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Roles of DNA topoisomerases in simian virus 40 DNA replication *in vitro*

(catenanes/topoisomerase inhibitors/chromosomal segregation)

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ABSTRACT **We examined the roles of DNA topoisomerases in the replication of simian virus 40 (SV40) DNA in a cell-free system composed of an extract from HeLa cells supplemented with purified SV40 tumor antigen. When the activities of both topoisomerase I (EC 5.99.1.2) and topoisomerase II (EC 5.99.1.3) in the extract were blocked with specific inhibitors or antibodies, DNA synthesis was decreased by a factor of 15–20. Addition of purified HeLa DNA topoisomerase II to extracts immunologically depleted of both topoisomerases completely restored replication, and the replication products consisted largely of monomeric daughter molecules. Addition of purified HeLa DNA topoisomerase I to depleted extracts restored DNA synthesis, but the primary products were multiply intertwined, catenated daughter molecules. We conclude that DNA topoisomerases have at least two roles in the replication of SV40 DNA. Either topoisomerase I or topoisomerase II is sufficient to provide the unlinking activity necessary for fork propagation during SV40 DNA replication. However, topoisomerase II is uniquely required for the segregation of newly synthesized daughter molecules.**

Two major mammalian DNA topoisomerases, topoisomerase I (EC 5.99.1.2) and topoisomerase II (EC 5.99.1.3), have been isolated and characterized (for recent reviews see refs. 1–3). In HeLa cells DNA topoisomerase I is a 100-kDa monomeric protein capable of relaxing both negative and positive superhelical twists by making a transient single-strand break (4). HeLa DNA topoisomerase II is a homodimer (monomer = 170 kDa) that catalyzes the passage of one duplex DNA segment through another via a transient double-strand break. The activity of topoisomerase II can lead to numerous topological interconversions of duplex circular DNA, including relaxation, formation of knots or catenanes, and resolution of knots or catenanes (5, 6).

Although there is good evidence in prokaryotic systems that topoisomerases play important roles in DNA replication, recombination, and transcription, much less is known about the functions of eukaryotic topoisomerases. Recently, yeast mutants defective in topoisomerase activities have been isolated and characterized (7–10). These genetic studies have implicated yeast DNA topoisomerase II in the segregation of daughter chromosomes at mitosis. Direct biochemical evidence for the involvement of eukaryotic topoisomerases in DNA replication or other cellular processes has been lacking.

The papovavirus simian virus 40 (SV40) has proven to be a useful model for studying the mechanisms of DNA replication in eukaryotic cells. The replication of SV40 DNA shares several features with the replication of cellular DNA (for recent reviews see refs. 11 and 12): (*i*) in the cell nucleus SV40 DNA is complexed with histones to form a minichromosome with a nucleoprotein structure that resembles cellular chromatin; (*ii*) SV40 DNA replication starts from a unique site and proceeds in a bidirectional fashion; (*iii*) SV40 DNA replication is mediated almost exclusively by cellular proteins, since the only required viral protein is the SV40 tumor (T) antigen. Recently, a cell-free system that supports SV40 DNA replication has been developed (13, 14). Replication in this system requires a DNA template containing the SV40 origin of DNA replication and is also dependent upon the presence of the SV40 T antigen. Cellular replication proteins are provided in the form of an extract derived from mammalian cells permissive for SV40 DNA replication *in vivo* (e.g., human or monkey). This system provides an approach to defining the functions of cellular proteins in DNA replication.

We have made use of the cell-free replication system to examine the possible roles of DNA topoisomerase I and DNA topoisomerase II in DNA replication. The availability of the purified topoisomerase proteins (4, 6) as well as specific inhibitors and antibodies (15–18) made it possible to study the ability of each topoisomerase to support DNA replication in the absence of the other. Our data indicate that either topoisomerase I or topoisomerase II can provide the topoisomerase activity necessary for efficient synthesis of progeny SV40 DNA molecules. However, topoisomerase II is uniquely required for the segregation of such newly synthesized molecules.

MATERIALS AND METHODS

Proteins and Enzymes. SV40 large T antigen was purified to homogeneity by immunoaffinity chromatography (19). Type I and type II HeLa DNA topoisomerases were purified to more than 95% homogeneity by modified procedures of Liu and Miller (4) and Miller *et al.* (6), respectively. Antiserum against purified DNA topoisomerase I from HeLa (human) cells was raised in rabbits, and IgG antibodies were purified from this serum by following the procedure of Halligan *et al.* (17). Antibodies against HeLa DNA topoisomerase II were prepared by using the same procedure.

