Topoisomerase I Is Preferentially Associated with Isolated Replicating Simian Virus 40 Molecules after Treatment of Infected Cells with Camptothecin

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Detergent extraction of simian virus 40 (SV40) DNA from infected monkey CV-1 cells, after a brief exposure to the drug camptothecin, yields covalent complexes between topoisomerase I and DNA that band with reduced buoyant densities in CsCl. The following lines of evidence indicate that the enzyme is preferentially associated with SV40 replicative intermediates. First, the percentage of the isolated labeled viral DNA that exhibited a reduced buoyant density is inversely proportional to the length of the labeling period and approximately parallels the percentage of replicative intermediates for each labeling time (5 to 60 min). Second, after labeling for 60 min, the isolated low-density material was found to be enriched for replicative intermediates as measured by sedimentation in neutral sucrose. Third, analysis of extracted viral DNA by equilibrium centrifugation in CsCl-propidium diiodide gradients that separate replicating molecules from completed form I DNA revealed that camptothecin pretreatment specifically caused the linkage of topoisomerase I to replicating molecules. In addition, analysis of the low-density material obtained under conditions when only the newly synthesized strands of the replicative intermediates were labeled showed that the enzyme was associated almost exclusively with the parental strands. Taken together, these observations indicate that topoisomerase I is involved in DNA replication, and they are consistent with the hypothesis that the enzyme provides swivels to allow the helix to unwind. The observed bias in the distribution of topoisomerase I on intracellular SV40 DNA could be the result of rapid encapsidation of replicated molecules that precludes the association of topoisomerase I with the DNA or, alternatively, the result of a specific association of the enzyme with replicative intermediates.

DNA topoisomerases catalyze the interconversion of topological forms of DNA by introducing transient breaks in either one or both strands of the DNA (see reference 36 for a review). The enzymes belonging to the type I subclass introduce a transient single-strand break in duplex DNA. The reaction involves a nicked intermediate containing enzyme covalently bound to one end of the broken strand (7). The type II enzymes create transient double-strand breaks in DNA by the staggered breakage of both strands with attachment of an enzyme subunit to one of the ends at each break site. The type II enzymes change the linking number of the DNA by passing another region of the same molecule through the temporary double-strand break.

Genetic studies combined with the results of experiments involving enzyme inhibitors have helped elucidate the roles of both types of topoisomerases in bacteria. It is clear that in *Escherichia coli* the DNA gyrase, a type II enzyme that couples the hydrolysis of ATP to the generation of negative supercoils, is responsible for maintaining torsional strain in the bacterial chromosome (12, 28). The type I enzyme acts to counteract the action of the gyrase and prevents excessive supercoiling of the DNA. In addition, the DNA gyrase is responsible for decatenating daughter DNA circles at the end of a replication cycle (33). Additional roles for one or both enzymes in providing swivels for DNA replication and transcription have been postulated, but clear-cut evidence in support of these hypotheses is lacking.

In eucaryotic cells the known type II enzyme appears to be responsible for decatenating newly replicated circular molecules (11, 37). The strand-passing activity of the type II enzyme is probably also responsible for resolving intertwined linear duplexes present at the end of chromosome

replication (19). The in vivo role of the type I enzyme in eucaryotes is less clear. At the time of the discovery of the eucaryotic type I enzyme, it was hypothesized that the activity might provide swivels to allow the DNA helix to unwind during DNA replication (9). Recent evidence is consistent with this hypothesis. In *Saccharomyces cerevisiae*, for example, the type I enzyme appears to be dispensable, but genetic evidence suggests that either the type I enzyme or the type II enzyme is required for DNA replication (6). Similarly, recent evidence implicates the type I enzyme in simian virus 40 (SV40) replication in vitro (37). The type I enzyme has been found associated with actively transcribed DNA (5, 6, 13, 15–17, 34), suggesting that it may also provide swivels for DNA transcription.

Camptothecin (CTT) is a highly cytotoxic alkaloid that inhibits both DNA and RNA synthesis in eucaryotic cells (1, 21). The cellular target of the drug appears to be the type I topoisomerase (2). The results of in vitro studies have revealed that the drug affects the interaction of the enzyme with its DNA substrate to greatly increase the proportion of enzyme molecules trapped in a covalent complex with DNA when the reactions are stopped with detergent (22). Although the drug inhibits the relaxing activity of the enzyme, substantially higher levels are required to observe inhibition than to observe an effect on the formation of the covalent complex after detergent treatment.

In the present study, CTT is used to examine the interaction of the monkey cell type I topoisomerase with SV40 DNA in vivo. The results indicate that the enzyme is associated with replicating SV40 DNA, providing direct evidence that it is involved in DNA replication in vivo.

MATERIALS AND METHODS

Cell culture and virus infections. The CV-1 African green monkey cell line was grown on plastic dishes (diameter, 5 cm; Lux Scientific Instrument Corp.) in 5 ml of Dulbecco modified Eagle medium containing 10% newborn calf serum (GIBCO Laboratories). Confluent plates were infected with SV40 (a clone of strain VA45-54 obtained from P. Tegtmeyer [35]) at a multiplicity of approximately 20 PFU per cell. DNA was labeled for the indicated times at 40 to 46 h postinfection by replacing the growth medium with 2 ml of serum-free medium containing 10 μCi of [^{3}H]thymidine per ml (20 Ci/mmol; Du Pont, NEN Research Products). CTT lactone (NSC 94600; Natural Products Division, National Cancer Institute, Bethesda, Md.) was stored as a 10 mM stock solution in dimethyl sulfoxide at $-20°$C and, when indicated, added to the labeling medium at a final concentration of 10 μM.

The procedure for isolating ^{3}H- or ^{14}C-labeled or unlabeled SV40 DNA has been described previously (10).

Extraction of viral DNA. The viral DNA was extracted by the method of Hirt (18). The cells were lysed on the plate by the addition of 0.4 ml of a solution containing 50 mM Tris hydrochloride (pH 7.5), 10 mM EDTA, and 0.6% sodium dodecyl sulfate (SDS). After 10 min at 20°C, 0.1 ml of 5 M NaCl was added. The solutions were mixed on the plate by gentle rocking, and the lysate was poured into a 1.5-ml Eppendorf tube. The cellular DNA was removed by centrifugation for 15 min in an Eppendorf microcentrifuge, and the Hirt supernatant was analyzed by equilibrium centrifugation as described below. When indicated, the Hirt supernatant was treated with proteinase K (50 μg/ml) for 30 min at 37°C prior to the subsequent analysis steps.

