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Camptothecin, a Specific Inhibitor of Type I DNA Topoisomerase, Induces DNA Breakage at Replication Forks

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The structure of replicating simian virus 40 minichromosomes, extracted from camptothecin-treated infected cells, was investigated by biochemical and electron microscopic methods. We found that camptothecin frequently induced breaks at replication forks close to the replicative growth points. Replication branches were disrupted at about equal frequencies at the leading and the lagging strand sides of the fork. Since camptothecin is known to be a specific inhibitor of type I DNA topoisomerase, we suggest that this enzyme is acting very near the replication forks. This conclusion was supported by experiments with aphidicolin, a drug that blocks replicative fork movement, but did not prevent the camptothecin-induced breakage of replication forks. The drug teniposide, an inhibitor of type II DNA topoisomerase, had only minor effects on the structure of these replicative intermediates.

DNA topoisomerases are essential enzymatic components of the DNA replication apparatus; type I topoisomerases are probably required to remove the superhelical tension which could accumulate in front of advancing replication forks, and type II topoisomerases can also participate in this reaction but normally function to separate the catenated products of replication cycles (reviewed in references 34 and 35).

Type I DNA topoisomerases release topological stress by breaking one strand of the DNA double helix and passing the other strand through the break. Mammalian type I topoisomerase, a protein of M_r 100,000, becomes covalently linked to the 3′ phosphate of a broken strand before the attached strand is then transferred to a 5′ OH receiving group (reviewed in references 34 and 35).

The cytotoxic antitumor drug camptothecin interferes with this reaction and stabilizes the intermediate enzyme-linked DNA breaks (17). Camptothecin appears to be specific for type I topoisomerase because other topoisomerases are not affected by the drug (17). The specificity of the drug is underlined by the finding that camptothecin-resistant cell lines possess a drug-resistant type I topoisomerase (2). Thus, camptothecin is a potentially valuable tool for assessing the role of type I topoisomerase in vivo.

Previous work has shown that camptothecin inhibits RNA and DNA synthesis in vivo (1, 2, 4, 16, 32). The extent of inhibition is correlated with breaks in cellular DNA (26). These breaks have been mapped in certain cases to specific sequences of transcribed genes (3, 9, 10, 29) as well as to regions of replicating DNA (23), in agreement with other experiments showing that type I topoisomerase may be involved in transcription and replication of eucaryotic DNA (34, 35).

Biochemical studies with in vitro DNA replication systems have shown that type I topoisomerase is involved in the fork movement reaction in replicating DNA, but this function is dispensable and can be replaced by a type II topoisomerase (36). Similar conclusions have been reached in studies of the behavior of yeast mutants with impaired type I and type II topoisomerase functions (5, 7, 11). Type II topoisomerase appears to be normally involved in a termination reaction (20), including the separation of catenated replication products (31).

Simian virus 40 (SV40) serves as a useful model of mammalian DNA replication because the size of the replicon is small, ca. 5,200 base pairs per circular double-stranded DNA (33); because the replicon is organized much like cellular chromatin, with 20 to 25 densely packed nucleosomes, the SV40 minichromosome (25, 33); and because SV40 DNA uses the replication machinery of the host cell, except for an initiator protein, the virus-coded T antigen, which unwinds the viral origin of replication and serves as a DNA helicase at replication forks (for a brief review, see reference 28). Except for T antigen, all other replication functions, including DNA topoisomerase, are supplied by the host cell.

In the present work, we have extracted replicating SV40 minichromosomes from camptothecin-treated infected host cells. The minichromosomes were cross-linked in vitro by using psoralen and UV light irradiation as already described (24). Electron microscopic examination, performed after deproteinization and DNA denaturation, is a very useful method to unambiguously distinguish between superhelical and relaxed sections of replicative intermediate DNA (25). We show below that camptothecin caused DNA strand breakage that preferentially occurred at replication forks.

MATERIALS AND METHODS

Cell culture and virus infection. African green monkey kidney cells (strain TC7) were grown on 14.5-cm plastic dishes in Dulbecco modified Eagle medium (DME medium; Gibco) supplemented with 5% fetal calf serum (Gibco). Cells were infected with SV40 strain SVS at a multiplicity of 10 to 20 PFU/cell. All experiments were carried out 42 to 45 h after infection.

