Topoisomerase activity is associated with purified SV40 T antigen

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ABSTRACT

Purified SV40 T antigen has been assayed for topoisomerase activity. The ability to relax negatively-supercoiled SV40 DNA was found in preparations of T antigen purified either from human 293 cells infected with Ad5-SVR111 virus or from insect Sf9 cells infected with recombinant baculovirus 941T. The T antigen-associated relaxing activity was stimulated by $MgCl_2$ and was not dependent on ATP, suggesting that it is not due to cellular topoisomerase II. The topoisomerase activity was immunoprecipitated by a monoclonal antibody specific for T antigen, but not by a control monoclonal antibody. In addition, immunoblotting of purified T antigen from human 293 cells with anti-human topoisomerase I and anti-human topoisomerase II antibodies failed to detect cellular topoisomerases I or II. Sedimentation analysis of purified T antigen revealed that the topoisomerase activity co-sedimented with the hexameric form of T antigen at 23S. The topoisomerase activity is, therefore, either inherent to T antigen or due to a cellular topoisomerase I tightly bound to, and co-purifying with, T antigen.

INTRODUCTION

Simian virus 40 (SV40) large tumor (T) antigen is essential both for initiation of SV40 DNA replication (1) and for elongation of the DNA (2,3) once synthesis has been initiated. The initiation and complete synthesis of Simian virus 40 (SV40) origin-containing plasmid DNA in an *in vitro* system (4) has recently been achieved using SV40 T antigen and purified proteins (5,6), thereby allowing for delineation of the essential components in the process. In initiating DNA replication, SV40 T antigen forms an ATP-dependent complex at the origin (7–9) and then induces localized melting in the early palindrome region as well as untwisting of the A/T region of the origin DNA (10–12). The next phase of replication involves the unwinding of the origin region by the ATP-dependent helicase activity of T antigen (13–15) in the presence of single-strand DNA binding protein. Once the origin region has been opened up, other proteins essential for DNA replication, such as DNA polymerase α -primase (5,6,16) associate with the origin and initiate synthesis of the daughter strands. Evidence has been found for the formation of a complex between SV40 T antigen and DNA polymerase α which is necessary for replication to proceed

(17–21). The data suggest that T antigen may be responsible for guiding the DNA polymerase α to both the origin and the replication forks.

In order for the events initiated by T antigen to lead to elongation of SV40 DNA, it is essential that topoisomerase I act on the DNA soon after initiation to relieve the positive superhelical tension generated as a result of the untwisting and unwinding at the origin. In the experiments conducted to date, topoisomerase I must be added to the *in vitro* DNA replication system in order to carry out this function (14,15), although topoisomerase II can substitute for topoisomerase I (22,23). The fact that topoisomerase has to be added to the *in vitro* replication system suggests that T antigen itself does not have topoisomerase activity. In fact, purified SV40 T antigen has been examined for inherent topoisomerase activity, but conflicting data obtained in different laboratories has not clarified the issue (24,25). Since the association of topoisomerase activity with purified SV40 nucleoprotein complexes (NPCs) has been shown to be temperature-sensitive in SV40 tsA58-infected monkey cells in my laboratory, it was considered important to re-examine purified SV40 T antigen for possible topoisomerase activity.

MATERIALS AND METHODS

Cells and viruses

TC7 cells were grown in Dulbecco-modified Eagle's minimum essential medium (DMEM) supplemented with 10% calf serum. TC7 cells were infected with 1 ml of SV40 wild-type virus at a 1 in 10 dilution (multiplicity of infection 5 to 10 PFU/cell) or tsA58 virus at a 1 in 5 dilution (multiplicity of infection = 1 PFU/cell).

Human 293 cells (26), transformed by adenovirus type 5 (Ad5), were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, 2 mM glutamine and 1 mM sodium pyruvate. Subconfluent 293 cells were infected with the recombinant Ad5-SV40 hybrid virus Ad5-SVR111 (27), originally obtained from Y. Gluzman.

Sf9 cells, derived from *Spodoptera frugiperda* (28), were grown in TNM-FH medium (Grace's medium supplemented with yeastolate and lactalbumin hydrolysate) plus 10% fetal calf serum. Subconfluent Sf9 cells were infected with either recombinant baculovirus 941T (originally obtained from Robert Lanford) to overexpress large T antigen (29) or recombinant baculovirus NPVp53 to overexpress mouse wild-type p53 (kindly provided by G. Mayr and P. Tegtmeyer).

