigen-p53 complex. *Cancer Cells* 4, 387–393.

Lanford, R. E. (1988). Expression of simian virus 40 T antigen in insect cells using a baculovirus expression vector. *Virology* 167, 72–81.

Li, C., Cao, L. G., Wang, Y. L., and Baril, E. F. (1993). Further purification and characterization of a multienzyme complex for DNA synthesis in human cells. *J. Cell. Biochem.* 53, 405–419.

Malkas, L. H., Hickey, R. J., Li, C., Pedersen, N., and Baril, E. F. (1990). A 21S enzyme complex from HeLa cells that functions in simian virus 40 DNA replication in vitro. *Biochemistry* 29, 6362–6374.

Mann, K. (1993). Topoisomerase activity is associated with purified SV40 T antigen. *Nucleic Acids Res.* 21, 1697–1704.

Marton, A., Jean, D., Delbecchi, L., Simmons, D. T., and Bourgaux, P. (1993). Topoisomerase activity associated with SV40 large tumor antigen. *Nucleic Acids Res.* 21, 1689–1695.

Mastrangelo, I. A., Hough, P. V. C., Wall, J. S., Dodson, M., Dean, F. B., and Hurwitz, J. (1989). ATP-dependent assembly of double hexamers of SV40 T antigen at the viral origin of DNA replication. *Nature (London)* 338, 658–662.

Melendy, T., and Stillman, B. (1991). Purification of DNA polymerase delta as an essential simian virus 40 DNA replication factor. *J. Biol. Chem.* 266, 1942–1949.

Melendy, T., and Stillman, B. (1993). An interaction between replication protein A and SV40 T antigen appears essential for primosome assembly during SV40 DNA replication. *J. Biol. Chem.* 268, 3389–3395.

Merino, A., Madden, K. R., Lane, W. S., Champoux, J. J., and Reinberg, D. (1993). DNA topoisomerase I is involved in both repression and activation of transcription. *Nature (London)* 365, 227–232.

Mole, S. E., and Lane, D. P. (1985). Use of simian virus 40 large T-β-galactosidase fusion proteins in an immunochemical analysis of simian virus 40 large T antigen. *J. Virol.* 54, 703–710.

Murakami, Y., Wobbe, C. R., Weissbach, L., Dean, F. B., and Hurwitz, J. (1986). Role of DNA polymerase α and DNA primase in simian virus 40 DNA replication in vitro. *Proc. Natl. Acad. Sci. USA* 83, 2869–2873.

Myers, R. M., and Tjian, R. (1980). Construction and analysis of simian virus 40 origins defective in tumor antigen binding and DNA replication. *Proc. Natl. Acad. Sci. USA* 77, 6491–6495.

Nasheuer, H. P., von Winkler, D., Schneider, C., Dornreiter, I., Gilbert, I., and Fanning, E. (1992). Purification and functional characterization of bovine RP-A in an in vitro SV40 DNA replication system. *Chromosoma* 102, S52–S59.

Oberosler, P., Hloch, P., Ramsperger, U., and Stahl, H. (1993). p53-catalyzed annealing of complimentary nucleic acids. *EMBO J.* 12, 2389–2396.

Prelich, G., Kostura, M., Marshak, D. R., Mathews, M. B., and Stillman, B. (1987). The cell-cycle regulated proliferating cell nuclear antigen is required for SV40 replication in vitro. *Nature (London)* 326, 471–475.

Roberts, J. (1989). Simian virus 40 (SV40) large tumor antigen causes stepwise changes in SV40 origin structure during initiation of DNA replication. *Proc. Natl. Acad. Sci. USA* 86, 3939–3943.

Scheffner, M., Wessel, R., and Stahl, H. (1989). Sequence independent duplex DNA opening reaction catalyzed by SV40 large T antigen. *Nucleic Acids Res.* 17, 93–106.

Shortle, D., and Nathans, D. (1979a). Mutants of SV40 with base substitutions at the origin of DNA replication. *Cold Spring Harbor Symp. Quant. Biol.* 43, 663–668.

Shortle, D., and Nathans, D. (1979b). Regulatory mutants of simian virus 40: Constructed mutants with base substitutions at the origin of DNA replication. *J. Mol. Biol.* 131, 801–817.

Simanis, V., and Lane, D. P. (1985). An immunoaffinity purification procedure for SV40 large T antigen. *Virology* 144, 88–97.

Simmons, D. T., Loeber, G., and Tegtmeyer, P. (1990a). Four major sequence elements of simian virus 40 large T antigen coordinate its specific and nonspecific DNA binding. *J. Virol.* 64, 1973–1983.