Drugs. Camptothecin (NSC 94600) was obtained from the Division of Cancer Treatment, Natural Products Branch, National Cancer Institute, and was dissolved in dimethyl sulfoxide (DMSO; Serva) at a concentration of 5 mg/ml. Aphidicolin (Boehringer Mannheim) was dissolved in DMSO at a concentration of 2 mg/ml.

Labeling and analysis. Replicating SV40 DNA was pulse-labeled with 1 mCi of [^{3}H]thymidine (Amersham) in 10 ml of

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DME medium for 4 min. The radioactive medium was removed after the pulse. The cells were washed twice with thymidine-depleted DME medium before chases were carried out in 7.5 ml of DME medium–5% fetal calf serum supplemented with 100 μM nonradioactive thymidine in either the absence or presence of 54 μM camptothecin or 59 μM aphidicolin or both.

For long-term labeling, 0.2 mCi of [^{3}H]thymidine per plate was used for 4, 19, or 49 min. Drugs were added 4 min after the start of the labeling period.

Viral DNA was extracted by the method of Hirt (15). The Hirt supernatant was treated with 50 μl of proteinase K (Boehringer Mannheim) per ml at 37°C for 1 h. The DNA was collected by ethanol precipitation.

Neutral sucrose gradients (7.5 to 25%) were performed exactly as described before (27).

Preparation of viral DNA for electron microscopy. SV40-infected TC7 cells were pulse-labeled with 50 μCi of [^{3}H]thymidine in 5 ml of DME medium for 18 to 20 min. Camptothecin was added 5 min after the start of the labeling period and left for 15 min. In experiments with aphidicolin, the drug was added 18 min after labeling and left for 30 min. In some experiments, both drugs were used in combination. Aphidicolin was added first, at 15 min, followed by camptothecin, added at 20 min after the start of the labeling period. Viral chromatin was extracted 25 min later.

SV40 chromatin was extracted in hypotonic buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid, pH 7.8], 5 mM potassium acetate, 0.5 mM magnesium acetate, 1 mM dithioerythritol) as described (27, 30) and purified in 5 to 30% sucrose gradients prepared in the same hypotonic buffer. Centrifugation was performed in the Beckman SW40 rotor for 110 min at 0°C and 39,000 rpm. The combined sucrose gradient fractions with replicating minichromosomes (80S to 95S) were treated with 4,5',8-trimethylpsoralen (Paul B. Elder Co. or Sigma) and UV-irradiated in open plastic dishes on ice exactly as described before (25). The psoralen-cross-linked SV40 replicating chromatin was collected by ethanol precipitation. The pellets were washed once with 100% ethanol. The dried pellets were resolved in TE (10 mM Tris [pH 7.8], 1 mM EDTA)–0.25% sodium dodecyl sulfate. After treatment with RNase A (500 μg/ml; Boehringer) for 10 min at 37°C and proteinase K (50 μg/ml) for 1 h at 37°C, the DNA was extracted by repeated phenol-chloroform treatments and precipitated in ethanol. The dried pellets were resolved in TE and spread for electron microscopy. Spreading and measuring DNA contour lengths were performed as described by Sogo et al. (24, 25).

RESULTS

Pulse-chase experiments. SV40 DNA, pulse-labeled with [^{3}H]thymidine, was chased with a 5,000-fold excess of nonradioactive thymidine in the presence or, in a parallel experiment, in the absence of camptothecin. The DNA was extracted and analyzed by neutral sucrose gradient centrifugations (Fig. 1). As expected from previous experiments (27), pulse-labeled replicating SV40 DNA sedimented in a broad peak ranging from 16S to 27S (Fig. 1a). During a normal chase, these structures were converted to mature superhelical form I (21S) and relaxed form II (16S) DNA (Fig. 1b). In contrast, a chase in the presence of camptothecin resulted in DNA forms sedimenting in a rather broad peak between 21S and 15S (Fig. 1c). This result indicates that camptothecin induced an aberrant pattern of ^{3}H-labeled replicating DNA.