SV40 DNA, plasmids and enzymes

SV40 DNA was Hirt-harvested from SV40-infected monkey TC7 cells and purified by cesium chloride-ethidium bromide density gradient centrifugation. The pUC.HSO and pUC.8-4 plasmids (30,31) were originally obtained from J.Li and T.Kelly. The pUC.HSO plasmid contains SV40 wild-type origin DNA from nucleotide 5171 to nucleotide 133 inserted into pUC19, whereas pUC.8-4 contains the same HindIII to SphI fragment of SV40 DNA except for a 4-nucleotide deletion from nucleotide 5239 to 5242. The p53MN plasmid (obtained from G. Mayr and P.Tegtmeyer) contains the wild-type p53 gene inserted into pBluescript KS+.

SV40 nucleoprotein complexes

SV40 nucleoprotein complexes (NPCs) were extracted from SV40-infected TC7 cells at 72 hours post-infection in the case of wild-type- and tsA58-infected cells grown at 32°C, according to the method of Su and DePamphilis (32), as described previously by Mann and Hunter (33). Viral NPCs were extracted from isolated nuclei in hypotonic buffer containing 10 mM HEPES (pH 7.8), 5 mM KCl, 0.5 mM MgCl₂, and 0.5 mM dithiothreitol at 4°C for 1 hr and sedimented on linear 5 to 30% neutral sucrose gradients at 49,000 rpm for 50 mins in a Beckman SW 50.1 rotor at 4°C.

To analyze the proteins present in each of the gradient fractions, SV40-infected TC7 cells were labeled in 2 ml DMEM minus methionine, containing 5% dialyzed calf serum and ³⁵S-methionine (specific activity greater than 1000 Ci/mmol) at a concentration of 100 uCi/ml for 6 hrs at 32°C.

Purification of SV40 T antigen

SV40 T antigen was purified from Ad5-SVR111-infected human 293 cells by the method of Dixon and Nathans (34), as previously described in detail (35). T antigen was stored at -20°C in a buffer containing 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), and 50% glycerol.

In contrast, SV40 T antigen was purified from baculovirus 941T-infected insect Sf9 cells by the method of Simianis and Lane (36), as modified by Ray and Tegtmeyer (37). After two sequential elutions of T antigen with 50% ethylene glycol in buffer containing 20 mM Tris-HCl (pH 8.5), 500 mM NaCl, 1 mM EDTA, 10% glycerol, the PAb419-protein A sepharose column was washed with 10 mM Tris-HCl (pH 8.5), 1 mM EDTA, 10% glycerol and then eluted with elution buffer containing 20 mM triethylamine (pH 10.8) and 10% glycerol. Each of the T antigen eluates was dialyzed separately and stored at -20°C in 10 mM PIPES (pH 7.0), 5 mM NaCl, 0.1 mM EDTA, 1 mM DTT, and 50% glycerol.

For neutral sucrose density gradient centrifugation, T antigen from the baculovirus expression system was eluted with the high pH buffer, concentrated and transferred into hypotonic buffer (see NPC section above) + 10% glycerol in a centricon-30 microconcentrator. The T antigen preparation was then diluted down with hypotonic to a final glycerol concentration of 4.5% and loaded on a 5%-20% or 5%-30% neutral sucrose gradient made up in hypotonic buffer. The gradients were centrifuged at 49,000 rpm for the requisite amount of time in a Beckman SW 50.1 rotor at 4°C.

Topoisomerase assays

The ability to relax 0.5-1 µg of SV40, pUC.HSO, or pUC.8-4 DNA was assayed by incubation of the DNA with 0.2-0.8 µg

of purified SV40 T antigen in a buffer containing 10 mM HEPES (pH 7.8), 170 mM KCl, 10 mM MgCl₂, and 1 mM DTT. The total reaction, with a volume of 30 µl, was incubated at 37°C for 30 mins. The reaction was stopped by the addition of 15 µl of 2× Tris-borate-EDTA (TBE), 5 mM EDTA, 5% SDS, 50% glycerol, and .03% bromophenol blue followed by 7.5 µl of β-mercaptoethanol and then incubation at 37°C for 15-30 mins, prior to analysis by agarose gel electrophoresis. A 15 µl aliquot of each sample was run on a 0.7% agarose-TBE gel and then visualized by staining with ethidium bromide.

In assaying for topoisomerase activity associated with SV40 NPCs, 10 µl aliquots of every two adjacent gradient fractions were pooled and added to ³H-SV40 DNA substrate to assay for topoisomerase activity across the sucrose gradient. After analysis of the products of the reaction by agarose gel electrophoresis, the agarose gels were subjected to fluorography in PPO-methanol, dried down, and exposed to Kodak XAR-5 film.