Neutral CsCl equilibrium centrifugation. The Hirt supernatant was diluted with 10 mM Tris hydrochloride (pH 7.5) and 1 mM EDTA to 4 ml, and 4.8 g of CsCl was added (final density, 1.67 g/ml). ^{14}C-labeled SV40 DNA was added as a marker, and the sample was centrifuged in a Beckman VTi 65 rotor at 220,000 $\times$ g and 20°C for 16 h. Fractions were collected onto Whatman 3MM filter disks (diameter, 2.3 cm). The filters were washed with 5% trichloroacetic acid followed by 95% ethanol, and the radioactivity was counted by using a liquid scintillation counter with a toluene-based scintillation fluid. The percentage of the labeled material exhibiting a reduced buoyant density (see Fig. 2a [shaded area]) was determined by cutting out the two areas from photocopies of the original graph and weighing them.

Centrifugation of samples for the preparative isolation of low-density material followed the same procedure, except that the fractions were collected into plastic centrifuge tubes and a sample was spotted onto 3MM filters to locate the radioactive peaks in the gradient. The fractions containing the low-density material were pooled, treated with proteinase K when indicated, and dialyzed to remove the CsCl. After phenol and chloroform extraction, unlabeled SV40 DNA or bovine serum albumin was added as a carrier, and the material was ethanol precipitated.

Alkaline CsCl equilibrium centrifugation. The fractions from the neutral CsCl gradient containing the low-density material were combined, and the DNA in 0.6 ml of the pool was denatured by the addition of 1.2 ml of 0.5 M Na$_3$PO$_4$ and 50 μl of 1 M NaOH. ^{14}C-labeled SV40 DNA cut with *Bam*HI (New England BioLabs, Inc.) was added as a single-strand marker, and the centrifuge tube was filled with a 60% (wt/wt) solution of CsCl in H$_2$O. The samples were centrifuged in a VTi 65 rotor at 220,000 $\times$ g and 20°C for 16 h. Fractions were

collected and processed for scintillation counting as described above.

CsCl-propidium diiodide equilibrium centrifugation. Propidium diiodide is an intercalating dye that binds to DNA in the same way as ethidium bromide but allows a greater separation of form I and form II DNAs when present during CsCl centrifugation (9). The Hirt supernatant was diluted to 3.6 ml with 10 mM Tris hydrochloride (pH 7.5) and 10 mM EDTA, and 3.5 g of CsCl was added. After the CsCl had dissolved, 0.63 ml of a 4-mg/ml propidium diiodide solution was added, and the samples were centrifuged to equilibrium in a VTi 65 rotor at 220,000 $\times$ g and 20°C for 16 h. Fractions were collected and processed for scintillation counting as described above.

SV40 replicative intermediates are distributed in a CsCl-propidium diiodide gradient at intermediate densities between closed-circular form I DNA and nicked form II DNA. The percentage of the total radioactive label in a CsCl-propidium diiodide gradient representing replicative intermediates was estimated by summing all of the counts banding at positions less dense than those of form I DNA. Since the replicative intermediates overlap the form II DNA, this method of determining the percentage of replicative intermediates for any given labeling period is an overestimate by an amount equal to the fraction of the total label present in the form II molecules. Although it is not experimentally possible to factor out the contribution of form II molecules to the total, estimates based on the amount of form II present in long labeling periods suggest that the error introduced by this method probably does not exceed 15%.

Neutral sucrose gradient centrifugation. The ethanol precipitate of the purified low-density material (see above) was dissolved in 0.10 ml of 10 mM Tris hydrochloride (pH 7.5)–10 mM EDTA. ^{14}C-labeled form I marker SV40 DNA was added to the sample, and the mixture was layered onto a 5 to 20% sucrose gradient containing 20 mM Tris hydrochloride (pH 7.5), 1 mM EDTA, and 1 M NaCl. In some experiments, the Hirt supernatant was treated with proteinase K and layered directly onto the sucrose gradient. The gradients were centrifuged in a Beckman SW 60 rotor at 311,000 $\times$ g and 20°C for 2.3 h. Fractions were collected and processed for scintillation counting as described above. Where two peaks overlapped, separate peaks were reconstructed on the basis of the total counts and the profile of the pure marker in the same gradient. The relative amounts of the two species were determined by cutting out the separate peaks from photocopies of the original graph and weighing them.

5' end labeling and alkaline agarose gel analysis. The low-density material from the neutral CsCl gradient was isolated as described above with bovine serum albumin as a carrier in the ethanol precipitation step. The DNA was denatured by making the sample 0.25 M NaOH. After neutralization, 5' ends were phosphorylated with polynucleotide kinase (U.S. Biochemical Corp.) and [γ-^{32}P]ATP (3,000 Ci/mmol; Du Pont, NEN) under the conditions recommended by the manufacturers, except that the reactions were carried out at 0°C to minimize the exchange reaction. The reactions were stopped by the addition of excess EDTA, and the mixtures were incubated at 65°C in 0.3 M NaOH for 20 min to hydrolyze any contaminating RNA fragments. The samples were precipitated with ethanol in the presence of 2 M ammonium acetate to remove unincorporated ATP.

Alkaline agarose gels were run as previously described (4). The lanes containing the unlabeled size markers (*Bam*HI-cut SV40 DNA and *Hin*fI fragments of SV40) were cut off, the gel was neutralized, and the marker bands were located by
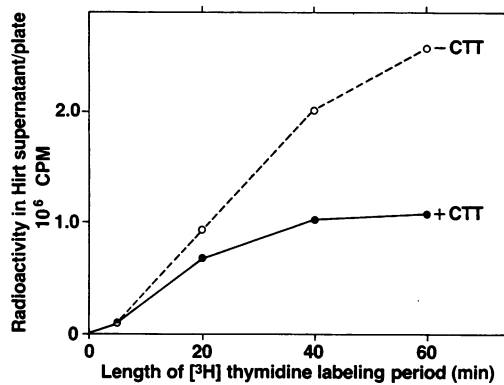
FIG. 1. Effect of CTT on SV40 DNA replication. SV40-infected CV-1 cells were labeled with [³H]thymidine in the presence (●) or absence (○) of 10 μM CTT for the indicated times. The total amount of labeled viral DNA in the Hirt supernatant was determined by trichloroacetic acid precipitation of a sample on a glass filter.

ethidium bromide staining. The remainder of the gel was dried and exposed to Kodak XAR-2 film for autoradiography.

