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Patterns of Strongly Protein-Associated Simian Virus 40 DNA Replication Intermediates Resulting from Exposures to Specific Topoisomerase Poisons†

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ABSTRACT: Exposure of infected CV-1 cells to specific type I and type II topoisomerase poisons caused strong protein association with distinct subsets of simian virus 40 (SV40) DNA replication intermediates. On the basis of the known specificity and mechanisms of action of these drugs, the proteins involved are assumed to be the respective topoisomerases. Camptothecin, a topoisomerase I poison, caused strong protein association with form II (relaxed circular) and form III (linear) viral genomes and replication intermediates having broken DNA replication forks but not with form I (superhelical) viral DNA or normal late replication intermediates which were present. In contrast, type II topoisomerase poisons caused completely replicated forms and late viral replication forms to be tightly bound to protein—some to a greater extent than others. Different type II topoisomerase inhibitors caused distinctive patterns of protein association with the replication intermediates present. Both intercalating and nonintercalating type II topoisomerase poisons caused a small amount of form I (superhelical) SV40 DNA to be protein-associated in vivo. The protein complex with form I viral DNA was entirely drug-dependent and strong, but apparently noncovalent. The protein associated with form I DNA may represent a drug-stabilized "topological complex" between type II topoisomerase and SV40 DNA.

Topoisomerase poisons stabilize a reaction intermediate in which the enzyme is covalently bound to DNA at the site of a DNA strand break (Liu, 1989). In the case of eukaryotic type II topoisomerase, the protein subunits are bound to the 5′ side of each DNA strand break, and the breaks may be either single stranded or double stranded. Eukaryotic topoisomerase I makes single-strand breaks exclusively, with the protein covalently attached on the 3′ side. Type II topoisomerase poisons such as etopside, teniposide, and adriamycin are standards in the arsenal of anticancer drugs (Liu, 1989). More recently, type I topoisomerase poisons have shown promise for the treatment of human colon cancer (Giovanella et al., 1989).

The antineoplastic action of topoisomerase poisons may be related to their ability to interfere with cellular processes which require topoisomerase action. The movement of DNA replication forks requires a topoisomerase to reduce the linkage of the parental DNA strands and remove positive superhelical stress ahead of the forks. This is known as the swivel function, and both theory (Champoux & Been, 1980) and a large body of experimental evidence (Brill et al., 1987; Goto & Wang, 1985; Uemura & Yanagida, 1984; Yang et al., 1987) indicate that either type I or a type II topoisomerase can serve as the "swivel". It is also clear that type II topoisomerase is required for the separation of newly replicated daughter DNA strands both in bacteria (Steck & Drlica, 1984) and in eukaryotes (Sundin & Varshavsky, 1981; Snapka, 1986; Snapka et al., 1988; DiNardo et al., 1984; Uemura & Yanagida, 1986; Holm et al., 1985).

The DNA tumor virus SV40 has been very useful for understanding the roles of topoisomerase in eukaryotic DNA replication. SV40 is considered a model for the mammalian replicon because of its extensive use of cellular DNA replication machinery and chromosomal proteins (DePamphilis et al., 1979). This system has been used to show that the type I topoisomerase poison camptothecin breaks replication forks at all stages of DNA replication (Snapka, 1986; Avemann et al., 1988), while type II topoisomerase poisons slow or block the replication of the last few hundred base pairs of the viral genome and the separation of daughter chromosomes (Snapka

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et al., 1988; Snapka, 1986; Richter & Strausfeld, 1988). These results are consistent with a model in which type I topoisomerase serves as the main swivel for most DNA replication fork movement, with type II topoisomerase assuming more of this role in the final stages of replication (Snapka et al., 1988; Richter & Strausfeld, 1988).

To learn more about the detailed interactions of topoisomerases with replicating and nonreplicating DNA, we have extended our study of the SV40 system to ask which viral replication intermediates are strongly protein-associated in the presence of specific topoisomerase poisons. The finding that different topoisomerase poisons cause different sets of viral DNA replication intermediates to be strongly protein-associated has implications for the details of topoisomerase functions in mammalian DNA replication.

The detection of a strong, noncovalent protein complex with superhelical viral genomes only in cells treated with topoisomerase II poisons suggests that stabilization of a noncovalent topoisomerase II–DNA complex may be a key factor in the mechanism of this class of drugs.