Immunoprecipitations

Purified T antigen was immunoprecipitated by resuspending 0.25 µg of T antigen in 22 µl of 10 mM HEPES (pH 7.8) containing 1% NP-40 and 0.001% SDS and incubating with 5 µl of PAb 416 or PAb 421 hybridoma supernatant for 30 mins at 4°C. This was followed by incubation with 10 µl of protein A sepharose for 30 mins at 4°C. After washing the immunocomplexes with 10 mM HEPES (pH 7.8) containing 1% NP-40 and 0.001% SDS, each pellet was resuspended and assayed for topoisomerase activity. The products of the reaction were analyzed by agarose gel electrophoresis.

Immunoblotting

Proteins to be analyzed were separated by electrophoresis on a 7.5% SDS-polyacrylamide gel. After electrophoresis, the gel was equilibrated for 30 mins in transfer buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 20% methanol v/v) and then electroblotted onto a nitrocellulose membrane (0.45 µm) at 12V overnight at room temperature. The nitrocellulose sheet was then incubated in 50% isopropanol for 30 mins, followed by two 30 min washes in TBS buffer (15 mM Tris-HCl, pH 7.8, 150 mM NaCl, 5 mM Na₂CO₃, 1 mM EDTA). A section of the nitrocellulose sheet was cut off, washed with distilled water for 1 hr, and stained with 20 ml of colloidal gold (BioRad) at room temperature for 2 hrs. The rest of the nitrocellulose sheet was incubated overnight in TBS buffer plus 0.1% gelatin and 100 µg of bovine serum albumin (immunoglobulin free) per ml (38,39). The sheet was then washed for 1 hr with TBS buffer plus 0.1% gelatin and 0.1% tween 20 (buffer GT) and incubated for 5 hrs with anti-human topoisomerase I serum from autoimmune scleroderma patients (Immunovision) or with rabbit anti-human topoisomerase II serum (kindly provided by L.Liu) in buffer GT at a 1:500 or 1:1000 dilution. Unbound antibodies were removed by washing the sheet for 1 hr in buffer GT. Bound antibodies were then detected by incubating the sheet with 5 µCi of ³⁵S-protein A (Amersham) in buffer GT for 1 hr. The unbound ³⁵S-protein A was removed by washing the sheet for 1 hr in buffer GT.

For immunodetection of SV40 T antigen, the nitrocellulose sheet was blocked with TBS buffer containing gelatin but no bovine serum albumin in order to enhance the detection of T antigen. After blocking, the sheet was washed with buffer GT and then incubated with PAb 416 at a 1:500 dilution in buffer GT for 5 hrs. The rest of the procedure was the same as for immunodetection of the topoisomerases.

RESULTS

Topoisomerase activity associated with purified SV40 nucleoprotein complexes

Analysis of sucrose gradient-purified SV40 nucleoprotein complexes (NPCs) from monkey cells infected with SV40 wild-type virus revealed an associated topoisomerase activity that was capable of relaxing exogenously added, negatively-supercoiled SV40 form I DNA to form Ir DNA, with the generation of a series of topoisomers (Fig. 1A). This topoisomerase activity peaked in the region of the gradient containing NPC-associated SV40 T antigen, as confirmed by analysis of the ^{35}S -methionine-labeled proteins in each gradient fraction.

The association of this topoisomerase activity with NPCs from SV40 tsA58-infected monkey cells was found to be temperature sensitive when infected cells were grown at 32°C and shifted up to 41°C for 1 hour prior to harvest of the NPCs (Fig. 1B). In contrast, the topoisomerase activity was not temperature sensitive in NPCs extracted from cells infected with SV40 wild-type virus (Fig. 1A). On the basis of these results, it was reasoned that the topoisomerase activity was either inherent to SV40 T antigen or alternatively due to a host cell enzyme normally associated with the SV40 NPCs but no longer present at the nonpermissive temperature due to cessation of SV40 DNA synthesis. In view of these results, it was important to determine if topoisomerase activity was detected in preparations of purified SV40 T antigen. It was reasoned that a topoisomerase-active T antigen would provide the means for a smooth transition between initiation and elongation in SV40 DNA replication.

Figure 1. NPC topoisomerase from SV40 wild-type-infected versus tsA48-infected monkey cells grown at 32°C versus 41°C. Crude NPCs from SV40-infected TC7 cells grown either at 32°C for 6 hrs or at 32°C for 5 hrs with a shift-up to 41°C for 1 hr prior to harvest were sedimented on 5–30% sucrose gradients containing 1 mM DTT. Every two gradient fractions were pooled and assayed with ^3H -SV40 DNA substrate in the presence of 170 mM KCl for 30 mins at 37°C. Channel #1 = fractions #9+10, through channel #10 = fractions #27+28; M = marker SV40 DNA.