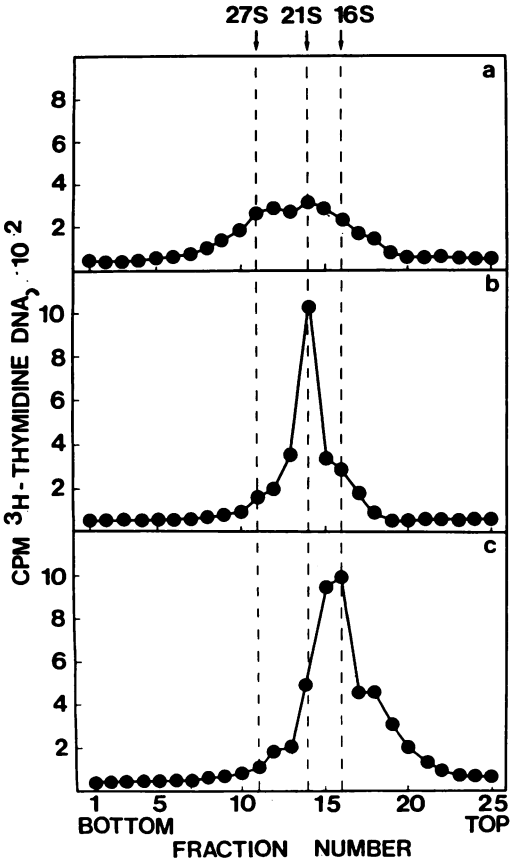


FIG. 1. Pulse-chase experiment. SV40-infected cells were pulse-labeled for 4 min with [^{3}H]thymidine (a) and chased with an excess of nonradioactive thymidine in the absence (b) or presence (c) of camptothecin. The extracted DNA was sedimented through neutral sucrose gradients (27). Under the centrifugation conditions, superhelical form I SV40 DNA and relaxed form II DNA have sedimentation coefficients of 21S and 16S, respectively. Late replicative intermediates sediment at 27S (27).

Pulse-chase experiments similar to those shown in Fig. 1 were also performed to investigate the ^{3}H-labeled DNA by agarose gel electrophoresis and fluorography. Pulse-labeled SV40 DNA appeared as a smear of radioactively labeled material representing replicating molecules in all stages of the replicating cycle up to late Cairns (LC) structures (data not shown [27]), which are known to accumulate before segregation of the replication products takes place (22). The chase products of the control experiments appeared mainly as mature form I and form II DNA, as expected from the data in Fig. 1 and previous work (27). When the chase was performed in the presence of camptothecin, an entirely different pattern of chase products was obtained, as described before by Snapka (23): only a small fraction of replicating DNA was converted into mature form I and form II replication products; some radioactively labeled DNA appeared to be linearized to give form III DNA, i.e., linear SV40 DNA of unit length; the remaining radioactivity was located in a region between the position of LC structures and form II DNA as a "compressed" smear of replicating intermediates with sharp boundaries on the cathode side and a smearing out on the anode side, exactly as described before by Snapka (23) (data not shown).

In conclusion, our pulse-chased experiment results are in agreement with earlier findings (see above) showing that

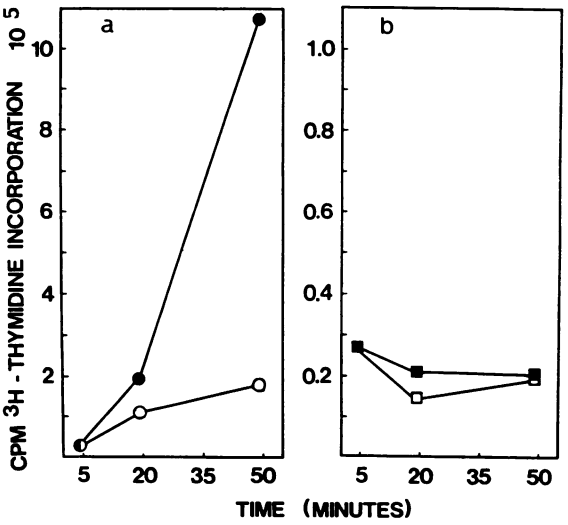


FIG. 2. Incorporation of [^{3}H]thymidine into SV40 DNA. (a) Long-term labeling conditions in the absence (●) or presence (○) of camptothecin. (b) Long-term labeling in the presence of aphidicolin (■) or aphidicolin and camptothecin (□).

camptothecin induced an aberrant pattern of SV40 DNA replication, causing an accumulation of structures with properties of unusual replicative intermediate DNA forms.

It was of interest to find out whether these structures were used as substrates for continued DNA synthesis.