RESULTS

CTT induces covalent attachment of protein to replicating or newly replicated SV40 DNA. CTT inhibits the incorporation of [³H]thymidine into SV40 DNA, with the inhibition being complete by about 40 min after addition of the drug (Fig. 1). Prolonged exposure of SV40-infected cells to CTT (15 min) has previously been shown to cause the accumulation of broken replicative intermediates as determined by agarose gel electrophoresis (31). In addition, treatment of cells for long periods with CTT causes the appearance of protein-free single-strand breaks (25; J. J. Champoux, unpublished results). These results are consistent with the early findings showing that CTT introduces single-strand breaks in chromosomal DNA (20, 32). To avoid what may be secondary effects of the drug in the experiments described below, infected cells were exposed to the drug for only 5 min just before the DNA was extracted with SDS. CTT was found to have no effect on the rate of SV40 DNA synthesis in the first 5 min after addition (Fig. 1).

CV-1 cells were infected with SV40 and pulse-labeled with [³H]thymidine for 5 min at 44 h after infection in either the presence or the absence of 10 μM CTT. It has been shown previously that only replicative intermediates are labeled in a 5-min pulse (24, 26). The cells were lysed with SDS by the procedure of Hirt (18), and the Hirt supernatant was banded to equilibrium in a CsCl gradient. The distribution of ³H radioactivity in the gradient for the sample labeled in the presence of CTT is shown in Fig. 2a. Approximately 60% of the ³H radioactivity banded in the gradient at a position less dense than that of the native ¹⁴C marker SV40 DNA (Fig. 2a, shaded area). In the control sample labeled in the absence of CTT, all of the ³H radioactivity cobanded with the ¹⁴C marker (data not shown; see Table 1). Virtually no label was released into the Hirt supernatant when uninfected CV-1 cells were similarly pulse-labeled in the presence of CTT, demonstrating that the observed label represents viral DNA. Treatment of the Hirt supernatant with proteinase K prior to the CsCl gradient analysis shifted all of the low-density material to the position of the marker DNA (Fig. 2b), demonstrating that bound protein was responsible for the
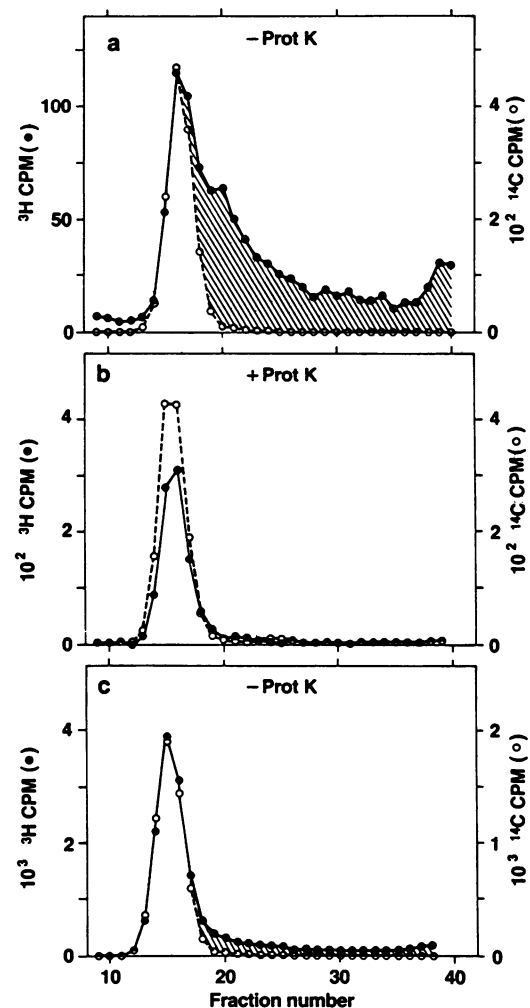

FIG. 2. Analysis of SV40 DNA-protein complexes by equilibrium centrifugation in neutral CsCl. SV40-infected cells were labeled with [³H]thymidine for 5 min in the presence of 10 μM CTT, and the labeled viral DNA was isolated by the Hirt extraction method. The Hirt supernatant was banded directly in a neutral CsCl gradient (a) or treated with proteinase K (Prot K) and banded in CsCl (b). Similarly, DNA was analyzed, without proteinase K treatment, from infected cells that had been labeled continuously for 60 min with the addition of 10 μM CTT during the last 5 min of the labeling period (c). The shaded areas identify the fractions containing ³H-labeled DNA (●) that exhibited a buoyant density less than that of the ¹⁴C-labeled marker SV40 DNA (○).

reduction in buoyant density. The most likely interpretation of these results is that SDS lysis of CTT-treated infected cells induced covalent attachment of the type I topoisomerase to the DNA, resulting in a DNA-protein complex with a buoyant density less than that of native DNA. Using a filter-binding assay, Mattern et al. (25) have similarly found that DNA extracted from CTT-treated cells contains bound protein.

The buoyant density of native SV40 DNA is 1.700 g/ml. Knowing the molecular weights of SV40 DNA (3.46×10^6) and topoisomerase I (ca. 1×10^5) and assuming a density of 1.29 g/ml for protein, it is possible to predict the buoyant density of a 1:1 complex between the enzyme and the DNA to be 1.685 g/ml (7). The shoulder at fraction 20 on the ³H-labeled peak in Fig. 2a falls at a density of 1.686 g/ml,

TABLE 1. Quantitation of low-density material as a function of labeling time

[³H]thymidine labeling period (min)	Time of CTT treatment (min)	Proteinase K pretreatment	Low-density material (% of total)^a	Proteinase K-sensitive material (% of total)^b
0–5		–	3	
0–5	0–5	–	61	56
0–5	0–5	+	5	
0–20		–	6	
0–20	15–20	–	52	42
0–20	15–20	+	10	
0–40		–	8	
0–40	35–40	–	35	28
0–40	35–40	+	7	
0–60		–	6	
0–60	55–60	–	26	14
0–60	55–60	+	12	

^{a} The amount of labeled DNA that banded at a buoyant density less than that of the marker SV40 DNA was determined (Fig. 2a, shaded area), and the results of a set of parallel experiments were tabulated as a percentage of the total label in the CsCl gradient. In repeated experiments, the observed values for the percentage of low-density material in the CTT-treated samples varied by ±5.

^{b} The percentage of the total label in the CsCl gradient that was due to bound protein was calculated by subtracting the amount of low-density labeled material present after proteinase K treatment from the amount of label in the untreated sample. These percentage values have an error of approximately ±10.