Topoisomerase activity associated with purified SV40 T antigen

To ascertain if topoisomerase activity was associated with purified SV40 T antigen, as well as with the SV40 nucleoprotein complexes, SV40 T antigen was purified from extracts of Ad5-transformed human 293 cells infected with recombinant Ad5-SVR111 virus (40). Purification of SV40 T antigen was achieved by immunoaffinity chromatography, through binding to PAb 419 covalently linked to protein A sepharose, washing with buffers containing 0.5 M salt at pH 8 and then pH 9, and finally elution of purified protein at pH 10.7 (34,35). Subsequent incubation of the purified SV40 T antigen with covalently-closed, double-stranded SV40 DNA resulted in relaxation of the negatively-supercoiled SV40 form I DNA (Fig. 2A). Increasing amounts of SV40 T antigen resulted in an increased number of topoisomers of lower linking number (Fig. 2A). Pre-incubation of T antigen with 1% NP-40 for 30 mins at 4°C before the addition of KCl, MgCl_2 , and SV40 DNA resulted in a stimulation of the topoisomerase activity.

To determine if a functional origin of replication was necessary for expression of the topoisomerase activity, purified T antigen was incubated either with pUC.HSO, a plasmid containing the wild-type SV40 DNA origin, or with pUC.8-4, an origin-defective plasmid with a 4-nucleotide deletion at the BglII site (30,31). Plasmid pUC.8-4 is defective for replication both *in vivo* and *in vitro*, while pUC.HSO is replicated efficiently in both cases (41). When incubated with purified SV40 T antigen, in the absence of competitor DNA, relaxation of both pUC.HSO and pUC.8-4 DNA occurred (Fig. 2B), suggesting that a functional origin of replication is not essential for action of the topoisomerase.

The ability to relax negatively-supercoiled DNA was also characteristic of SV40 T antigen purified from the baculovirus expression system (Fig. 3). Sf9 insect cells were infected with recombinant baculovirus expressing either SV40 T antigen or mouse wild-type p53. Extracts from the insect cells expressing mouse wild-type p53 were subjected to the same purification protocol on PAb 419-protein A sepharose as extracts from the cells expressing SV40 T antigen. PAb 419 is a monoclonal

Figure 2. Relaxation of negatively-supercoiled DNA by SV40 T antigen purified from human 293 cells infected with Ad5-SVR111 virus. (A) Incubation of SV40 DNA (1 µg) with different amounts of purified SV40 T antigen: buffer alone (lane a), 280 ng T antigen (lane b), 560 ng T antigen (lane c), 840 ng T antigen (lane d). (B) Incubation of 0.5 µg SV40 T antigen (lanes a, c, e) or buffer (lanes b, d, f) with 0.5 µg of SV40 DNA (lanes a and b), pUC.HSO DNA (lanes c and d), or pUC.8-4 DNA (lanes e and f).

Figure 3. Topoisomerase activity associated with SV40 T antigen purified from insect Sf9 cells infected with baculovirus 941T. (A) Colloidal gold-stained Western blot of protein extracted from infected Sf9 cells expressing either SV40 T antigen or p53 and eluted off of PAb 419-protein A sepharose columns with two sequential elutions of ethylene glycol (EG) or two sequential elutions of high pH (PH) buffer. Lane M = marker proteins with molecular masses of 196, 106, 71, 44, and 28 kDa. (B) Incubation of p53MN DNA (0.45 μ g) with buffer (lane M), ethylene glycol-eluted (EG1 and EG2) or high pH-eluted (PH1 and PH2) fractions of either purified SV40 T antigen or a control p53 preparation.

antibody specific for T antigen, not for p53. This control was done to determine if the topoisomerase detected in the purified T antigen preparation was due to a cellular enzyme sticking to the PAb 419-protein A sepharose and thereby contaminating the preparation. It should be noted that a different purification scheme (36,37) was used for T antigen purified from the baculovirus system than for T antigen purified from the human 293 cells infected with the hybrid virus. Two sequential ethylene glycol elutions of protein bound to PAb 419-protein A sepharose were followed by two sequential high pH elutions.

When analyzed by SDS-polyacrylamide gel electrophoresis and colloidal gold staining of Western blots, highly purified T antigen from the baculovirus expression system was found in all four eluted fractions, while no proteins were detected in the control p53 fractions (Fig. 3A). Colloidal gold staining of proteins in Western blots has been found to be as sensitive as silver staining. Topoisomerase activity was detected in the ethylene glycol-eluted fractions of the PAb 419-purified p53 control preparation as well as in the SV40 T antigen preparation (Fig. 3B). In contrast, the topoisomerase activity was only present in the two high pH-eluted fractions of purified T antigen, and not in either of these fractions in the control preparation (Fig. 3B). This suggests that the topoisomerase activity detected in the ethylene glycol-eluted fractions is due to a contaminating cellular topoisomerase, whereas that found in the high pH-eluted fractions is due either T antigen itself or alternatively to a cellular topoisomerase co-purifying with T antigen.