Inhibition of [^{3}H]thymidine incorporation. To determine whether camptothecin caused inhibition of SV40 DNA synthesis, we added camptothecin to SV40-infected cells 4 min after the start of long-term labeling with [^{3}H]thymidine. Immediately before and 15 and 45 min after addition of the drug, SV40 DNA was extracted and sedimented through neutral sucrose gradients. No drug was added in a parallel control experiment. Replicating intermediates were observed after the 4-min pulse-labeling period. With continuous labeling, mature form I and form II DNA accumulated in the control experiment, whereas broad peaks of 15S to 21S DNA appeared in drug-treated cells (data not shown; see Fig. 1). The sum of the radioactivity recovered in the SV40 DNA fraction of these gradients was determined and plotted as a function of labeling time. [^{3}H]thymidine was incorporated at an essentially linear rate in the control experiment, and [^{3}H]thymidine incorporation was severely inhibited in camptothecin-treated cells (Fig. 2a). The effects of camptothecin on [^{3}H]thymidine uptake and on the conversion of thymidine to dTTP are not known. We therefore propose that the drug drastically inhibited SV40 DNA replication.

In Fig. 2b, we show the results of similar experiments with aphidicolin and with aphidicolin-camptothecin-treated SV40-infected cells. It is apparent that aphidicolin caused an immediate stop of SV40 DNA synthesis, in agreement with previous observations (8).

Breakage at replication forks. Replicating SV40 minichromosomes were extracted from camptothecin-treated infected cells as well as from untreated cells and from cells treated with DMSO, the solvent for camptothecin. The replicating minichromosomes were purified by sucrose gradient centrifugation and subjected to the psoralen-UV-irradiation procedure outlined in Materials and Methods. After deproteinization and denaturation, the DNA molecules were spread for electron microscopy. Several hundred replicating SV40 DNA molecules from the control and drug-treated samples were analyzed.

In Fig. 3 we show examples of intact replicative intermediate minichromosomes as controls. The molecule shown in Fig. 3a contained on both replicated branches arrays of single-stranded DNA bubbles corresponding to the location of nucleosomes (25). The unreplicated section in the molecule appeared as double-stranded DNA because the unreplicated part of replicative intermediate SV40 DNA is normally in a superhelical conformation (19, 21) and is therefore resistant to denaturation (25). In contrast, the molecule shown in Fig. 3b contained an unreplicated part of a relaxed configuration, probably due to a single-stranded break some place in the unreplicated duplex. For this reason, denaturation of the unreplicated section was possible and also resulted in an array of single-stranded bubbles corresponding to the location of the nucleosomes.

In the control samples (no camptothecin treatment, DMSO treatment), we found that the majority (>85%) of all replicating intermediates were intact and possessed the structural features described above (Fig. 3). The remainder

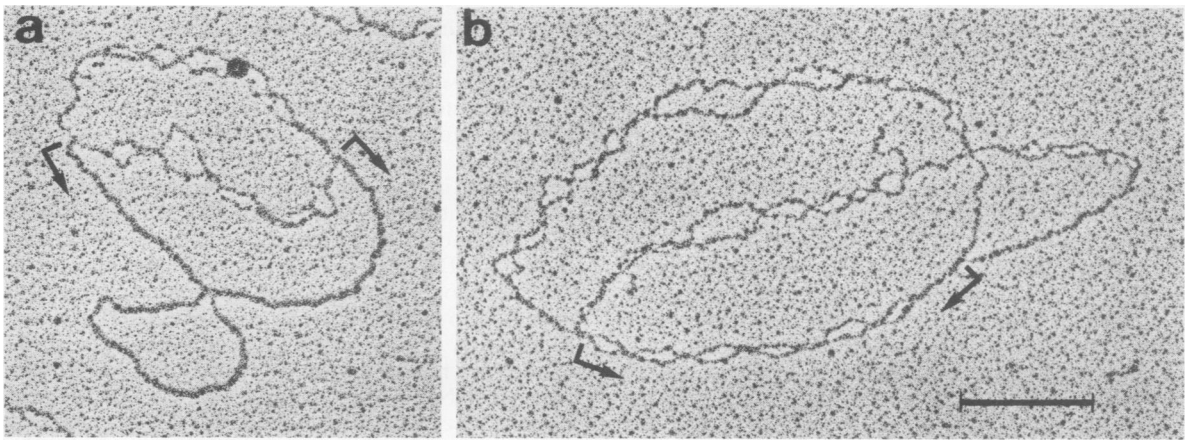
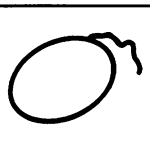
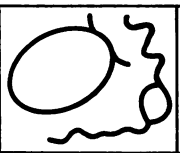
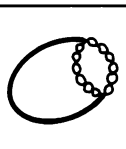
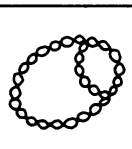


FIG. 3. Electron micrographs of replicating SV40 DNA prepared from psoralen cross-linked minichromosomes. Examples of unbroken replicative intermediates from camptothecin-treated infected cells. (a) Unreplicated parental section (between arrows) is in a covalently closed superhelical configuration. (b) Parental DNA strand is nicked. The unreplicated section (between arrows) shows the typical array of denatured, single-stranded DNA bubbles.