TABLE 2. Comparison of DNA-protein complexes with replicative intermediates

[³H]thymidine labeling time (min)	Percent DNA-protein complexes^a	Percent replicative intermediates^b	Ratio^c
5	56	94	0.6
20	42	69	0.6
40	28	38	0.7
60	14	18	0.8

^{a} These data are taken directly from Table 1. For each time point, CTT was present during the last 5 min of the labeling period.

^{b} The percentage of the label present in replicative intermediates for each labeling period was estimated from the radioactivity that banded in a CsCl-propidium diiodide gradient at densities less than than of form I DNA (see Materials and Methods). No CTT treatment was used in these determinations. In repeated determinations, the observed values for the percentage of replicative intermediates varied by approximately ±10.

^{c} The ratio of the percentages of DNA-protein complexes to replicative intermediates was calculated for each labeling period.

consistent with the presence of 1:1 DNA-enzyme complexes in the gradient. However, the majority of the DNA-protein complexes banded with a lower buoyant density, indicating that they contained more than one bound topoisomerase molecule or possibly some other protein in addition to the topoisomerase. Assuming that topoisomerase I is the only bound protein, the buoyant density of the material banding near the top of the gradient would be consistent with the presence of approximately seven bound topoisomerase molecules per DNA molecule (see below).

In a separate experiment, infected cells were labeled for a total of 60 min, with the addition of CTT during the last 5 min of the labeling period. The results (Fig. 2c) reveal that under these conditions a much smaller proportion of the labeled DNA exhibited a reduced buoyant density. The percentage of labeled viral DNA that banded with a reduced buoyant density for different total labeling times is presented in Table 1. In every case, addition of CTT during the last 5 min of the labeling period resulted in the appearance of material with a reduced buoyant density as compared with the untreated controls. However, the percentage of proteinase K-sensitive, low-density material (Fig. 2, shaded areas) was inversely proportional to the length of the labeling period (Table 1). Furthermore, this percentage approximately paralleled the percentage of replicative intermediates for the same labeling times (no CTT present) as determined by equilibrium centrifugation in the presence of propidium diiodide (Table 2) (see Materials and Methods). These results suggested that topoisomerase I might be preferentially associated with replicating SV40 DNA.

Protein-bound DNA is nicked and contains free 5' hydroxyls. If the in vivo target of CTT is topoisomerase I, SDS lysis of the infected cells should trap the topoisomerase in a covalent linkage with SV40 DNA. Furthermore, the protein-bound DNA molecules should contain single-strand breaks

at the point of protein attachment, with the free end at the nick being a 5' hydroxyl.

To determine whether the DNA molecules containing bound protein also contained single-strand breaks, the low-density material was isolated from a CsCl gradient, treated with proteinase K to remove any bound protein, and rebanded in a CsCl gradient containing propidium diiodide (an analog of ethidium bromide) to separate closed circles from nicked circles. More than 90% of the isolated labeled DNA banded at the position of nicked form II molecules (data not shown).

To test whether the free ends of the broken strands contained 5' hydroxyls, the isolated low-density material was denatured and treated with polynucleotide kinase in the presence of [γ-³²P]ATP to label any free 5' ends. The products were subjected to electrophoresis in an alkaline agarose gel; the autoradiogram of the gel is shown in Fig. 3. Strands the size of SV40 unit-length linear DNA, as well as smaller fragments, are clearly present in the sample isolated after treatment of the cells with CTT for 5 min (Fig. 3, lane 1). The 5' hydroxyls are associated with DNA molecules containing bound protein, since treatment of the Hirt supernatant with proteinase K prior to centrifugation in neutral CsCl greatly reduced the number of free 5' ends identified in the low-density region of the gradient (lane 2). Furthermore, the nicks are likely to be associated with the topoisomerase-induced nicks, since only a small number of free 5' ends were observed in the control sample isolated from cells that had not been treated with CTT (lane 3).

From the results presented in Fig. 2, it appeared that the majority of the DNA molecules were associated with more protein than could be accounted for by the presence of one bound topoisomerase I per SV40 DNA molecule. If all of the bound protein were topoisomerase I, there should be up to seven nicks per replicative intermediate. Assuming that the nicks are randomly located on the DNA, one can predict that in addition to unit-length linear DNA, an array of smaller fragments should be labeled by the polynucleotide kinase in the experiment just described. The radioactivity in lane 1 of Fig. 3 was quantitated by counting the appropriate regions of the dried gel, and it was found that only 26% of the total label was present in the 5.2-kilobase-pair band of linear DNA; the remainder of the label was found in the lower region of the gel, where smaller fragments migrate. This result suggests that the broad spectrum of lower buoyant densities observed for the DNA-protein complexes shown in Fig. 2a is due to the presence of multiple topoisomerase I molecules bound per DNA molecule.
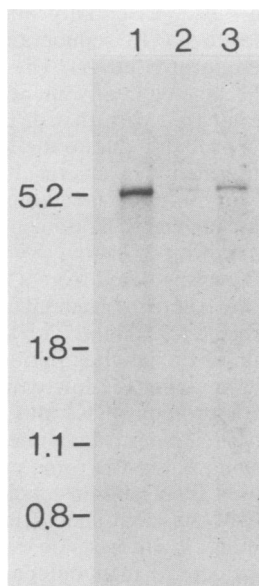
FIG. 3. Alkaline agarose gel analysis of 5'-end-labeled low-density DNA. SV40-infected cells were treated with 10 μM CTT for 5 min, and the DNA in the Hirt supernatant banded to equilibrium in CsCl in the presence of ^{3}H-labeled SV40 DNA as a marker. The fractions corresponding to the low-density material were pooled, treated with proteinase K, dialyzed, and extracted with phenol and chloroform. The DNA was ethanol precipitated with bovine serum albumin as a carrier. It was then denatured with alkali, and the 5' ends were labeled with polynucleotide kinase as described in Materials and Methods. The labeled DNA was analyzed by electrophoresis in alkaline agarose (lane 1). In two parallel control experiments, the comparable fractions from CsCl gradients were similarly analyzed for samples which had been treated with proteinase K prior to the CsCl gradient (lane 2) or in which the extracts had been made from cells that had not been treated with CTT (lane 3). The sizes of the marker fragments run on the same gel are given to the left side of the autoradiogram in kilobase pairs.

CsCl-propidium diiodide gradient analysis of SV40 DNA after CTT treatment.