Immunoprecipitation of topoisomerase activity by antibodies specific for T antigen

The data presented in Figure 3 show that the topoisomerase associated with high pH-eluted T antigen is not the result of a cellular topoisomerase simply binding to the matrix or the antibody. Immunoprecipitations were done to determine if the

Figure 4. Immunoprecipitation (IP) of topoisomerase activity by a monoclonal antibody specific for T antigen. SV40 T antigen (0.2 μ g) purified from 293 cells infected with Ad5-SVR111 virus was immunoprecipitated with protein A sepharose (PAS) alone (lane c), PAb 421 + PAS (lane d), or PAb 419 + PAS (lane e). Relaxation activity was assayed by incubating SV40 DNA (0.3 μ g) with buffer alone (lane a), with T antigen prior to IP (lane b), or with the immunoprecipitated pellets after resuspension in assay buffer (lanes c,d,e).

relaxation activity was due to a non-specific contaminating topoisomerase loosely bound to T antigen or rather to a topoisomerase specifically and tightly associated with T antigen. It was considered unlikely that a loose association between a cellular topoisomerase and T antigen would survive the high salt washes and high pH elution of T antigen. Once dissociated from T antigen, such a topoisomerase would no longer be immunoprecipitated by an antibody specific for T antigen.

Purified T antigen was incubated with PAb 419, specific for an antigenic site on T antigen, versus a control antibody (PAb 421) specific for an antigenic site on p53, followed by protein A sepharose. When the immunoprecipitates were assayed for topoisomerase activity, it was found that the relaxation activity was immunoprecipitated by PAb 419, but not by PAb 421 (Fig. 4). Analysis of the proteins present in the immunoprecipitates by SDS-polyacrylamide gel electrophoresis confirmed the presence of T antigen in the PAb 419 immunoprecipitate, but not in the PAb 421 immunoprecipitate. On the basis of this result, it can be concluded that the topoisomerase activity is not due to a loosely-bound, contaminating protein present in the purified T antigen preparation. Instead, the topoisomerase must be inherent to T antigen itself or else due to a cellular topoisomerase tightly bound to T antigen. Either possibility is considered to be of significance.

Characteristics of the topoisomerase associated with T antigen

Experiments were conducted to determine if the topoisomerase in the purified T antigen preparation had unique requirements for enzymatic activity as compared to the requirements of known cellular topoisomerases. It was reasoned that the finding of unique properties would argue in favor of the relaxation activity being due to SV40 T antigen rather than to a cellular topoisomerase. Both eukaryotic topoisomerase I and topoisomerase II are known to be capable of relaxing supercoiled, double-stranded DNA. However, relaxation of DNA by topoisomerase II is ATP-dependent and Mg^{2+} -dependent (42,43), whereas relaxation by topoisomerase I is independent of ATP (44,45). Relaxation of DNA by topoisomerase I can be stimulated by Mg^{2+} , but Mg^{2+} is not an absolute requirement for activity (44,45).

Figure 5. Characteristics of the topoisomerase associated with purified SV40 T antigen. Incubation of 0.5 μ g SV40 DNA with buffer (lane a) or 0.5 μ g SV40 T antigen in the presence of 170 mM KCl + 10 mM $MgCl_2$ (lane b); 170 mM KCl + 10 mM $MgCl_2$ + 3 mM ATP (lane c); or 170 mM KCl + 10 mM EDTA (lane d).

In my assay, the topoisomerase activity associated with T antigen was independent of exogenously added ATP (Fig. 5), suggesting that it is not due to a cellular type II topoisomerase. To examine the effect of Mg^{2+} concentration on the topoisomerase activity associated with T antigen, the relaxation activity under standard conditions (10 mM $MgCl_2$) was compared with the activity in the absence of Mg^{2+} (no added $MgCl_2$ + 1.5 mM EDTA). In the absence of Mg^{2+} , relaxation of substrate DNA by purified T antigen was no longer detected (Fig. 5). Thus, the T antigen-associated topoisomerase activity can be stimulated by the addition of Mg^{2+} , as reported for eukaryotic type I topoisomerases (44–46). Under my assay conditions, residual enzyme activity in the absence of Mg^{2+} may have been below the limits of detection. Therefore, it is not possible to conclude if the T antigen-associated topoisomerase differs from cellular topoisomerase I with regard to a strict Mg^{2+} dependency.