Conditions for DNA Replication *in Vitro*. HeLa cytosolic extracts with protein concentrations of 15–20 mg/ml were prepared as described by Li and Kelly (14). Standard reaction mixtures (25 μl) (14) were incubated at 37°C for 90 min. Reactions were stopped by adding 20 μl of 2% $NaDodSO_4$/20 mM EDTA, containing proteinase K at 500 μg/ml and incubated at 37°C for an additional 60 min. DNA was precipitated twice by adding 30 μl of 7.5 M ammonium acetate, pH 7.5, and 120 μl of 95% (vol/vol) ethanol. Samples were washed twice with 95% ethanol and resuspended in 40 μl of TE buffer (10 mM Tris·HCl, pH 8.0/1 mM EDTA). In experiments in which the reaction products were enzymatically modified (Figs. 2–4), the products were further purified by extracting once with 1 vol of phenol and once with 5 vol

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Abbreviations: SV40, simian virus 40; T antigen, tumor antigen.

of diethyl ether. The DNA was then precipitated with ethanol and washed as described above.

Immunological Depletion of Topoisomerases I and II from HeLa Extracts. To 100 μl of extract (17 mg/ml), 50 μl of topoisomerase I antibody (rabbit IgG against purified HeLa topoisomerase I, 1 mg/ml) and 50 μl of topoisomerase II antibody (rabbit IgG against purified HeLa topoisomerase II, 1 mg/ml) were added. The mixture was incubated on ice for 20 min. Fifty microliters of staphylococcal protein A-Sepharose CL-4B (Pharmacia) with a binding capacity of 8 mg of human IgG per ml was added, and the incubation was continued for 30 min. After spinning 7 min in an Eppendorf centrifuge at 4°C, the supernatant was saved. The above steps were repeated and the final supernatant (4.2 mg/ml) was used in the assay.

Nicking of Multiply Intertwined Catenated Dimers. *Nicking the DNA by using DNase I.* Reaction mixtures (25 μl) contained 20 ng of purified replication products, 2 μg of control DNA (supercoiled pSV2 DNA), 10 μg of ethidium bromide, 1 μg of DNase I, 50 mM Tris·HCl at pH 7.5, 10 mM $MgCl_2$, 0.1 mM dithiothreitol, and bovine serum albumin at 50 μg/ml. Digestion was carried out at 25°C for 30 min and stopped by adding 5 μl of 5 M NaCl.

Nicking the DNA by using topoisomerase I and camptothecin. The procedure described by Hsiang *et al.* (15) was followed. A 25-μl reaction mixture containing 20 ng of purified replication products, 4 μg of topoisomerase I, 100 μM camptothecin, 50 mM Tris·HCl at pH 7.5, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA, and bovine serum albumin at 30 μg/ml was incubated at 37°C for 30 min and stopped by adding 1 μl of 20% $NaDodSO_4$. The mixture was treated with 10 μg of proteinase K at 37°C for 90 min and then extracted with phenol.

RESULTS

Topoisomerase Activity Is Required for SV40 DNA Replication *in Vitro*. To assess the possible roles of DNA topoisomerases I and II in the replication of SV40 DNA, we initially studied the effects of specific topoisomerase inhibitors and antibodies on viral DNA synthesis *in vitro*. For this purpose we made use of a cell-free system capable of replicating plasmid DNA templates that contain the SV40 origin of DNA replication (13, 14). This system consists of a cytosolic HeLa cell extract supplemented with purified SV40 large T antigen. In a preliminary experiment we measured the effects of the inhibitors and antibodies on topoisomerase activity in the HeLa cell extract. The untreated extract contains approximately 20% of the total nuclear topoisomerase I and II activities (data not shown). Addition of 100 μM camptothecin, a specific inhibitor of mammalian DNA topoisomerase I (15), reduced topoisomerase I activity by a factor of approximately 25 (data not shown). Similarly, when 100 μM epipodophyllotoxin VM-26, a specific inhibitor of mammalian DNA topoisomerase II (16, 18) was added to the extract, the topoisomerase II activity declined by a factor of approximately 50 (data not shown). These results indicate that the inhibitors can significantly reduce the activities of topoisomerases I and II in the HeLa cell extracts.