Figure 4a shows the CsCl-propidium diiodide gradient profile for a sample that had been labeled for 20 min with the addition of CTT during the last 5 min of the labeling period. Results of pretreating the sample shown in Fig. 4a with proteinase K (Fig. 4b) confirmed that the material banding at positions less dense than those of the form II marker peak contained bound protein. The fact that proteinase K treatment shifted the low-density material to the position of form II DNA (rather than to the position of form I DNA) confirmed that the DNA molecules containing bound protein also contained single-strand breaks.

Figure 4c shows the results of a CsCl-propidium diiodide gradient analysis of the Hirt supernatant from infected cells that had been labeled for 20 min in the absence of CTT. The material banding at positions intermediate between those of form I and form II DNA has been shown previously to represent replicative intermediates in all stages of replication (23, 29, 30). The intermediate density arises from the fact that dye binding to the unreplicated, closed portion of the molecules is restricted in relation to binding to the replicated arms. By comparing the distribution of label between form I DNA and replicative intermediates from CTT-treated cells (Fig. 4a) and the untreated control cells (Fig. 4c), it can be seen that the replicative intermediates were preferentially shifted to lower densities when the DNA was extracted from
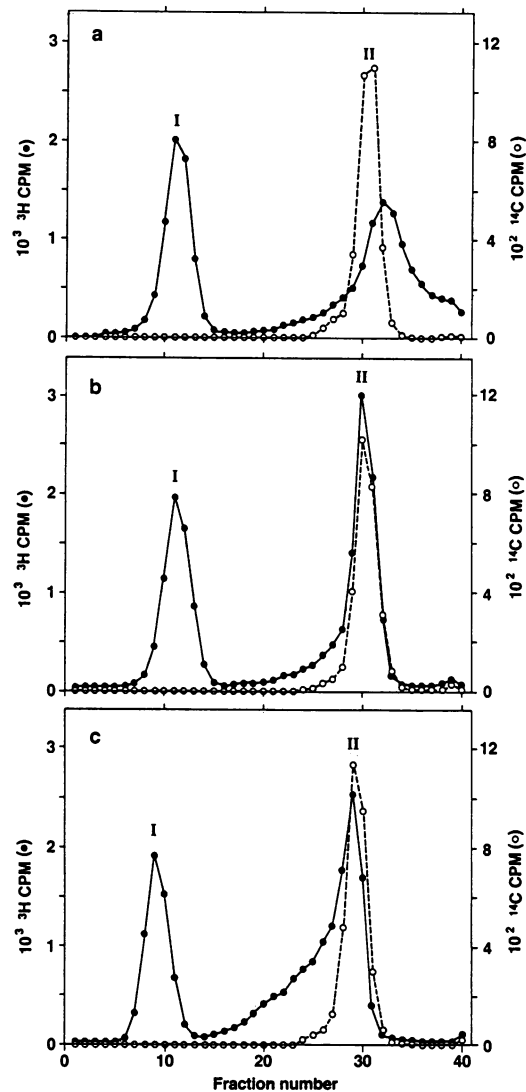

FIG. 4. CsCl-propidium diiodide equilibrium centrifugation of viral DNA isolated from CTT-treated and untreated cells. SV40-infected cells were labeled for 20 min with [^{3}H]thymidine, with the addition of 10 μM CTT during the last 5 min of the labeling period. A portion of the ^{3}H-labeled viral DNA (●) in the Hirt supernatant was analyzed directly by CsCl equilibrium centrifugation in the presence of propidium diiodide (a). A second portion was treated with proteinase K prior to the gradient analysis (b). ^{14}C-labeled SV40 nicked circular DNA (○) was included as a buoyant density marker for form II DNA (II); the ^{3}H-labeled high-density peak marked the position of closed-circular form I SV40 DNA (I). A parallel culture was labeled with [^{3}H]thymidine for 20 min without the addition of CTT and similarly analyzed (c).

CTT-treated cells. Therefore, CTT treatment appears to specifically trap topoisomerase I molecules on replicating SV40 molecules.

Sucrose gradient sedimentation analysis of low-density material.

More than 90% of the label incorporated into viral DNA in a 5-min pulse of [^{3}H]thymidine is found in replicating molecules (Table 2). Apparently, a slow step near the end of SV40 replication precludes the appearance of exogenous label in completed form I molecules during the first 5 min of labeling (24, 26). Pulse-labeled replicating SV40 DNA molecules can be distinguished from nonreplicating form I
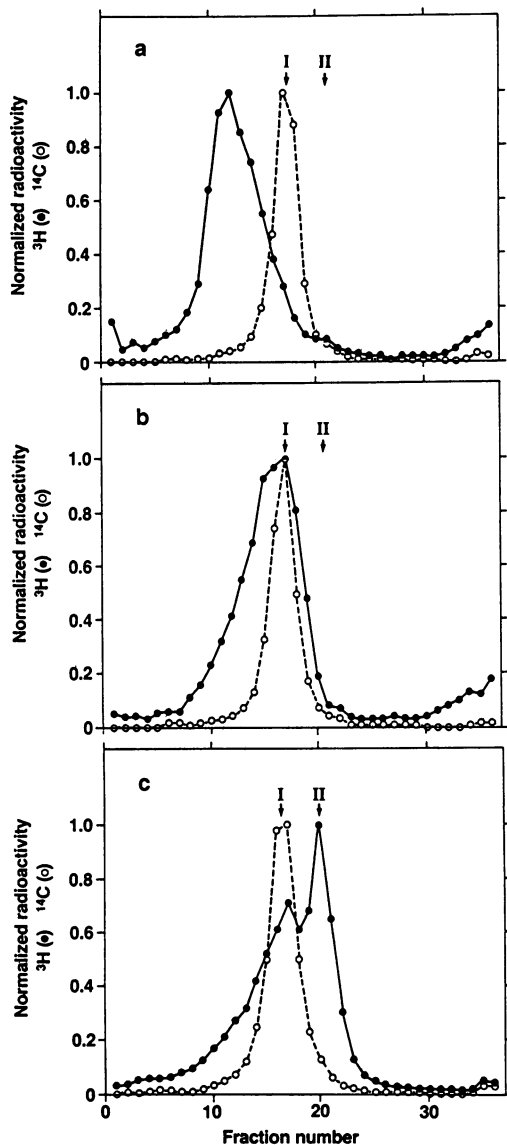
FIG. 5. Neutral sucrose gradient analyses. SV40-infected cells were labeled for 5 min with [^{3}H]thymidine in the absence of CTT (a) or in the presence of 10 μM CTT (b) or for 60 min with the addition of 10 μM CTT during the last 5 min (c). For panels a and b, the Hirt supernatants were treated with proteinase K and analyzed directly by sedimentation in neutral sucrose. For panel c, the Hirt supernatant was banded to equilibrium in CsCl, and the fractions containing the low-density material were pooled (corresponding to fractions 21 to 38 for the gradients shown in Fig. 2), treated with proteinase K, dialyzed to remove the CsCl, and further purified as described in Materials and Methods. The sedimentation profile of the ^{3}H-labeled DNA (●) is shown, along with the profile for the marker ^{14}C-labeled SV40 form I DNA (○). The position of the slower-sedimenting form II DNA is indicated on the graph.