Experimental Procedures

Cell Culture and Virus Infection. African green monkey kidney cells (CV-1) were grown in Eagle's minimal essential medium (Gibco) supplemented with 5% calf serum, 14 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes),[1] pH 7.2, and 4 mM $NaHCO_3$. The cells were infected with a single plaque isolate of SV40 strain 777 at a multiplicity of infection of 10.

Drugs. Camptothecin (NSC 94600) was obtained from the National Cancer Institute, Division of Cancer Treatment, Natural Products Branch, and was dissolved in dimethyl sulfoxide. VP-16 (etopside, NSC 141540) was obtained from the same source in a solvent composed of 2 mg/mL citric acid, 30 mg/mL benzyl alcohol, 80 mg/mL polysorbate 80/Tween 80, 650 mg/mL poly(ethylene glycol)300, and 30.5% (v/v) absolute ethanol. The National Cancer Institute, Division of Cancer Treatment, Drug Synthesis and Chemistry Branch, provided *m*-AMSA (NSC 24992), which was dissolved in dimethyl sulfoxide. Ellipticine, proflavine, and actinomycin D were purchased from Sigma. Solvents used were ethanol (actinomycin D), 0.01 N HCl (ellipticine), dimethyl sulfoxide, and deionized water (proflavine). None of the solvents used in the study affects SV40 DNA replication or causes protein association of viral replication intermediates.

Radiolabeling and Drug Treatment. Experiments were done at 36-h postinfection. Unless stated otherwise, cells were labeled for 30 min with [*methyl*-^{3}H]thymidine (250 μCi/mL), and drugs were added directly to the labeling media 15 min after the start of label. Labeling and drug treatment were stopped by removal of media and addition of Hirt lysing solution (Hirt, 1967).

Glass Filter Binding of Protein and Protein–DNA Complexes. The use of glass filters (Whatman GF/C, 2.4 cm) for in vivo assay of topoisomerase poisons and topoisomerase antagonists has been described (Shin et al., 1990). Briefly, 20 μL of Hirt lysate supernatant is added to 1 mL of protein binding buffer (0.4 M GuHCl, 10 mM Tris-HCl, pH 8.0, 10 mM NaEDTA, 0.01% sarkosyl, and 0.3 M NaCl). This is then filtered through a prewetted GF/C filter, and the filter is rinsed first with 4 mL of GuHCl buffer and then with ice-cold 95% ethanol. Binding of Hirt-extracted SV40 DNA to the filter under these conditions is dependent on in vivo exposure of infected cells to drugs or chemicals known to cause covalent protein–DNA cross-links [for instance, formaldehyde or topoisomerase poisons, (Shin et al., 1990)]. For elution and electrophoretic analysis, the volumes are increased, with up to 10 mL of 0.4 M GuHCl being used and with sample size scaled up proportionately. To elute bound protein–DNA complexes, the filters were placed in 0.7-mL conical microtubes, covered with 0.4 mL of elution buffer (0.1% SDS, 10 mM Tris-HCl, pH 7.5, 1 mM NaEDTA, and 100 mM NaCl), and incubated at 60 °C for 15 min with frequent vortexing. A small pinhole was made in the bottom of the elution tube, which was then placed in a 1.7-mL centrifuge tube. The eluted material was collected by brief centrifugation to force the elution buffer through the pinhole and into the bottom of the larger tube.

Glass Powder Binding and Elution of Protein–DNA Complexes. Glass powder was purchased as a 50% (w/v) suspension in deionized H_2O (Glassmilk, Bio 101, Inc., La Jolla, Ca). Protein binding buffer and elution buffer were identical with those described above for glass filter binding. To bind proteins selectively to glass powder, 50 μL of glass powder suspension and 0.2 mL of Hirt supernatant were mixed with 1 mL of protein binding buffer. The mixture was shaken for 15 min at room temperature and then centrifuged briefly. The supernatant was discarded, and the glass powder pellet was resuspended and washed twice in protein binding buffer and then once in 10 mM Tris-HCl, pH 7.5, 1 mM NaEDTA, and 3 N NaCl. Finally, 0.1 mL of elution buffer was added to the glass powder pellet, which was then resuspended and incubated at 60 °C for 15 min. The glass was pelleted by brief centrifugation, and the supernatant was poured off. A second elution of the pellet was then done, and the second supernatant was added to the first. The combined supernatant typically contained more than 90% of the labeled protein–DNA complexes which bound the glass powder in protein binding buffer.