DNA replication in the cell-free system was assayed by using the plasmid pJLO, which contains the complete SV40 origin of DNA replication. In the absence of any inhibitors two major species of replication products were observed by gel electrophoresis (Fig. 1, lane B). Both of these species have been characterized previously (13, 14, 20, 21). The faster-migrating species represent circular monomers of pJLO, and they appear as a ladder of topological isomers ranging from relaxed to more highly negatively supercoiled forms. These products have successfully completed all the stages of replication, including initiation, elongation, termination, and separation of the daughter molecules. The high

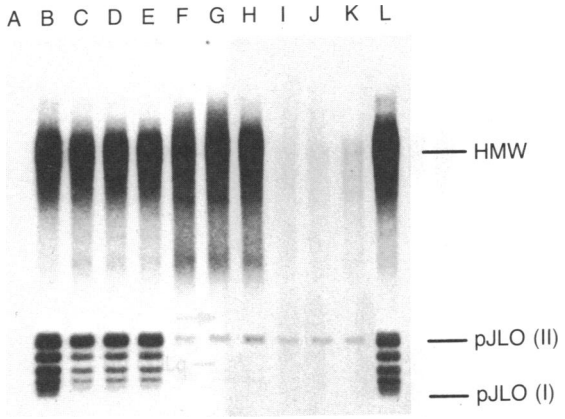

FIG. 1. Inhibition of SV40 DNA replication *in vitro* by inhibitors of DNA topoisomerases I and II. SV40 DNA was synthesized in standard reaction mixtures containing T antigen, HeLa extract, and pJLO DNA. The reaction mixtures were incubated 90 min in the presence or absence of topoisomerase inhibitors. Lane A, T antigen omitted; lane B, no inhibitors added: lane C, 100 μM camptothecin; lane D, 33 μM camptothecin; lane E, 11 μM camptothecin; lane F, 100 μM VM-26; lane G, 33 μM VM-26; lane H, 11 μM VM-26; lane I, 100 μM camptothecin and 100 μM VM-26; lane J, 33 μM camptothecin and 33 μM VM-26; lane K, 11 μM camptothecin and 11 μM VM-26; lane L, 1.5% (vol/vol) dimethyl sulfoxide, the solvent for both inhibitors. Electrophoresis was carried out in 0.8% agarose in 80 mM Tris phosphate, pH 8.0/2 mM EDTA at 25°C at 1 V/cm for 10 hr. HMW, high molecular weight products; pJLO (II), nicked pJLO DNA; pJLO (I), highly supercoiled pJLO DNA.

molecular weight DNA above the monomer ladder consists largely of rolling-circle molecules (14). We have suggested that these molecules represent side-products of the normal replication cycle, which may be generated by the action of nucleases present in the HeLa cell extract (14).

As shown in Fig. 1, inhibition of topoisomerase I with camptothecin resulted in a slightly reduced level of DNA replication in the cell-free system. However, both circular monomers and high molecular weight products were observed among the products (Fig. 1, lanes C–E). Inhibition of topoisomerase II with VM-26 resulted in a profound decrease in the amount of circular monomers and a similar increase in the amount of slowly migrating DNA forms (Fig. 1, lanes F–H). When both topoisomerase inhibitors were added simultaneously, DNA synthesis in the cell-free system was abolished (Fig. 1, lanes I–K). Qualitatively similar results were obtained in parallel experiments using anti-topoisomerase antibodies (data not shown). These data demonstrate that topoisomerase activity is essential for SV40 DNA replication *in vitro*.

DNA Topoisomerase II Can Unlink the Two Parental Strands During the Entire Process of DNA Replication. To analyze more thoroughly the individual roles of each topoisomerase in SV40 DNA replication, we depleted HeLa cell extract of both topoisomerases by using anti-topoisomerase antibodies. We then studied the ability of the depleted extract to support SV40 DNA replication *in vitro* in the presence of highly purified topoisomerase I or topoisomerase II. Direct analysis of the topoisomerase activities confirmed the effectiveness of the immunological depletion technique (data not shown). The final levels of topoisomerase I and topoisomerase II activities in the depleted extract were less than 5% of the levels in the original extract. As expected from the inhibitor studies, the depleted extract was unable to support significant SV40 DNA replication *in vitro* (Fig. 2, compare lanes B and F). However, the addition of purified HeLa topoisomerase II to the depleted extract restored efficient DNA replication (Fig. 2, lane J). Furthermore, a major fraction of the products of this reconstituted reaction consisted of