Lack of evidence for co-purification of topoisomerases I or II with T antigen

As mentioned above, it was reasoned that the topoisomerase activity associated with purified SV40 T antigen could be either an intrinsic activity of T antigen or alternatively due to a cellular topoisomerase tightly bound to T antigen and co-purifying with it in a complex on PAb 419-protein A sepharose. It was important, therefore, to examine the purified T antigen preparation both by staining and immunoblotting for the presence of other proteins, which might be potential candidates for a cellular topoisomerase. The purity of T antigen purified from human 293 cells was checked by SDS–polyacrylamide gel electrophoresis, Western blotting onto nitrocellulose, and staining with colloidal gold. Staining revealed that more than 95% of the protein was present as a single major band (Fig. 6, panel 1), confirmed to be T antigen by immunoblotting (Fig. 6, panel 2). A series of minor bands were also detected by PAb 416 plus ^{35}S -protein A (Fig. 6, panel 2), indicating that they were related to SV40 T antigen, presumably as breakdown products.

Figure 6. Lack of evidence for co-purification of topoisomerases I or II with T antigen. Purified SV40 T antigen preparations (lanes a and b) and Ad5-SVR111-infected 293 human cell extracts (lanes c and d), either after adsorption (lane c) or before adsorption (lane d) to PAb 419-protein A sepharose, were blotted onto nitrocellulose. Lane 'm' = marker proteins with molecular masses of 97.5, 66, 45, and 31 kDa. The blots were stained with colloidal gold (panel 1) or immunoblotted with T antigen-specific monoclonal antibody PAb 416 (panel 2), anti-human topoisomerase I (panel 3), or rabbit anti-human topoisomerase II (panel 4), followed by ^{35}S -protein A.

Immunoblotting was used to determine if human topoisomerase I or topoisomerase II was present in the purified T antigen preparation. Replica blots were probed either with anti-human topoisomerase I from scleroderma patients or with rabbit anti-human topoisomerase II, followed by ^{35}S -protein A. We have previously used these same antibodies successfully to detect topoisomerases I and II in immunoblots of extracts from SV40-infected monkey cells (47,48). The autoradiograms of the immunoblots have been deliberately overexposed in order to increase the probability of detecting low levels of topoisomerases I and II, if present, in the purified T antigen preparations (Fig. 6, panels 3 and 4). A shorter exposure of these autoradiograms revealed only the major bands in the crude extracts (lanes c and d in Fig. 6, panels 3 and 4), indicating that the antisera are quite specific for the human topoisomerases. Some of the minor bands in the overexposed immunoblots are presumed to be the result of weak, nonspecific adherence of the antibodies to proteins not related to the topoisomerases in the cellular extracts. In addition, some of the minor bands in these same tracts are probably topoisomerase-related, due to the fact that the topoisomerases are very susceptible to breakdown in cellular extracts (46). In the purified T antigen preparations (lanes a and b, Fig. 6, panels 3 and 4), no evidence could be found for proteins migrating in a position similar to any of the major, or even minor, bands seen in the crude extracts, suggesting that neither topoisomerase I nor topoisomerase II was present. The weak detection of SV40 T antigen in the purified preparations by both antibodies (Fig. 6, panel 3) is thought to be the result of nonspecific adherence of the antibodies to the large amounts of purified protein.

Co-sedimentation of topoisomerase and hexameric T antigen

An alternative method, that of density gradient centrifugation, was used to determine if the topoisomerase activity could be uncoupled from SV40 T antigen. It was reasoned that similar sedimentation values (S-values) of the topoisomerase and purified

Figure 7. Co-sedimentation of topoisomerase activity and purified T antigen on neutral sucrose gradients. **Top left panel:** Colloidal gold-stained Western blot of SV40 T antigen purified from the baculovirus expression system (PH2-eluted fraction) and sedimented in a 5%-20% neutral sucrose gradient for 4 hrs at 49,000rpm. **Top right panel:** Topoisomerase assay of gradient fractions shown in top left panel. Aliquots (7.5 μ l) of every 2 fractions of the gradient were pooled and assayed with p53MN DNA (0.45 μ g) in the presence of 170 mM KCl + 10 mM MgCl₂. Topoisomerase assays of control p53MN DNA without any added gradient fraction, the pellet fraction, and the purified PH2 T antigen loaded on the gradient are also shown. **Bottom left panel:** Colloidal gold-stained Western blot of purified SV40 T antigen (PH2 fraction) sedimented in a 5%-30% neutral sucrose gradient for 1 hr 50 mins at 49,000rpm. **Bottom right panel:** Topoisomerase assay of gradient fractions shown in bottom left panel. For the assay, aliquots (7.5 μ l) of every 2 fractions were pooled, except in the T-antigen-peak region of the gradient where 15 μ l of each fraction was used.