Electrophoretic Analysis of SV40 DNA Replication Intermediates. Agarose gel electrophoresis of SV40 replication intermediates was carried out as described (Sundin & Varshavsky, 1980; Snapka, 1986). Samples for electrophoresis were digested with proteinase K (1 mg/mL) in 0.6% SDS for 5 h at 45 °C, extracted in chloroform/2-propanol (24:1), and ethanol-precipitated before being taken up in gel loading buffer.

Results

DNA does not bind to glass in 0.4 M GuHCl (protein binding conditions, see Experimental Procedures) unless it is tightly associated with protein (Coombs & Pearson, 1978; Shin et al., 1990). In the absence of topoisomerase poisons, Hirt-extracted SV40 DNA replication intermediates in 0.4 M GuHCl did not bind to glass powder (Figure 1, lanes 1–5). When infected cells were exposed to VP-16 before extraction, several viral replication intermediates were bound to glass powder (Figure 1, lane 6). The binding was abolished by pretreatment with proteinase K (lane 8). This indicates that these intermediates were strongly protein-associated as a result of exposure to VP-16. VP-16 is a specific type II topoisomerase poison which traps the enzyme in a covalent complex with DNA at the site of a DNA strand break (Liu, 1989). This complex is rendered irreversible by SDS denaturation. Thus, the protein associated with these intermediates as a result of VP-16 treatment is assumed to be topoisomerase II. Approximately one-third of the pulse-labeled viral DNA in Hirt

[1] Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-aniside; VP-16, etopside; NaEDTA, sodium ethylenediaminetetraacetate; GuHCl, guanidine hydrochloride; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; SDS, sodium dodecyl sulfate.