supercoiled molecules (^{14}C-labeled marker DNA) by their faster sedimentation in neutral sucrose (Fig. 5a) (24, 30). SV40 DNA isolated by Hirt extraction after a 5-min pulse in the presence of CTT was treated with proteinase K and analyzed directly by sedimentation in sucrose. The DNA sedimented as a broad peak that overlapped and moved slightly faster than the form I marker DNA (Fig. 5b). Consistent with the fact that only replicating molecules are

labeled in a 5-min pulse, essentially none of the material isolated in the presence of CTT sedimented at the position of SV40 nicked circles (form II DNA). The slower sedimentation of the molecules labeled in a 5-min pulse in the presence of CTT (Fig. 5b) compared with that of the control without the drug (Fig. 5a) is probably due to the loss of supercoiling of the replicative intermediates as a result of the presence of CTT-induced nicks in the DNA (see above).

To investigate further whether topoisomerase I is preferentially associated with replicating SV40 molecules, the low-density material was isolated from a neutral CsCl gradient and analyzed by sucrose gradient sedimentation. Infected cells were labeled for 60 min, with the addition of CTT during the last 5 min of the labeling period. The fractions of the CsCl gradient containing the low-density material were pooled, treated with proteinase K, and sedimented in sucrose (Fig. 5c). If replicative intermediates had been present in the low-density material in the same proportion as in the total population, then for a 60-min labeling time (Table 2) approximately 18% of the label should have sedimented as replicative intermediates. In fact, 60% of the label sedimented as a broad peak (62% in an independent experiment), with a sedimentation rate similar to the replicative intermediates isolated in the presence of CTT (Fig. 5b). Quantitatively similar results were obtained when DNA was extracted after the infected cells had been labeled for 20 or 40 min (data not shown). Although the replicative intermediates sedimented at approximately the same rate as supercoiled form I molecules in these experiments, the results cited above, showing that essentially all of the DNA in the low-density material contained one or more nicks, exclude the possibility that a significant fraction of the rapidly sedimenting DNA in Fig. 5c is form I DNA. Thus, in the experiment shown, the low-density material was enriched for replicative intermediates by at least a factor of 3. Since the method used for analyzing the distribution of label in a CsCl-propidium diiodide gradient somewhat overestimates the percentage of replicative intermediates (see Materials and Methods), this enrichment factor represents a minimal value (see Discussion). The remainder of the label sedimented at the position of form II molecules. It is possible that these form II molecules were derived from recently replicated DNAs that had not yet lost their complement of topoisomerase I.

Association of topoisomerase I with parental versus nascent strands. As shown above, labeled thymidine is incorporated exclusively into replicative intermediates during a 5-min pulse. It follows that virtually all of the pulse label will be present in nascent or newly replicated strands and that the template parental strands will be unlabeled. To determine whether the bound topoisomerase was associated with the nascent strands, cells were pulse-labeled for 5 min, and the low-density material was isolated from a neutral CsCl gradient and rebanded in an alkaline CsCl gradient. Although more than 95% of the isolated material rebanded in a neutral gradient at a position less dense than that of form II (data not shown), only 10% had a reduced buoyant density in alkaline CsCl (Fig. 6a). There are two reasons why the failure to observe a larger proportion of low-density material in the alkaline gradient was not due to dissociation of the protein from the DNA by the high pH. First, the small amount of low-density material observed in Fig. 6a was due to bound protein, since it was not present in a sample that had been pretreated with proteinase K (data not shown). Second, after a 40-min labeling period (with CTT present during the last 5 min), during which some of the parental strands should have
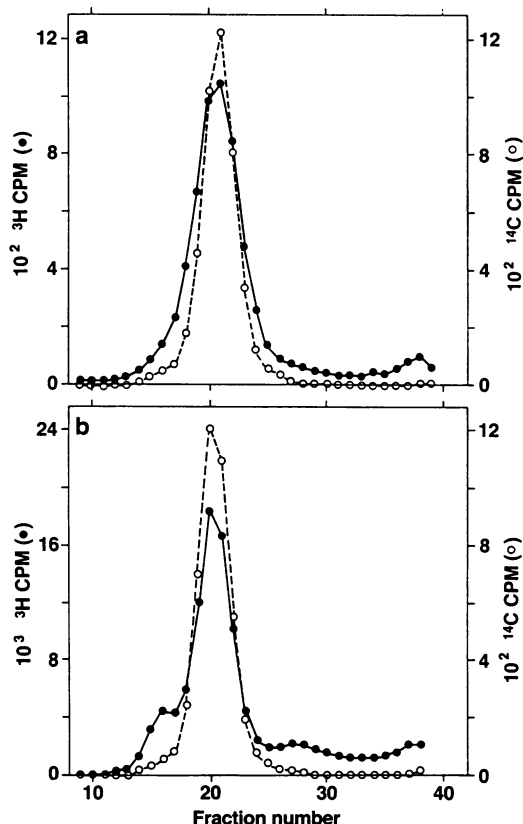
FIG. 6. Analysis of low-density material by equilibrium centrifugation in alkaline CsCl. SV40-infected cells were labeled either for 5 min in the presence of 10 μM CTT (a) or for 60 min with the addition of CTT during the last 5 min of the labeling period (b). The viral DNA in the Hirt supernatant was banded to equilibrium in neutral CsCl, and the fractions containing the low-density material were pooled without proteinase K treatment. After the pH of the samples had been adjusted to >12.5, ^{14}C-labeled SV40 linear DNA (O) was added as a marker and the ^{3}H-labeled DNA (●) was centrifuged to equilibrium in alkaline CsCl. When rebanded in neutral CsCl, more than 95% of the DNA shown in panel a and 80% of the DNA shown in panel b contained bound protein, as evidenced by their reduced buoyant densities. The shoulder on the dense side of the peak in panel b represents closed-circular SV40 DNA that contaminated the pooled low-density fractions from the neutral CsCl gradient.

been labeled by virtue of two or more cycles of replication, approximately 20% of the low-density material exhibited a reduced buoyant density in alkaline CsCl (Fig. 6b). The stability of the protein-DNA linkage in alkali is consistent with what is known about the structure of the intermediate in the topoisomerase I reaction (8). Therefore, 90% of the protein bound to the replicative intermediates appears to be associated with parental strands rather than the newly replicated DNA strands in the replicative intermediates.