Simmons, D. T., Upson, R., Wun-Kim, K., and Young, W. (1993). Biochemical analysis of mutants with changes in the DNA-binding domain of simian virus 40 tumor antigen. *J. Virol.* 67, 4227–4236.

Simmons, D. T., Wun-Kim, K., and Young, W. (1990b). Identification of simian virus 40 T antigen residues important for specific and nonspecific binding to DNA and for helicase activity. *J. Virol.* 64, 4858–4865.

Smale, S., and Tjian, R. (1986). T-antigen-DNA polymerase alpha complex implicated in simian virus 40 DNA replication. *Mol. Cell. Biol.* 6, 4077–4087.

Sompayrac, L., and Danna, K. J. (1991). The amino-terminal 147 amino acids of SV40 large T antigen transform secondary rat embryo fibroblasts. *Virology* 181, 412–415.

Srinivasan, A., Peden, K. W. C., and Pipas, J. M. (1989). The large tumor antigen of simian virus 40 encodes at least two distinct transforming functions. *J. Virol.* 63, 5459–5463.

Stewart, L., Ireton, G. C., Parker, L. H., Madden, K. R., and Champoux, J. J. (1996). Biochemical and biophysical analyses of recombinant forms of human topoisomerase I. *J. Biol. Chem.* 271, 7593–7601.

Stillman, B., Bell, S. P., Dutta, A., and Marahrens, Y. (1992). DNA replication and the cell cycle. *Ciba Found. Symp.* 170, 147–156.

Tegtmeyer, P. (1972). Simian virus 40 deoxyribonucleic acid synthesis: The viral replicon. *J. Virol.* 10, 591–598.

Tsurimoto, T., and Stillman, B. (1989). Multiple replication factors augment DNA synthesis by the two eukaryotic DNA polymerases, α and δ. *EMBO J.* 8, 3883–3889.

Tsurimoto, T., and Stillman, B. (1991a). Replication factors required for SV40 DNA replication *in vitro.* II. Switching of DNA polymerase α and δ during initiation of leading and lagging strand synthesis. *J. Biol. Chem.* 266, 1961–1968.

Tsurimoto, T., and Stillman, B. (1991b). Replication factors required for SV40 DNA replication *in vitro.* I. DNA structure-specific recognition of a primer-template junction by eukaryotic DNA polymerases and their accessory proteins. *J. Biol. Chem.* 266, 1950–1960.

Tsurimoto, T., Melendy, T., and Stillman, B. (1990). Sequential initiation of lagging and leading stranded synthesis by two different polymerase complexes at the SV40 DNA replication origin. *Nature (London)* 346, 534–539.

Waga, S., and Stillman, B. (1994). Anatomy of a DNA replication fork revealed by reconstitution of SV40 DNA replication in vitro. *Nature (London)* 369, 207–212.

Wessel, R., Schweizer, J., and Stahl, H. (1992). Simian virus 40 T-antigen DNA helicase is a hexamer which forms a binary complex during bidirectional unwinding from the viral origin of DNA replication. *J. Virol.* 66, 804–815.

Wiekowski, M., Schwarz, M. W., and Stahl, H. (1988). Simian virus 40 large T antigen DNA helicase: Characterization of the ATPase-dependent DNA unwinding activity and its substrate requirements. *J. Biol. Chem.* 263, 436–442.

Wold, M. S., Li, J. J., and Kelly, T. J. (1987). Initiation of simian virus 40 DNA replication in vitro: Large-tumor-antigen and origin-dependent unwinding of the template. *Proc. Natl. Acad. Sci. USA* 84, 3643–3647.

Wu, Y., Hickey, R., Lawlor, K., Wills, P., Yu, F., Ozer, H., Starr, R., Quan, J. Y., Lee, M., and Malkas, L. (1994). A 17S multiprotein form of murine cell DNA polymerase mediates polyomavirus DNA replication in vitro. *J. Cell. Biochem.* 54, 32–46.

Wun-Kim, K., Upson, R. H., Young, W., and Simmons, D. T. (1993). The DNA binding domain of simian virus 40 large T antigen has multiple functions. *J. Virol.* 67, 7608–7611.

Zahradka, P. (1992). Dideoxynucleoside triphosphates inhibit a late stage of SV40 DNA replication in vitro. *Mol. Cell. Biochem.* 110, 65–73.

Zhu, J., Rice, P. W., Chamberlain, M., and Cole, C. N. (1991). Mapping the transcriptional transactivation function of simian virus 40 large T antigen. *J. Virol.* 65, 2778–2790.