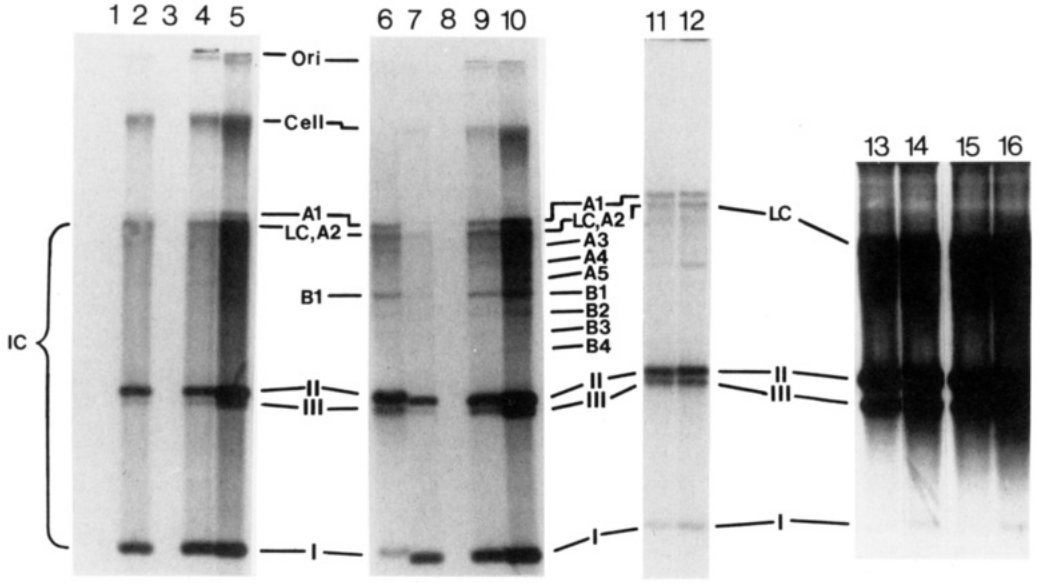

FIGURE 1: Glass powder binding of pulse-labeled SV40 replication intermediates in the presence of VP-16. Lanes 1–5, SV40 intermediates from control cells (no drug); lanes 6–10, SV40 intermediates from cells exposed to 100 μM VP-16. Lanes 5 and 10 were loaded with DNA from unfractionated Hirt supernatants of pulse-labeled SV40-infected cells. Identical Hirt supernatants, treated with proteinase K, were mixed with glass powder under protein binding conditions. The glass powder supernatants (containing unbound material) were run in lanes 4 and 9, and the elutes from the glass powder were run in lanes 3 and 8. Hirt supernatants not treated with proteinase K were also carried through glass powder fractionation, with unbound material in lanes 2 and 7 and glass powder eluates in lanes 1 and 6. Lanes 11 and 12, same as lane 6, except that the material was eluted from glass fiber filters rather than glass powder. Lanes 13–16, reversal of the VP-16-induced protein association with form I SV40 DNA. Lanes 14 and 16, samples same as in lane 6, but overexposed; lanes 13 and 15, same but treated with 1% SDS at 65 °C for 15 min before glass powder binding. Abbreviations: Ori, origin of electrophoresis; Cell, cellular DNA; I, form I (superhelical) SV40 DNA; II, form II (relaxed circular) SV40; III, form III (linear) SV40; LC, latest Cairns structure; IC, intermediate Cairns structures; A, fully relaxed catenated SV40 dimers; B, catenated SV40 dimers with one member relaxed and one superhelical. For catenated dimers, the catenation linking number is indicated by the number following the letter.

supernatants of VP-16-treated cells bound to and eluted from glass powder under protein binding conditions. Comparing the bound material (lane 6) with the unbound material (lane 7), it is apparent that there is relatively less form I DNA (completely replicated, superhelical genomes) and more of the late Cairns structure and catenated dimers in the bound material. The late Cairns structure is a "theta-form" replication intermediate with approximately 200 base pairs unreplicated (Tapper & DePamphilis, 1980). Intermediate Cairns structures are less completely replicated θ replication intermediates. In one-dimensional gels, they are seen as a smear extending from the form I band to the late Cairns structure band (IC in Figure 1). Catenated dimers are newly replicated daughter chromosomes which remain intertwined (catenated) due to the absence of topoisomerase II action (Sundin & Varshavsky, 1981; DiNardo et al., 1984; Snapka, 1986). The catenation is thought to arise due to a failure of topoisomerases to reduce the linkage of the parental strands to zero during replication (Sundin & Varshavsky, 1980). There are three families of catenated dimers: the A family in which both circles are relaxed; the B family in which one circle is relaxed and one is superhelical; and the C family in which both circles are superhelical. These gels can resolve the A and B catenated dimer families into ladders based on the catenation linking number of each dimer (Sundin & Varshavsky, 1980). Thus, "A1" indicates a band of fully relaxed singly intertwined dimers, "A2" indicates doubly intertwined completely relaxed dimers, "B1" indicates dimers in which a superhelical circle and a relaxed circle are intertwined one time, etc. The VP-16-dependent association of form I DNA with protein (presumably topoisomerase II) was unexpected since the only known drug-stabilized reaction intermediate consists of topoisomerase subunits covalently attached to DNA at the site of a DNA strand break (Liu, 1989). A DNA strand break in form I (superhelical) DNA converts it to form II (relaxed circular) DNA.

Since glass binding of protein-associated SV40 DNA has been most thoroughly characterized for glass fiber filters (Shin et al., 1990), we also eluted the viral forms bound to GF/C filters. Lanes 11 and 12 in Figure 1 show that the same viral replication intermediates from VP-16-treated infected cells were selectively bound to GF/C filters. Glass powder and glass fiber filters show essentially identical binding behavior, but elution of filters requires more manipulation and larger volumes of elution buffer.

To explore the unexpected protein association of form I viral genomes with protein in the presence of VP-16, we attempted to reverse the binding by heating in higher concentrations of SDS. As shown in Figure 1, lanes 13–16, this treatment abolished the glass binding of form I DNA. This suggests that VP-16 causes a strong but probably noncovalent association of form I DNA with protein in vivo.

A different pattern of protein-associated SV40 replication intermediates was seen when infected cells were exposed to the type I topoisomerase poison camptothecin (Figure 2, lanes 1–4). In contrast to VP-16, neither form I nor the late Cairns structure was bound to glass as a result of camptothecin treatment. As with VP-16, approximately one-third of the pulse-labeled viral replication intermediates in Hirt supernatants of camptothecin-treated cells bound to and eluted from glass powder under protein binding conditions. The SV40 intermediates bound were forms II (relaxed circular), III (linear), and two short smears of replication intermediates. These aberrant replication intermediates, designated LC′and LC″, are composed of SV40 replication intermediates with broken replication forks (σ structures) (Snapka, 1986). Note that the form III band is relatively more prominent in the glass-bound material.