TABLE 1. Structure of replicative intermediate SV40 DNA in camptothecin-treated cells[a]

				Total no. of analyzed molecules		
Camptothecin	187 (66%)	18 (6%)	80 (28%)	285	31 (39%)	49 (61%)
Untreated cells	27 (10%)	7 (3%)	227 (87%)	261	173 (76%)	54 (24%)

[a] The data from two independent experiments were combined. (Left panel) The analyzed structures were classified as sigma forms, randomly broken molecules (broken in either the unreplicated or replicated parts of the molecule), and unbroken forms as indicated. (Right panel) The unbroken replicative intermediates from camptothecin-treated and control cells were classified according to the topological state of the unreplicated region: superhelical in the left and relaxed in the right column (see Fig. 3).

of the molecules were broken at random sites (see Tables 1, 3, and 4). In contrast, in SV40 DNA extracted from camptothecin-treated cells, 66% of all branched molecules considered to represent replicative intermediates were broken at one replication fork; 6% were broken at random sites in the unreplicated parental or the replicated section; and only 28% of all replicating intermediates remained apparently intact (Table 1). In Fig. 4, we show typical examples of putative broken replicating intermediates from camptothecin-treated cells. A characteristic sigma (σ)-type structure was seen in these and other cases. They were characterized by a branch attached to a DNA circle of unit length. We interpret this to mean that the circles were composed of a replicated and an unreplicated section, whereas the branch was assumed to correspond to one of the newly synthesized daughter strands broken off at one replication fork. We determined the size distribution of the branches and compared it with the size distribution of replicated branches in intact replicative intermediates. The branches ranged from a few hundred base pairs to almost the unit length of about 5,200 base pairs in length (Fig. 5). No significant differences between these data and the size distribution of replicated branches in intact replicating intermediates could be detected (Fig. 5). These results indicate that the broken molecules were not a subset of replicative intermediates. They included instead the entire spectrum of replicating molecules from early to late replicative forms. Since the lengths of the broken replicated branches must be equal to the lengths of the replicated parts of the circles, it was possible to localize the putative sites to which the open ends of the branches could have been attached before breakage (arrows in Fig. 4). In most cases no conspicuous structures could be detected at these sites. However, a short single-stranded tail was occasionally found sticking out close to these sites (e.g. Fig. 4a and c). These tails are most likely part of a broken nucleosomal bubble (25).

We tried to find out whether the breakages occurred at the leading or the lagging side of a replication fork. For this purpose we determined whether the linkage between the broken branch and the circle was double stranded (open arrowhead in Fig. 4b) or single stranded (solid arrowheads in Fig. 4a and c). A total of 89 broken replicative intermediates were analyzed (Table 2). About 63% of these had a small single-stranded region at the attachment site of the branch to the circle. In the remaining analyzable sigma forms (37%), the linkage region of the branch was double stranded. The difference between these numbers was not significant. We therefore conclude that strand breakages occurred at about equal frequencies on the leading and the lagging strand sides of the replication forks (Table 2).

Single-strand breaks outside replication forks. As shown in Table 1, in camptothecin-treated cells 28% of the branched molecules had two intact branch points, as in normal replicative intermediates. We determined whether the unreplicated sections in these molecules were in a superhelical or a relaxed configuration (Fig. 3). As summarized in Table 1, more than 60% of these molecules were found to be relaxed, in contrast to the control, in which only 24% of all replicative intermediates had a relaxed unreplicated section. This result shows that under our conditions, camptothecin also induced at least one single-strand break in the parental DNA strand of more than half of the molecules with intact replication forks, suggesting that topoisomerase I may act elsewhere on the parental strand.