T antigen would argue strongly in favor of T antigen being a topoisomerase. Purified SV40 T antigen was sedimented on neutral sucrose gradients, and the gradient fractions were analyzed for their ability to relax supercoiled SV40 DNA. In addition, each fraction was analyzed by SDS-polyacrylamide gel electrophoresis, Western blotting, and colloidal gold staining for the presence of SV40 T antigen. When purified PH2-eluted T antigen was run on a 5-20% neutral sucrose gradient at 49,000 rpm for 4 hours, the majority of the topoisomerase activity and T antigen were found in the pellet (Figure 7, top panels). In order to determine the S-value of T antigen and the topoisomerase that had pelleted, it was necessary to sediment some more purified PH2-eluted T antigen on a 5-30% neutral sucrose gradient for a shorter period of time. Under these conditions, it was found that the topoisomerase activity co-sedimented with the peak of SV40 T antigen (Figure 7, bottom panels). There was neither topoisomerase activity nor T antigen found in the pellet fraction of this gradient. It should be noted that the high molecular weight protein seen in fractions #19-#24 of the gradient was recognized by PAb 416 in an immunoblot, indicating that it is a form of T antigen.

Using SV40 DNA and ribosomal subunits as markers, the S-value of the topoisomerase-active T antigen was found to be 23S, indicating that T antigen in this region of the gradient was present as an oligomer. In fact, when T antigen in the topoisomerase-active peak was cross-linked with 0.1% glutaraldehyde and subjected to SDS-polyacrylamide gel electrophoresis (49), it migrated as a hexamer. The co-sedimentation results strongly

suggest that the topoisomerase activity is an inherent property of SV40 T antigen. However, it should be noted that an alternative, though less probable, explanation is that cellular topoisomerase I specifically co-purifies with T antigen and remains tightly bound to T antigen throughout the centrifugation process.

DISCUSSION

In this study, topoisomerase activity has been shown to be associated with purified SV40 T antigen. Topoisomerase-active SV40 T antigen was purified from two different cellular systems, that is from human cells infected with Ad5-SVR111 and from insect cells infected with baculovirus 941T. In addition, two different methods of purification resulted in a T antigen preparation capable of relaxing negatively-supercoiled SV40 DNA *in vitro*. It is clear that it is necessary to wash the immunoaffinity column with a high salt (0.5 M LiCl) buffer of at least pH 8.5 to remove cellular topoisomerases that have non-specifically adhered to the column (Fig. 3B). There are two possible explanations for the topoisomerase activity observed in the high pH-eluted T antigen fractions: either a cellular topoisomerase co-purifies in a complex with SV40 T antigen, or SV40 T antigen itself has topoisomerase activity. If the activity is due to a cellular topoisomerase, then the interaction of T antigen with an insect topoisomerase, as well as with a human topoisomerase, would require conservation of the topoisomerase sequence in the region that binds to T antigen.

At the T antigen:DNA ratio of 1:1 (wt/wt) typically used in the topoisomerase assay, origin-specific binding of T antigen would be expected to predominate (35). However, the experimental data suggest that binding of SV40 T antigen to the core origin of SV40 DNA replication is not essential for relaxation of the DNA (Fig. 2B). The 4-nucleotide deletion in pUC.8-4 interferes with the binding of T antigen to the core origin (50), presumably to the central pentanucleotides in site II, and yet relaxation by the topoisomerase is still evident. The lack of any difference in topoisomerase activity when using pUC.HSO versus pUC.8-4 DNA as a substrate would suggest that the expression of the topoisomerase is a result of T antigen binding to site I or to sites within the vector. Relaxation of p53MN DNA by T antigen purified from the baculovirus system (Fig. 3B) also indicates that specific binding to SV40 DNA is not required for expression of the topoisomerase, although this plasmid does contain nucleotides 4710–4100 and 2770–2533 of the SV40 genome.

No evidence could be found for the presence of cellular topoisomerase I or topoisomerase II in the purified T antigen preparations when Western blots were probed with antibodies specific for these topoisomerases (Fig. 6). This could be a matter of sensitivity, although when studying the subcellular localization of topoisomerases I and II in monkey cells, we found that proteins below the level of detection with colloidal gold staining could be visualized by immunoblotting (48). In SV40-infected monkey cells subjected to subcellular fractionation, both topoisomerase I and II are preferentially associated with the chromatin (48), whereas the majority of SV40 T antigen is present in the NP-40 cytoplasmic/nucleoplasmic extract, with only a minor amount present in the chromatin (51,52). This suggests that if in fact SV40 T antigen is associated with one of the cellular topoisomerases within the nucleus, then it is only a minor subpopulation of T antigen molecules which participates in such an interaction. If a cellular topoisomerase is co-purifying in a complex with SV40 T antigen, then it is below the level of detection by immunoblotting. The expression of relaxation activity in the absence of exogenously added ATP and the lack of stimulation by ATP (Fig. 5) distinguish the T antigen-associated topoisomerase from all known eukaryotic type II topoisomerases, which require ATP for relaxation of negatively supercoiled DNA (42–44). In addition, immunoprecipitation of the T antigen-associated topoisomerase by PAb 419, but not by PAb 421 (Fig. 4), suggests that the topoisomerase is an inherent activity of SV40 T antigen, although it is feasible that a cellular topoisomerase I initially co-purifying with T antigen is now being immunoprecipitated in a complex with T antigen.