Actinomycin D has been reported to be a type II topoisomerase poison (Tewey et al., 1984).[2] We found that ex-

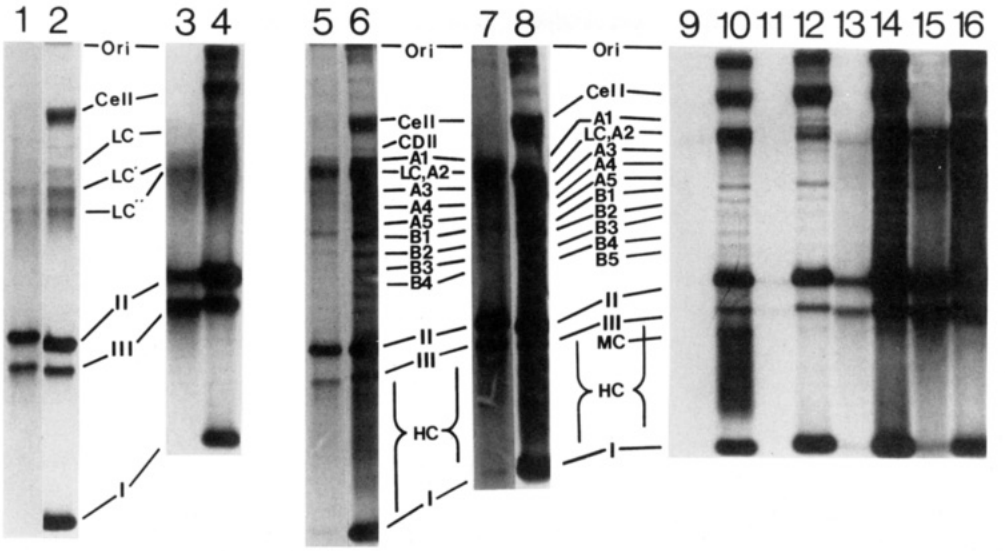

FIGURE 2: SV40 DNA replication intermediates tightly associated with protein after exposure of infected cells to type I or type II topoisomerase inhibitors. Lanes 1–4, SV40 replication intermediates from pulse-labeled, SV40-infected cells treated with 40 μM camptothecin: DNA from unfractionated Hirt supernatants was run in lanes 2 and 4, while DNA from identical Hirt supernatants which bound to glass powder under protein binding conditions was run in lanes 1 and 3. Lanes 5–8, SV40 replication intermediates from cells treated with 200 μM actinomycin D: lanes 6 and 8 contain material from unfractionated Hirt supernatants, and lanes 5 and 7 contain material from identical supernatants which was bound by glass powder. The glass powder bound material and unfractionated Hirt supernatants respectively are shown in lanes 9 and 10 for cells treated with 40 μM ellipticine, in lanes 11 and 12 for cells treated with 40 μM proflavine, in lanes 13 and 14 for cells treated with 40 μM *m*-AMSA, and in lanes 15 and 16 for cells treated with 100 μM VP-16. Abbreviations: HC, highly catenated SV40 dimers, LC′, Cairns replication intermediates with one broken replication fork (σ structures); LC″, Cairns replication intermediate with two broken replication forks. Other abbreviations same as in Figure I.

posure of SV40-infected CV-1 cells to actinomycin D caused accumulations of highly catenated SV40 daughter chromosomes (Figure 2, lanes 5 and 6). Production of catenated daughter chromosomes is a signature of type II topoisomerase inhibition (Sundin & Varshavsky, 1981; Snapka et al., 1988; DiNardo et al., 1984). The pattern of protein association seen for these viral replication intermediates was similar to that seen for the nonintercalating type II topoisomerase poison VP-16, except that the catenation linking numbers of the daughter chromosomes were higher in the actinomycin D treated cells. Higher catenation levels are always seen with the intercalating type II topoisomerase inhibitors (Snapka et al., 1988). A small amount of form I DNA was always protein-associated as a result of actinomycin D treatment.