Breaks at arrested replication forks. It was also possible that the topoisomerase was located some place ahead of the replication forks, allowing DNA synthesis to continue despite the presence of camptothecin until the growth points ran into a strand discontinuity. In order to investigate this possibility, we performed experiments with aphidicolin, a drug which inhibits mammalian replicative DNA polymerases (18), stopping growth point movement after the unwinding of a maximum of 100 base pairs of parental DNA (8). Consequently, SV40-infected cells were treated first with aphidicolin and 5 min later with camptothecin. If the proportion of sigma-type structures were reduced drastically under these conditions, the topoisomerase could be located at distances of more than ca. 100 base pairs ahead of the replication fork. We found instead that more than 50% of the replicating intermediates extracted from aphidicolin- and camptothecin-treated cells were in the shape of sigma structures just like those shown in Fig. 4 (Table 3). These data are in agreement with the proposition that type I DNA topoisomerases are located in proximity to the growth points in replicating SV40 DNA molecules.

Inhibition of type II DNA topoisomerase. Studies with in vitro replication systems have shown that type I topoisomerase is normally involved in the chain elongation reaction of replicating SV40 DNA, removing the positive superhelical tension which would otherwise accumulate ahead of the replication fork. But these studies have also shown that type I topoisomerase can be replaced by a type II topoisomerase