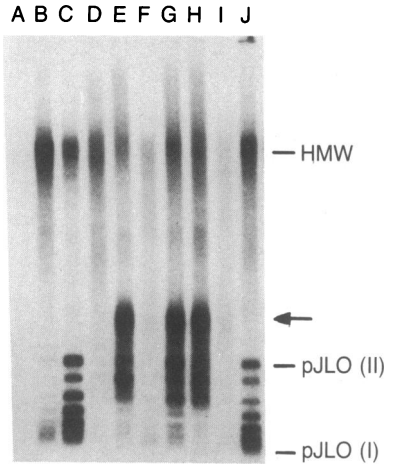

FIG. 2. Reconstitution of replication activity in topoisomerase-depleted extracts by addition of purified HeLa DNA topoisomerases. Aliquots of HeLa cell extract were depleted of topoisomerase I and II activities by using anti-topoisomerase antibodies and protein A-Sepharose. Standard replication reaction mixtures containing T antigen and pJLO DNA were incubated in the presence or absence of purified HeLa topoisomerase as indicated. In some reconstitution experiments camptothecin or VM-26 was included in the reaction mixture to inhibit any residual topoisomerase I or topoisomerase II activity, respectively. Reaction mixtures shown in lanes A–E contained mock-depleted extract. Lane A, T antigen omitted; lane B, no topoisomerase added; lane C, 500 units (40 ng) of purified topoisomerase I; lane D, 100 μM VM-26 (topoisomerase II inhibitor); lane E, 500 units of purified topoisomerase I plus 100 μM VM-26. Reaction mixtures shown in lanes F–J contained depleted extract. Lane F, no topoisomerase added; lane G, 500 units of purified topoisomerase I; lane H, 500 units of purified topoisomerase I and 100 μM VM-26; lane I, 100 μM VM-26; lane J, 20 units (160 ng) of purified topoisomerase II and 100 μM camptothecin (topoisomerase I inhibitor). Electrophoresis was carried out in 1% agarose in Mg-TBE buffer (89 mM Tris borate, pH 8.0/5 mM $MgCl_2$/2 mM EDTA) at 4°C at 3 V/cm for 20 hr.

circular monomers. We conclude that topoisomerase II is sufficient to provide all of the topoisomerase functions required for the complete replication of SV40 DNA. It is of interest that the extent of replication observed in the reconstituted reaction was significantly greater than that generally observed with a standard HeLa cell crude extract (e.g., Fig. 2, lane B). This observation suggested that the extent of replication in crude extracts is limited by the level of topoisomerase activity. In confirmation of this suggestion, we have shown that addition of purified topoisomerase I or topoisomerase II stimulates the replication activity of such extracts to a level comparable to that observed in the reconstituted reaction mixture (e.g., Fig. 2, lane C).

DNA Topoisomerase I Alone Is Insufficient for the Segregation of Newly Synthesized Daughter Molecules. The addition of purified HeLa topoisomerase I to the depleted extract also resulted in restoration of efficient DNA synthesis in the cell-free system (Fig. 2, lane G). In fact, the level of DNA synthesis attained with topoisomerase I was approximately the same as that previously observed with topoisomerase II (Fig. 2, compare lanes G and J). However, in contrast to the results obtained with topoisomerase II, the major products synthesized in reaction mixtures reconstituted with topoisomerase I did not display the characteristic ladder of bands expected for circular monomers of pJLO. Rather, they consisted of a heterogeneous population of molecules whose average mobility was significantly less than that of the monomers. Similar products were observed when reaction mixtures containing either depleted or undepleted extract were incubated in the presence of purified topoisomerase I and VM-26, the inhibitor of topoisomerase II (Fig. 2, lanes E and H). Thus, the generation of this novel class of replication products required both the presence of topoisomerase I and the absence of topoisomerase II. Moreover, DNA synthesis in all of these reaction mixtures was completely dependent upon the presence of T antigen and a template containing the SV40 origin of DNA replication (data not shown). We conclude from these results that topoisomerase I is sufficient to allow extensive SV40 DNA synthesis, but is not sufficient to allow the formation of monomeric products. [The residual monomer replication products observed when topoisomerase I was added to depleted extracts (Fig. 2, lane G) were most likely due to the presence of residual topoisomerase II activity, since the monomers were eliminated when VM-26 was added (Fig. 2, lane H).]