DISCUSSION

Lysis of SV40-infected cells with SDS in the presence of CTT generates viral DNA-protein complexes that band at a less dense position than free DNA in neutral CsCl. It is likely that the protein bound to the DNA is the type I topoisomerase for the following reasons. First, it has been demonstrated that in vitro, CTT increases the probability of trapping the topoisomerase I in a covalent complex with nicked

DNA when reactions are stopped with detergent (22). By analogy, one expects a similar reaction in vivo. Mattern et al. (25) present similar data on the formation of DNA-protein complexes in the presence of CTT and interpret their results as evidence that topoisomerase I is an intracellular target of the drug. Second, topoisomerase I is likely to be the target for the drug, since Andoh et al. (2) have isolated a CTT-resistant human cell line and have shown that the mutant cells have an altered type I topoisomerase. Third, the association between the protein and the DNA is what one would expect if the protein were topoisomerase I. Thus, the linkage is most probably a covalent one, since it is stable to alkali. Furthermore, the stability to alkali is consistent with the chemistry of a phosphoester bond between the end of the DNA chain and a tyrosine residue in the topoisomerase (8). Fourth, breakage by topoisomerase I leaves a free 5' hydroxyl on one end of the broken strand. Fifth, we have begun mapping, to the nucleotide level, the breaks present in the low-density material, and we have found that most of the observed break sites conform to the consensus sequence previously identified for the eucaryotic topoisomerase I (3; S. E. Porter and J. J. Champoux, unpublished results).

The fact that the parental strands of SV40 replicative intermediates are covalently closed led to the concept that transient rather than permanent single-strand breaks must provide the swivels required for unwinding the helix during DNA replication. At the time of its discovery, it was hypothesized that topoisomerase I was the logical enzyme to provide the requisite swivels during DNA replication (9). Since that time, very little evidence has accumulated to either support or disprove this hypothesis. Recently Yang et al. (37) investigated the roles of topoisomerases in SV40 replication in vitro. They showed that either topoisomerase I or topoisomerase II could allow fork movement during replication, presumably by acting to allow helix unwinding. Furthermore, they showed that topoisomerase II was required for decatenating the products of replication. The data presented here that show a preferential association of topoisomerase I with replicating SV40 molecules provide direct in vivo evidence implicating the enzyme in DNA replication. Since SV40 is highly dependent on cellular machinery for its replication, these findings also point to a likely involvement of the topoisomerase in cellular DNA replication. In addition, it has been demonstrated that the enzyme found associated with replicative intermediates is acting primarily on the parental rather than the newly replicated strands. This is the expected result if the enzyme is indeed providing swivels to allow the strands to unwind during replication. The results of Snapka (31) that show breakage of SV40 replicative intermediates in the presence of CTT are consistent with the results presented here.

What is the basis for the preferential association of topoisomerase I with replicating SV40 DNA molecules? Two alternative possibilities are considered here. First, it is possible that, soon after replication, most of the labeled molecules enter the pathway toward encapsidation and become inaccessible to topoisomerase I. From the work of Garber et al. (14), it appears that after 60 min of labeling, as much as 50% of the label is on the pathway to becoming virions, with the remaining DNA constituting the free pools of replicating and nonreplicating DNA. For the 60-min labeling time, it was estimated that less than 18% of the total label is in replicative intermediates, and thus less than 36% (18%/50%) of the free DNA (not on the pathway toward encapsidation) is expected to represent replicating DNA. The observed value of 60% replicative intermediates in the

low-density material for the 60-min labeling time is somewhat greater that the predicted value of 36% if no enrichment had occurred. However, given the approximate nature of these estimates, one cannot rule out the possibility that encapsidation is a significant factor in precluding the association of topoisomerase I with the DNA in these experiments. If encapsidation is a major contributing factor to the observed preferential association of topoisomerase I with replicating DNA, the pool of free, nonreplicating DNA is probably relatively small.

Alternatively, it is possible that some structural feature of replicating molecules causes the preferential association observed here. For example, it may be that the superhelical state of the DNA identifies the preferred substrates for topoisomerase I in the cell. Thus, the torsional strain in the form of positive supercoils that is introduced into the SV40 parental strands during replication may be the feature of the DNA that is recognized by topoisomerase I. DNA in actively transcribed regions may similarly be under torsional strain that attracts topoisomerase I to act on the DNA and provide swivels for transcription. In this regard it is noteworthy that supercoiled DNA has been reported to be preferentially cleaved by the avian type I topoisomerase (27). Alternatively, some protein may recognize or be associated with replication or transcription machinery and, by virtue of protein-protein interactions, may direct the topoisomerase I to the appropriate sites on the DNA for its action.

ACKNOWLEDGMENTS

LITERATURE CITED

1. Abelson, H. T., and S. Penman. 1973. Induction of alkali labile links in cellular DNA by camptothecin. Biochem. Biophys. Res. Commun. 50:1048–1054.

2. Andoh, T., K. Ishii, Y. Suzuki, Y. Ikegami, Y. Kusunoki, Y. Takemoto, and K. Okada. 1987. Characterization of a mammalian mutant with a camptothecin-resistant DNA topoisomerase I. Proc. Natl. Acad. Sci. USA 84:5565–5569.

3. Been, M. D., R. R. Burgess, and J. J. Champoux. 1984. Nucleotide sequence preference at rat liver and wheat germ type 1 DNA topoisomerase breakage sites in duplex SV40 DNA. Nucleic Acids Res. 12:3097–3114.

4. Been, M. D., and J. J. Champoux. 1983. Cutting of M13mp7 phage DNA and excision of cloned single-stranded sequences by restriction endonucleases. Methods Enzymol. 101:90–98.

5. Bonven, B. J., E. Gocke, and O. Westergaard. 1985. A high affinity topoisomerase I binding sequence is clustered at DNAase I hypersensitive sites in Tetrahymena R-chromatin. Cell 41:541–551.

6. Brill, S. J., S. DiNardo, K. Voelkel-Meiman, and R. Sternglanz. 1987. Need for DNA topoisomerase activity as a swivel for DNA replication and for transcription of ribosomal RNA. Nature (London) 326:414–416.