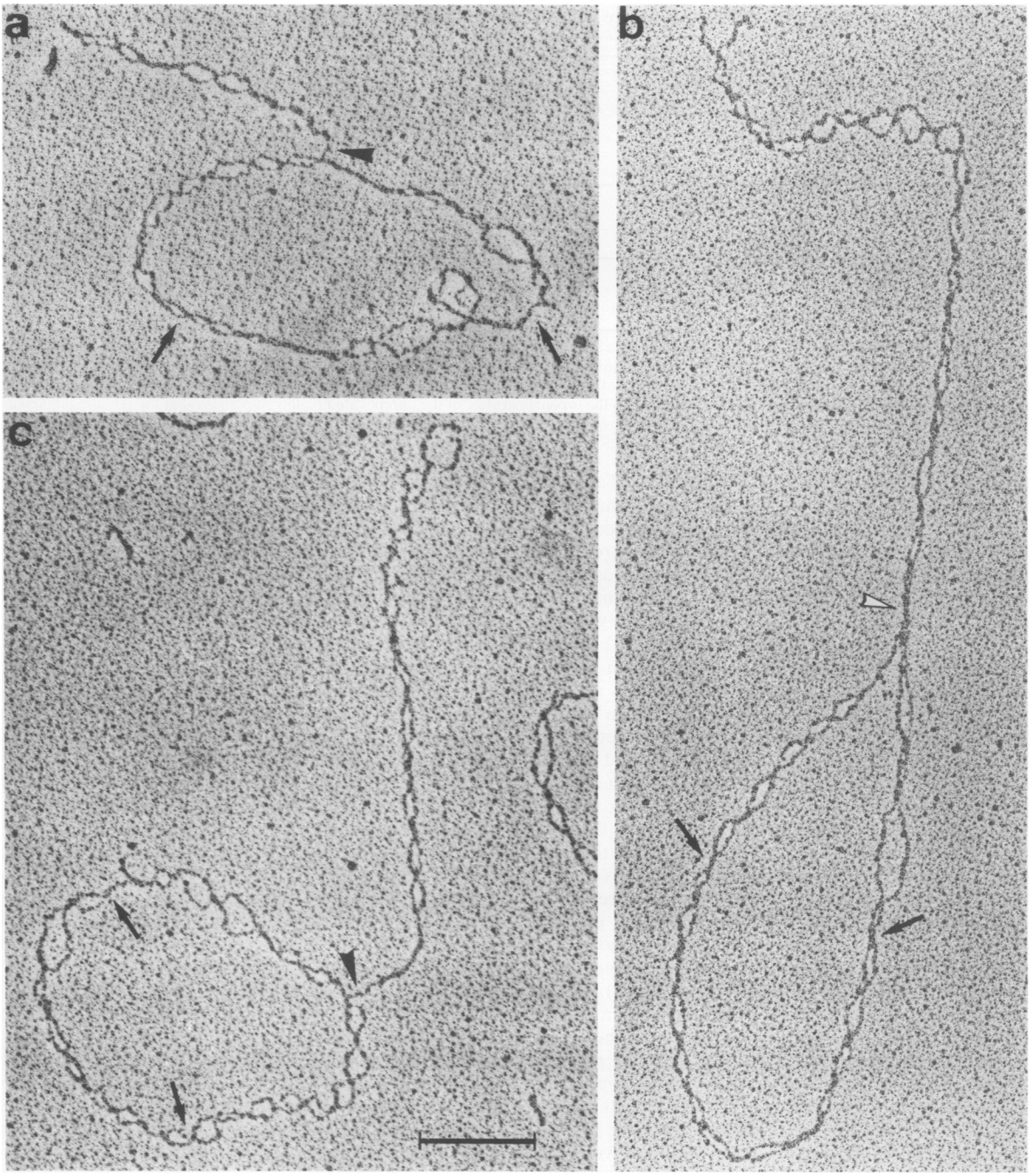


FIG. 4. Broken replicative intermediate SV40 DNA from camptothecin-treated cells. Examples of sigma forms (unit-length DNA circles attached to linear branches) at various stages of the replication cycle. In the structures shown in panels a and c, the branch is attached to the circle via a short single-stranded region (solid arrowheads), interpreted to be the lagging-strand side of the fork. Consequently, breakage is likely to have occurred at the leading side of the fork. The structure shown in panel b has a double-stranded region (open arrowhead) at the branch point; in this case, breakage is likely to have occurred at the lagging strand of the fork. The arrows (a, b, and c) indicate possible break points on the circular part of the structure.

when the type I enzyme is removed or inhibited (5, 7, 11, 36). Previous studies with the specific type II topoisomerase inhibitor teniposide (VM26), an epipodophyllotoxin derivative (6, 37), suggested that type II topoisomerase was mainly involved in a reaction converting late replicating intermediates (LC structures) into mature replication products (20). However, the participation of type II topoisomerase in chain elongation reactions could not be excluded entirely (20). To investigate this possibility, we treated SV40-infected cells with VM26 dissolved in DMSO under the conditions described previously (20). DNAs in replicating SV40 minichromosomes were cross-linked, extracted, and denatured as described above. Several hundred replicating intermediates were investigated and classified as intact molecules, sigma forms (broken at one growth point), or randomly broken molecules. The data were compared with results from a

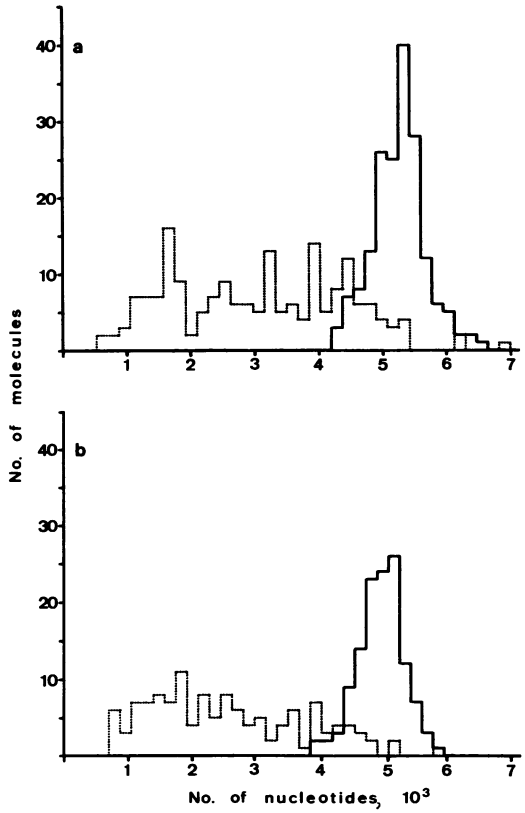


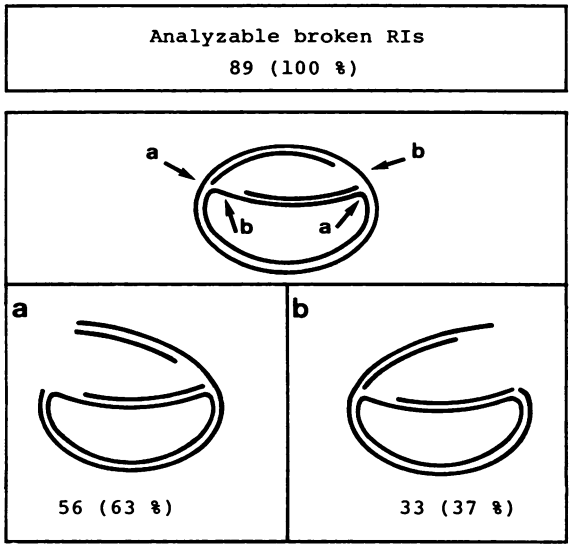
FIG. 5. Size distribution of replicative intermediates. (a) A total of 187 unselected sigma structures from camptothecin-treated cells were analyzed. The average size of the circles (solid line) was determined to be 5,241 ± 419 nucleotides. The sizes of the attached tails (broken line) were randomly distributed over a range from a few hundred base pairs to full-length SV40 DNA. (b) A total of 128 unbroken replicative intermediates from camptothecin-treated cells were investigated. The average size of the circles (solid line) was found to be 4,963 ± 363 nucleotides, corresponding to the sum of the unreplicated parental strand and one of the replicated sections (see Fig. 3). The broken line represents the random distribution of the second replicated section. No significant difference in the size distribution of replicated sections in unbroken replicative forms and the attached branches in sigma forms was detected.