We also examined protein association patterns for SV40 DNA replication intermediates exposed to other type II topoisomerase inhibitors. Ellipticine treatment produced highly catenated dimers, but unlike the catenated dimers seen in the presence of VP-16 and actinomycin D, they were not protein-associated (Figure 2, lanes 9 and 10). Forms I, II, and III and the late Cairns structure were also protein-free in the presence of ellipticine. Proflavine, another strong intercalator, also produced highly catenated SV40 daughter chromosomes. Small amounts of forms II and III were protein-associated in the presence of proflavine, but the catenated dimers and form I DNA were not detectable in the glass-bound material. The intercalating type II topoisomerase poison *m*-AMSA caused significant protein association of forms II and III and a low level of protein association with form I DNA and the late Cairns structure (Figure 2, lanes 13 and 14). Most of the catenated dimers produced by exposure to *m*-AMSA were in the unbound (protein-free) material, with only faint traces of catenated dimers being apparent in the glass-bound material.

To examine drug-dependent protein association with SV40 genomes in the absence of DNA replication, a pulse–chase experiment was done. When the pulse label was chased into

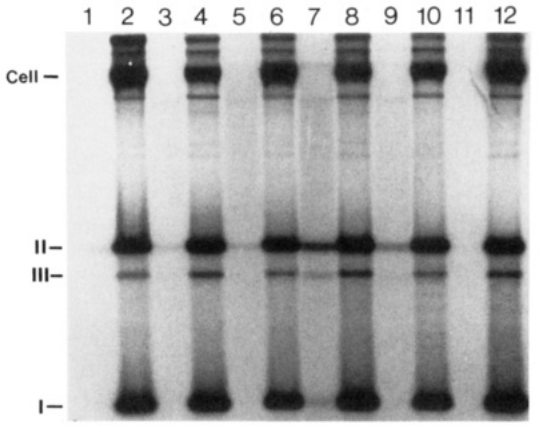

FIGURE 3: Association of completely replicated SV40 genomes with protein after exposure of infected cells to topoisomerase inhibitors. Completely replicated SV40 genomes were labeled with [^{3}H]thymidine for 30 min (250 μCi/mL), then the label was removed, and 20 mM unlabeled thymidine was added for 3 h. Drugs were added 15 min before extraction. The glass powder bound material and the unfractionated Hirt supernatant respectively are in lanes 1 and 2 for cells treated with 40 μM ellipticine, lanes 3 and 4 for 40 μM proflavine, lanes 5 and 6 for 40 μM *m*-AMSA, lanes 7 and 8 for 100 μM VP-16, lanes 9 and 10 for 40 μM camptothecin, and lanes 11 and 12 for cells not treated with any drug (control). Abbreviations same as in Figure 1.

[2] Actinomycin D has also been reported to weakly stabilize the covalent complex of topoisomerase I with DNA (Trask & Muller, 1988). This same report indicated that actinomycin D had no effect on DNA cleavage by topoisomerase II. As shown in this report, our results support the finding of Tewey et al. (1984) that actinomycin D is a topoisomerase II poison. Like other intercalating topoisomerase II poisons, it produces highly catenated dimers, a signature of topoisomerase II inhibition (Sundin & Varshavsky, 1981; Snapka, 1986; Snapka et al., 1988; DiNardo et al., 1984; Yang et al., 1987). In contrast to camptothecin, actinomycin D does not cause significant replication fork breakage (this report). Wasserman et al. (1990) have reported that actinomycin D stimulates both topoisomerase I and topoisomerase II cleavages on DNA. This supports the idea that there are no sharp divisions between different classes of topoisomerase inhibitors (Shin et al., 1990).

completed SV40 forms and the cells were extracted without exposure to topoisomerase inhibitors, all of the labeled forms were found in the protein-free material which did not bind glass powder (Figure 3, lanes 11 and 12). Exposure to ellipticine did not result in detectable protein association for any of the completely replicated forms (lanes 1 and 2). As in the case of replicating viral genomes, proflavine treatment caused some of the form II DNA to bind glass powder, but form III was not detectable. Exposure to *m*-AMSA caused a small amount of form II and a trace of form I DNA to bind to glass powder (lanes 5 and 6). VP-16 treatment resulted in glass powder binding by forms I, II, and III (lanes 7 and 8). Exposure of completely replicated viral genomes to camptothecin resulted in protein association of only form II DNA (lanes 9 and 10).