Several experiments were performed to define the nature of the novel products synthesized in reaction mixtures reconstituted with topoisomerase I. As shown in Fig. 3, incubation of the isolated reaction products with topoisomerase II quantitatively converted them to relaxed circular monomers. This result demonstrates that the products consist of newly synthesized pJLO molecules that are topologically linked. When the same product molecules were nicked by incubation with DNase I or with topoisomerase I in the presence of camptothecin (15), they were converted to a ladder of bands migrating between the high molecular weight DNA and the circular monomer DNA (Fig. 4*A*, lanes C and F). Partial treatment of the nicked samples with topoisomerase II caused a shift in the distribution within the ladder toward species with lower mobilities (Fig. 4*A*, lanes D and

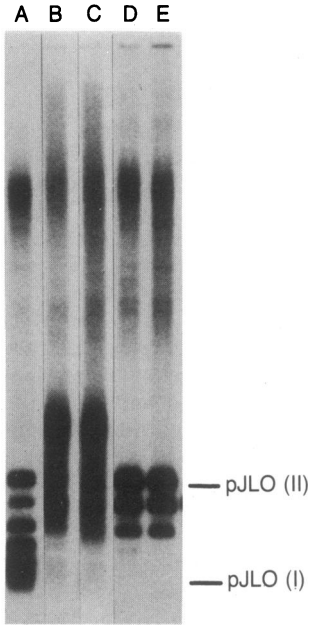

FIG. 3. The novel replication products synthesized in the presence of topoisomerase I alone are topologically linked circular monomers. The products of replication reaction mixtures containing topoisomerase I as the only topoisomerase activity (samples shown in Fig. 2, lanes E and H) were isolated and treated with topoisomerase II. Lane A, mobility markers (products of a standard replication reaction supplemented with purified topoisomerase I); lane B, products of reaction mixture containing undepleted HeLa cell extract, purified topoisomerase I, and the topoisomerase II inhibitor VM-26 (same sample as Fig. 2, lane E); lane C, products of reaction mixture containing depleted HeLa cell extract, purified topoisomerase I, and the topoisomerase II inhibitor VM-26 (same sample as Fig. 2, lane H); lane D, sample shown in lane B after exhaustive treatment with topoisomerase II (15 units of enzyme at 37°C for 60 min); lane E, sample shown in lane C after exhaustive treatment with topoisomerase II. Electrophoresis conditions were as described in the legend of Fig. 2.