7. Champoux, J. J. 1977. Strand breakage by the DNA untwisting enzyme results in covalent attachment of the enzyme to DNA. Proc. Natl. Acad. Sci. USA 74:3800–3804.

8. Champoux, J. J. 1981. DNA is linked to the rat liver DNA nicking-closing enzyme by a phosphodiester bond to tyrosine. J. Biol. Chem. 256:4805–4809.

9. Champoux, J. J., and R. Dulbecco. 1972. An activity from mammalian cells that untwists superhelical DNA-A possible swivel for DNA replication. Proc. Natl. Acad. Sci. USA 69:143–146.

10. Champoux, J. J., and B. L. McConaughy. 1975. Priming of superhelical SV40 DNA by Escherichia coli RNA polymerase for in vitro DNA synthesis. Biochemistry 14:307–316.

11. Dinardo, S., K. Voelkel, and R. Sternglanz. 1984. DNA topoisomerase II mutant of Saccharomyces cerevisiae: topoisomerase II is required for segregation of daughter molecules at the termination of DNA replication. Proc. Natl. Acad. Sci. USA 81:2616–2620.

12. Dinardo, S., K. A. Voelkel, R. Sternglanz, A. E. Reynolds, and A. Wright. 1982. Escherichia coli DNA topoisomerase I mutants have compensatory mutations in DNA gyrase genes. Cell 31:43–51.

13. Fleischmann, G., G. Pflugfelder, E. K. Steiner, K. Javaherian, G. C. Howard, J. C. Wang, and S. C. R. Elgin. 1984. Drosophila DNA topoisomerase I is associated with transcriptionally active regions of the genome. Proc. Natl. Acad. Sci. USA 81:6958–6962.

14. Garber, E. A., M. M. Seidman, and A. Levine. 1978. The detection and characterization of multiple forms of SV40 nucleoprotein complexes. Virology 90:305–316.

15. Garg, L. C., S. Diangelo, and S. T. Jacob. 1987. Role of DNA topoisomerase I in the transcription of supercoiled rRNA gene. Proc. Natl. Acad. Sci. USA 84:3185–3188.

16. Gilmour, D. S., and S. C. R. Elgin. 1987. Localization of specific topoisomerase I interactions within the transcribed region of active heat shock genes by using the inhibitor camptothecin. Mol. Cell. Biol. 7:141–148.

17. Gilmour, D. S., G. Pflugfelder, J. C. Wang, and J. T. Lis. 1986. Topoisomerase I interacts with transcribed regions in Drosophila cells. Cell 44:401–407.

18. Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. 26:365–369.

19. Holm, C., T. Goto, J. C. Wang, and D. Botstein. 1985. DNA topoisomerase II is required at the time of mitosis in yeast. Cell 41:553–563.

20. Horwitz, M. S., and S. B. Horwitz. 1971. Intracellular degradation of HeLa and adenovirus type 2 DNA induced by camptothecin. Biochem. Biophys. Res. Commun. 45:723–727.

21. Horwitz, S. B., C. Chang, and A. P. Grollman. 1971. Studies on camptothecin. I. Effects on nucleic acid and protein synthesis. Mol. Pharmacol. 7:632–644.

22. Hsiang, Y.-H., R. Hertzberg, S. Hecht, and L. F. Liu. 1985. Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. J. Biol. Chem. 260:14873–14878.

23. Jaenisch, R., A. Mayer, and A. J. Levine. 1971. Replicating SV40 molecules containing closed circular template DNA strands. Nature (London) New Biol. 233:72–75.

24. Levine, A. J., H. S. Kang, and F. E. Billheimer. 1970. DNA replication in SV40 infected cells. I. Analysis of replicating SV40 DNA. J. Mol. Biol. 50:549–568.

25. Mattern, M. R., S.-M. Mong, H. F. Bartus, C. K. Mirabelli, S. T. Crooke, and R. K. Johnson. 1987. Relationship between the intracellular effects of camptothecin and the inhibition of DNA topoisomerase I in cultured L1210 cells. Cancer Res. 47:1793–1798.

26. Mayer, A., and A. J. Levine. 1972. DNA replication in SV40 infected cells. VIII. The distribution of replicating molecules at different stages of replication in SV40 infected cells. Virology 50:328–338.

27. Muller, M. T. 1985. Quantitation of eukaryotic topoisomerase I reactivity with DNA. Preferential cleavage of supercoiled DNA. Biochim. Biophys. Acta 824:263–267.

28. Pruss, G. J., S. H. Manes, and K. Drlica. 1982. Escherichia coli DNA topoisomerase I mutants: increased supercoiling is corrected by mutations near gyrase genes. Cell 31:35–42.

29. Roman, A., J. J. Champoux, and R. Dulbecco. 1974. Characterization of the replicative intermediates of polyoma virus. Virology 57:147–160.

30. Sebring, E. D., T. J. Kelley, Jr., M. M. Thoren, and N. P. Salzman. 1971. Structure of replicating simian virus 40 deoxyribonucleic acid molecules. J. Virol. 8:478–490.

31. Snapka, R. M. 1986. Topoisomerase inhibitors can selectively interfere with different stages of simian virus 40 DNA replica-

tion. Mol. Cell. Biol. 6:4221–4227.

32. **Spataro, A., and D. Kessel.** 1972. Studies on camptothecin-induced degradation and apparent reaggregation of DNA from L1210 cells. Biochem. Biophys. Res. Commun. 48:643–648.

33. **Steck, T. R., and K. Drlica.** 1984. Bacterial chromosome segregation: evidence for DNA gyrase involvement in decatenation. Cell 36:1081–1088.

34. **Stewart, A. F., and G. Schutz.** 1987. Camptothecin-induced in vivo topoisomerase I cleavages in the transcriptionally active

tyrosine aminotransferase gene. Cell 50:1109–1117.

35. **Tegtmeyer, P., C. Dohan, and C. Reznikoff.** 1970. Inactivating and mutagenic effects of nitrosoguanidine on simian virus 40. Proc. Natl. Acad. Sci. USA 66:745–752.

36. **Wang, J. C.** 1985. DNA topoisomerases. Annu. Rev. Biochem. 54:665–697.

37. **Yang, L., M. S. Wold, J. J. Li, T. J. Kelly, and L. F. Liu.** 1987. Roles of DNA topoisomerases in simian virus 40 DNA replication in vitro. Proc. Natl. Acad. Sci. USA 84:950–954.