Discussion

Although these topoisomerase II inhibitors all slow completion of the late Cairns structure and decatenation of completed daughter chromosomes (Snapka, 1988), the patterns of protein association with replication intermediates were found to be quite varied. VP-16 caused some degree of protein association with all the intermediates present (with different intermediates showing different ratios of protein-bound to protein-free). In contrast, ellipticine and proflavine caused accumulations of late replicating forms including highly catenated dimers, yet these forms showed no protein association. For proflavine, only a trace of form II DNA was protein-associated. These observations are consistent with our earlier classification of these drugs as predominantly topoisomerase antagonists[3] (Snapka et al., 1988; Shin et al., 1990). Actinomycin D also caused accumulations of highly catenated dimers, but in this case, they were protein-associated. The late Cairns structure and forms II and III were also strongly protein-associated in the presence of actinomycin D. The well-characterized intercalating topoisomerase II poison *m*-AMSA caused still another pattern of protein association. There was a significant accumulation of label in the late Cairns and catenated dimer bands, but only forms II and III showed pronounced protein association. The late Cairns and form I DNA were only weakly protein-associated in cells treated with *m*-AMSA.

Protein-associated form I DNA was seen in all cells treated with topoisomerase II poisons. The amount of this form I DNA–protein complex was low in comparison to the total amount of labeled form I DNA in the Hirt supernatants. The protein association with form I viral DNA was entirely dependent on treatment with specific topoisomerase II poisons (actinomycin D, VP-16, *m*-AMSA), and DNA replication was not required. Form I DNA was not protein-associated in untreated control cells, in cells treated with the type I topoisomerase poison camptothecin, or in cells treated with type II topoisomerase antagonists. The drugs causing strong protein association with form I DNA are structurally unrelated and include both intercalators (*m*-AMSA and actinomycin D) and the nonintercalator VP-16. The only thing these drugs have in common is their activity as topoisomerase II poisons.

A general equation for topoisomerase II action is

$$\text{enzyme} + \text{DNA} \rightleftarrows \text{enzyme·DNA(noncovalent)} \rightleftarrows \text{enzyme·DNA(covalent)}$$

Here, free topoisomerase reversibly binds DNA to form a noncovalent topoisomerase–DNA complex. This complex is next converted to a covalent topoisomerase–DNA complex in which one or both of the topoisomerase subunits is covalently attached to the DNA on the 5′ side of a DNA strand break (Liu et al., 1983). This covalent topoisomerase–DNA complex is an intermediate in the strand passing reaction. Denaturation, for instance by SDS, renders this complex irreversible (Liu et al., 1983). The two strand breaks can occur independently (Zechiedrich et al., 1989), and it is thought that topoisomerase poisons stabilize the covalent complex by inhibiting the religation reaction (Osheroff, 1989). Since the drug-stabilized covalent topoisomerase–DNA complex necessarily involves at least one DNA strand break, it cannot occur on form I DNA. Strand breaks will convert form I to forms II or III.

Polyamines have been reported to stabilize a noncovalent association of topoisomerase II with form I SV40 DNA in vitro (Pommier et al., 1989). Our data indicate that type II topoisomerase poisons stabilize a strong but noncovalent complex between protein and form I SV40 DNA in vivo. Because of the specificity of the type II topoisomerase poisons, it is likely that this protein is topoisomerase II. Since no DNA strand break is involved, it is also likely that the complex is topological in nature. Our results suggest that type II topoisomerase poisons as a class stabilize this "topological complex" in vivo. If the topological complex corresponds to the second step in the reaction above, this stabilization would tend to shift the reaction to the right, and ultimately increase the amount of covalent complex. Thus, stabilization of the noncovalent (topological) topoisomerase–DNA intermediate in the reaction may play a significant role in the mechanisms of type II topoisomerase poisons. It may be that topoisomerase II poisons interact with the topoisomerase II and DNA in a manner analogous to polyamines. Investigations intended to unambiguously determine the identity of the form I DNA-associated protein are currently underway.