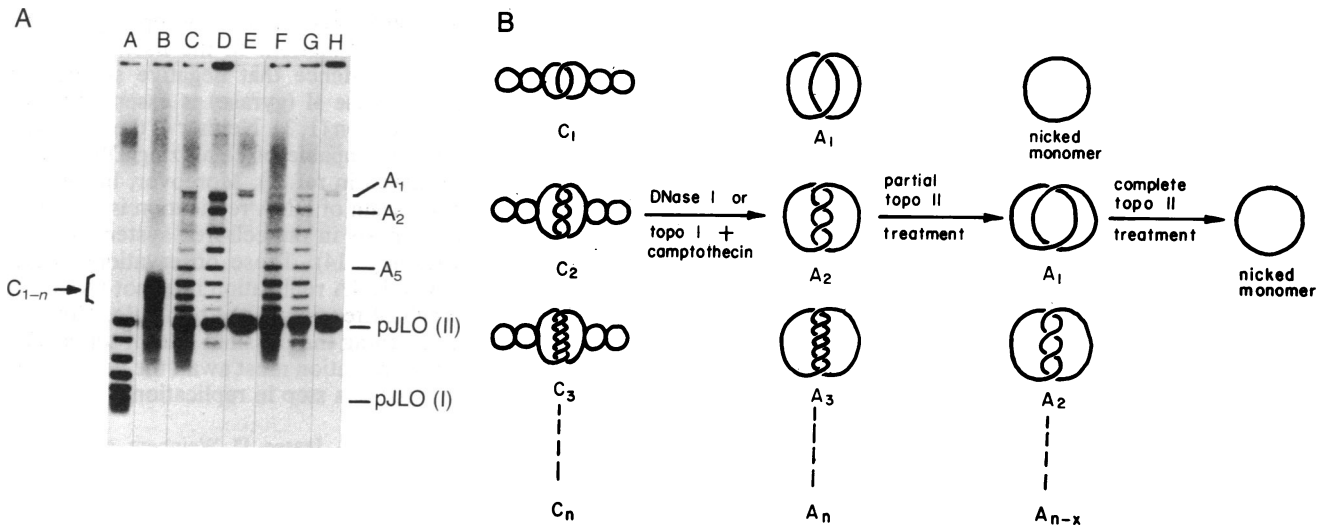

FIG. 4. Identification of the novel replication products as multiply intertwined catenated dimers. (*A*) Characterization of the catenated replication products synthesized in the presence of topoisomerase I alone. The products of a replication reaction mixture containing depleted HeLa cell extract, purified topoisomerase I, and the topoisomerase II inhibitor VM-26 (the same reaction mixture as in Fig. 2, lane H) were isolated and treated as indicated. Lane A, mobility markers (products of a standard replication reaction mixture supplemented with purified topoisomerase I); lane B, untreated sample (same as Fig. 2, lane H); lane C, sample shown in lane B after nicking with DNase I; lane D, sample shown in lane C after partial treatment with topoisomerase II (2 units of enzyme at 37°C for 30 min); lane E, sample shown in lane C after complete treatment with topoisomerase II (15 units of enzyme at 37°C for 60 min); lane F, sample shown in lane B after nicking by treatment with topoisomerase I and camptothecin; lane G, sample shown in lane F after partial treatment with topoisomerase II; lane H, sample shown in lane F after complete treatment with topoisomerase II. A_1, A_2, and A_5 indicate nicked daughter molecules with one, two, and five intertwines, respectively. C_{1-n} indicates covalently closed daughter molecules with one to *n* intertwines. Electrophoresis conditions were as described in the legend of Fig. 2. (*B*) Schematic interpretation of the experiment described in *A* (see text for details).

G), while prolonged treatment with topoisomerase II converted virtually all of the molecules in the ladder to a single species migrating at the position of nicked circular monomers (Fig. 4*A*, lanes E and H). These results strongly suggest that the novel products synthesized in replication reaction mixtures containing topoisomerase I as the only topoisomerase activity are covalently closed, catenated dimers. Molecules with identical properties were previously identified by Sundin and Varshavsky (22, 23) in studies of SV40 DNA replication *in vivo* under hypertonic conditions. As illustrated in the interpretive drawing of Fig. 4*B*, nicking of supercoiled catenated dimers (form C molecules of Sundin and Varshavsky) converts the molecules to open forms that migrate more slowly during gel electrophoresis (form A molecules). Because the dimers differ in the degree of intertwining between their two constituent monomers, they can be resolved into a ladder of bands with the least highly intertwined molecules migrating with the lowest mobility. Partial treatment of the form A molecules with topoisomerase II decreases the average degree of intertwining and, therefore, shifts the distribution within this ladder toward the lower-mobility forms. Complete treatment with topoisomerase II removes all interlocking and quantitatively converts the form A molecules to nicked monomer circles.

The identification of the novel reaction products as multiply intertwined catenated dimers indicates that DNA topoisomerase I can support all of the steps in SV40 DNA replication up to and including the completion of the two daughter molecules. However, when topoisomerase I is the only topoisomerase activity present, the newly synthesized progeny molecules remain topologically linked. Thus, the segregation of daughter molecules appears to be a unique function of topoisomerase II.

DISCUSSION

Replication of circular DNA molecules presents a topological problem that is independent of the process of DNA polymerization. Two intertwined parental DNA strands must be completely unlinked in order to give rise to two separate daughter molecules. There are two distinct aspects to this problem. First, the advancement of the replication fork(s) must be accompanied by a progressive decrease in the linking number of the parental strands. Otherwise the accumulation of superhelical turns induced by unwinding of the parental helix would make further fork movement energetically unfavorable. Second, there must be a mechanism to ensure the efficient segregation of the newly synthesized daughter duplexes. If the links between the two parental strands are not completely removed prior to the completion of DNA synthesis, the immediate products of replication will be catenated dimers, which must then be resolved into separate monomers. The same considerations may hold for the replication of eukaryotic chromosomes. Although chromosomal DNA molecules are linear, their extreme length, coupled with the existence of multiple replicons, raises topological problems that are similar to those encountered by closed circular molecules. In both prokaryotic and eukaryotic cells two classes of topoisomerases have been identified that are potentially capable of mediating the topological interconversions essential for DNA replication.