It is assumed that if such an association of T antigen with topoisomerase I exists, then it is likely to have a functional role in the cell during SV40 DNA replication. Both T antigen, acting as a helicase (53), and type I DNA topoisomerase (54) have been shown to be present at SV40 DNA replication forks, but have not been shown to form a complex with one another. In addition to acting directly on the DNA at the replication forks as a helicase during elongation (2,3,13), T antigen may act indirectly by correctly positioning enzymes required for elongation, such as DNA polymerase α and topoisomerase, at the forks. In fact, it has been shown that DNA polymerase α binds to SV40 T antigen on a PAb 419-protein A sepharose immunoaffinity column and can be eluted with 50 to 150 mM KPO_4 (17). However, it should be noted that the topoisomerase-active T antigen is purified under conditions which are much more stringent, and therefore

less likely to maintain protein–protein interactions, than those used for the isolation of the T antigen-DNA polymerase α complex.

The co-sedimentation of the topoisomerase activity with purified T antigen on neutral sucrose gradients provides the strongest evidence that T antigen is a topoisomerase. If instead the relaxation activity were due to a cellular topoisomerase, the topoisomerase would have had to remain bound to T antigen not only throughout purification, but also throughout sedimentation. This is considered unlikely since it would mean that such a T-antigen-topoisomerase complex would have remained intact during binding to PAb 419-protein A sepharose, several high salt washes with 0.5M LiCl, ethylene glycol elutions, high pH elution at pH 10.8, and finally high speed centrifugation on a neutral sucrose gradient. Since the majority of T antigen purified from the baculovirus expression system by high pH elution is in the hexameric form, it is not clear if topoisomerase activity is dependent on T antigen being present as a hexamer or if it is also a property of monomeric and other oligomeric forms of T antigen. A study on T antigen extracted from SV40-transformed cells has shown that the monomeric versus dimeric and tetrameric forms of T antigen do in fact differ from one another in functional activity, at least with respect to SV40 DNA-binding and ATPase activity (55). Interestingly, T antigen assembles as hexamers and double hexamers at the SV40 origin of replication (12,56) and is present as a hexamer when unwinding a single replication fork or as a double hexamer when unwinding two replication forks during bidirectional DNA synthesis (53).

It should be noted that, after density gradient centrifugation, the topoisomerase activity peaks in fractions #21–22, whereas SV40 T antigen peaks in fractions #22–23, nearer the top of the gradient (Figure 7, bottom panels). There are several possible explanations for the peak of topoisomerase not exactly coinciding with the SV40 T antigen peak. If one molecule of a cellular topoisomerase is tightly complexed with some (but not all) of the T antigen hexamers, then a shift in density of these hexamers away from those not complexed with a cellular topoisomerase would be expected. However, a larger shift in density might have been expected than was actually observed, since topoisomerase I has a molecular weight in the range of 100 kDa (46) and topoisomerase II in the range of 170 kDa (57). Alternatively, the shift in density could be the result of a specific modification of some (but not all) of the T antigen hexamers. This modification in turn may be essential for activation of the topoisomerase activity of T antigen. If only a fraction of the T antigen molecules are appropriately modified, this would explain why stoichiometric amounts of T antigen appear to be necessary for relaxation of the substrate DNA (Figure 2). It may be significant that the topoisomerase activity peaks in the same region of the gradient as the high molecular weight form of T antigen noted previously (Figure 7, bottom panels). Clearly, this high molecular weight T antigen, representing a minor fraction of the total population of T antigen molecules, must be present in an unusual form since it is resistant to denaturation by boiling in SDS and β -mercaptoethanol.

In conclusion, the association of topoisomerase activity with SV40 T antigen is significant in that it provides the means by which initiation of SV40 DNA synthesis can undergo the transition to elongation and then the means by which elongation can proceed. The data argue in favor of the topoisomerase activity being inherent to T antigen. However, it is not yet possible to definitively conclude if T antigen itself is a topoisomerase or if

T antigen serves a mediator role in binding cellular topoisomerase I and locating it at the replication forks of elongating SV40 DNA. In view of the essential function provided by this relaxation activity in DNA replication, either possibility is considered important and worth further pursuit.

Addendum

These results have previously been reported in preliminary form (DNA Tumor Virus Meeting, Cold Spring Harbor, 1990). Similar results have recently been described by P.Bourgaux (DNA Tumor Virus Meeting, Cambridge, 1991).

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