Exposure of SV40-infected cells to the type I topoisomerase poison camptothecin resulted in protein association with a different set of viral replication intermediates. On the basis of the known specificity of camptothecin for type I topoisomerase (Eng et al., 1988; Hsiang et al., 1985; Andoh et al., 1987; Bjornsti et al., 1989), we assume that the protein associated with viral DNA as a result of camptothecin exposure is type I topoisomerase. Form II viral DNA was protein-associated after exposure of either replicating or nonreplicating viral genomes to camptothecin. This is expected, since camptothecin traps topoisomerase I in a covalent complex with DNA on the 3′ side of a single-strand DNA break (Hsiang et al., 1985). Two short "smears" of replication intermediates known as LC′and LC″ are produced by breakage of replication forks by camptothecin-stabilized topoisomerase I–DNA complexes (Snapka, 1986). Both LC′and LC″ are protein-associated after camptothecin exposure. This finding confirms and extends an earlier report that replicating SV40 genomes are protein-associated in the presence of camptothecin (Champoux, 1988). Camptothecin has been reported to greatly increase the amount of pulse-label in form III SV40 DNA both in vivo

[3] Like hypertonic shock, strong intercalators (acridine orange, proflavine) prevent topoisomerase II action without significant trapping of protein–DNA complexes (Snapka et al., 1988; Shin et al., 1990). These drugs also prevent trapping of topoisomerase II–DNA complexes by specific topoisomerase II poisons (Shin et al., 1990). By definition, drugs which block the action of specific enzymes or which counteract the action of other drugs are antagonists. Drugs which stabilize covalent topoisomerase–DNA complexes are topoisomerase poisons (Liu, 1989). Topoisomerase inhibitors include both poisons and antagonists. Tewey et al. (1984) reported that ellipticine was a topoisomerase II poison at low concentrations. In our experience, it has behaved as a topoisomerase antagonist at all concentrations (Snapka et al., 1988; Shin et al., 1990). These different experiences with ellipticine may be related to the different sources of the drug or to the differences between in vitro and in vivo conditions.

(Snapka et al., 1988; Snapka, 1986) and in vitro (Hsiang et al., 1989). We have shown here that form III (linear) SV40 genomes were protein-associated after camptothecin exposure of replicating viral genomes but not after camptothecin exposure of nonreplicating genomes. This suggests that the increase in form III is not due to a set of closely spaced topoisomerase I sites on opposing strands (which would give a double-strand break) but must be due to some action unique to replicating chromosomes. We have recently shown that this increase in form III is due to complete detachment of DNA replication bubbles from late Cairns structures as a result of leading- and lagging-strand replication fork breaks caused by camptothecin-stabilized topoisomerase I–DNA complexes (Shin & Snapka, 1990). The late Cairns structure itself was present but was not significantly protein-associated. This suggests that topoisomerase I is not randomly trapped on replicating structures but may act preferentially near replication forks.

We (Snapka et al., 1988) and others (Varshavsky et al., 1983; Richter & Strausfeld, 1988; Fields-Berry & DePamphilis, 1989) have suggested that type I topoisomerase might not be able to act efficiently on the unreplicated portion of the late Cairns structure either because of stearic hindrance from converging DNA replication complexes or because of the presence of a "hyperstable" nucleosome at this site (Poljak & Gralla, 1987). This could explain the apparent increased role of topoisomerase II in replication of the late Cairns structure. A swivel activity would still be required, and unlike topoisomerase I, topoisomerase II can theoretically carry out the swivel function by acting behind the replication forks (Champoux & Been, 1980). However, the terminus region is rich in topoisomerase I sites (Porter & Campoux, 1989), and the fact that camptothecin exposure efficiently breaks replication forks in late replication structures suggests that topoisomerase I can act efficiently at replication forks at late stages of DNA replication in vivo. The results reported here are consistent with the idea that there is a shift from predominant topoisomerase I action to topoisomerase II action at the point where replication forks converge, but the reason for this apparent shift is not clear.

The ability to bind protein–DNA complexes to glass powder has enabled us to rapidly purify SV40 DNA viral replication intermediates uniquely associated with protein as a result of exposure to specific topoisomerase poisons. This approach may also be useful for the isolation of cellular DNA sequences uniquely associated with proteins after exposures to cytotoxic drugs.

Registry No. VP-16, 33419-42-0; *m*-AMSA, 51264-14-3; camptothecin, 7689-03-4; ellipticine, 519-23-3; proflavin, 92-62-6; actinomycin D, 50-76-0; topoisomerase, 80449-01-0.

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