The investigation reported in this paper is a direct biochemical examination of the roles of topoisomerases in eukaryotic DNA replication. Our results, summarized in Fig. 5, indicate that either DNA topoisomerase I or DNA topoisomerase II is sufficient to provide the unlinking activity

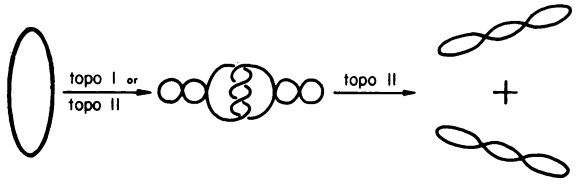

FIG. 5. Simple model for the roles of DNA topoisomerases in SV40 DNA replication *in vitro*.

required for fork propagation during SV40 DNA replication. In fact, in the presence of either topoisomerase, replication can proceed to the completion of covalently closed progeny circles. However, the newly synthesized daughter molecules are normally multiply intertwined and their resolution into separate monomer circles can be accomplished only by topoisomerase II. Thus, topoisomerase II is capable of mediating all of the topological interconversions required during DNA replication, while topoisomerase I lacks only the ability to resolve the catenated products of replication.

Our data on the roles of mammalian topoisomerases in DNA replication are in accord with the known enzymatic properties of these enzymes (1, 3). However, it should be noted that eukaryotic topoisomerase I, like its prokaryotic counterpart, is capable of resolving catenated dimers into separate circular monomers providing that at least one of the two circles contains a single-strand nick or gap (24). This pathway for decatenation does not appear to be utilized to any significant extent during SV40 DNA replication in the cell-free system, although we cannot rule out the possibility that the pathway might be operative under other experimental conditions (25).

The biochemical data presented in this paper are consistent with recent genetic studies of topoisomerase function in yeast. Analysis of temperature-sensitive topoisomerase II (*top2*) mutants has demonstrated that topoisomerase II is essential for viability (7, 9, 26). When synchronized mutant cells are grown at the nonpermissive temperature they undergo only a single round of DNA synthesis and then become arrested at medial nuclear division. The 2-μm plasmid DNA isolated from such arrested cells consists of multiply intertwined catenated dimers (7). These results suggest that topoisomerase II is required for segregation of daughter chromosomes in yeast. Similar analysis of yeast topoisomerase I (*top1*) mutants has not revealed any essential function for this enzyme (8, 9). However, inactivation of both topoisomerases in a *top1 top2* double mutant results in an immediate arrest of cell growth independent of the stage of the cell cycle (9, 26, 27), suggesting that some form of topoisomerase activity is continuously required for cell viability.

We have observed that the extent of DNA replication in the cell-free SV40 DNA replication system is limited by the level of topoisomerase activity. Addition of either topoisomerase to the crude extract stimulates DNA replication *in vitro*. It seems likely that this effect is due to an enhanced rate of chain elongation resulting from an increased efficiency of unlinking of the parental DNA strands; however, we cannot rule out more complex explanations. The relatively low levels of topoisomerase activity in crude extracts probably also explain why large numbers of catenated dimers are not observed when the topoisomerase II inhibitor VM-26 is added to standard replication reaction mixtures (Fig. 1, lanes F–H). In the presence of the inhibitor the reduced rate of chain elongation presumably results in a reduced rate of accumulation of completed molecules. This possibility is supported by the observation that addition of purified topoisomerase I to VM-26-treated extracts results in substantial accumulation of catenated dimers (Fig. 2, lane E).

While it seems clear that DNA topoisomerases are involved in the unlinking of the parental strands during chain elongation and in the segregation of daughter duplexes, our data do not provide direct information concerning a possible role for these enzymes in the initiation of SV40 DNA replication. However, if DNA topoisomerases are involved in the initiation reaction, it is evident from the experiments reported here that either topoisomerase I or topoisomerase II can provide the required function. In the *Escherichia coli* system there is good evidence that negative supercoiling catalyzed by topoisomerase II (gyrase) is essential for the initiation of DNA replication (1, 3). Neither of the eukaryotic topoisomerases appears capable of catalyzing DNA supercoiling *in vitro* (reviewed in ref. 3). Moreover, linear DNA containing the SV40 origin of DNA replication is capable of supporting DNA synthesis in the cell-free system, albeit at relatively low efficiency (14). These observations suggest that initiation of SV40 DNA replication may not require the presence of superhelical tension in the template. However, direct evidence concerning the possible role of topoisomerases or supercoiling in initiation must await the development of an assay specific for this step in replication.

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