parallel experiment, a control in which SV40-infected cells were treated with DMSO, the solvent for VM26 (and the other drugs in this study). As shown in Table 4, most of the replicative intermediates (59%) remained intact in VM26-treated cells. These structures largely retained the superhelical configuration of the unreplicated part, as in replicative intermediates from control cells. But it is also obvious from the data in Table 4 that the fraction of sigma forms and of randomly broken DNA structures was somewhat higher in VM26-treated than in DMSO-treated control cells but clearly not as high as in camptothecin-treated cells (Table 1).

DISCUSSION

The pulse-chase experiment (Fig. 1) clearly demonstrated that camptothecin treatment of SV40-infected cells caused pronounced changes of the structure of SV40 DNA replicative intermediates. Similar results have been reported before for polyomavirus (12, 13) and for SV40 (23). The DNA forms extracted from camptothecin-treated cells and identified by sucrose gradient sedimentation and agarose gel electrophoresis, have been interpreted as broken replicative intermediates (23). We show now that camptothecin indeed causes DNA breakage. One preferential site of cleavage is clearly close to the replication fork, but possible cleavage elsewhere on the parental side of the replicative intermediates is not excluded by the data in Table 1. The data immediately suggest a possible mechanism for the cytotoxic effects that camptothecin exerts on proliferating cells. They also give insights into the role that type I topoisomerase plays in replicative chain elongation.

TABLE 2. Localization of break points[a]

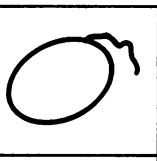
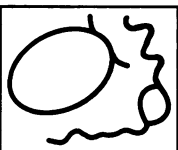
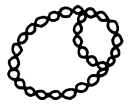


[a] The drawing in the center indicates possible break points (a) at the leading strand side of the fork and (b) at the lagging side. A total of 187 sigma-form molecules were screened for a single-stranded or double-stranded linkage of the branch to the circle. In 89 molecules, the results were unambiguous, as in Fig. 4. This number was taken as 100%. The other 98 molecules could not be analyzed. RI, Replicative intermediate.

In camptothecin-treated cells, a considerable portion of replicating SV40 minichromosomes were broken at one of the two replication forks, giving rise to sigma-type structures consisting of a linear replicated branch attached to a circular DNA molecule of unit length. This finding does not necessarily mean that breakage always occurred at one fork only. But replicating molecules broken at both replication forks remained undetected. If breakage occurred at the leading strand of one fork and at the lagging strand of the second fork, one reaction product would be a circular DNA, indistinguishable from normal nonreplicating DNA, and the other product would be an unidentifiable short linear DNA fragment. If breakage occurred simultaneously at the leading (or the lagging) strands of both forks (see drawing in Table 2, arrows a and b, respectively) a linear DNA of more than unit size would be produced. If such molecules were produced, they must have been rare, as we did not detect them. Moreover, replicating molecules broken at the leading and at the lagging strand of one fork would be converted to Y-type structures, which we detected in small numbers among the SV40 molecules from camptothecin-treated cells (data not shown).

It could be argued that the partial single-strandedness of replication forks made this part of the replicative intermediate vulnerable to unspecific strand breakages, particularly when the progression of the replication fork was impeded, as

TABLE 3. Analysis of replicative SV40 DNA intermediates from aphidicolin-treated and aphidicolin-camptothecin-treated cells[a]

				Total no. of analyzed molecules		
Aphidicolin	13 (8%)	1 (1%)	144 (91%)	158	112 (78%)	32 (22%)
Aphidicolin + Camptothecin	168 (51%)	29 (9%)	130 (40%)	327	62 (48%)	68 (52%)
Untreated cells	20 (11%)	7 (4%)	156 (85%)	183	120 (77%)	36 (23%)

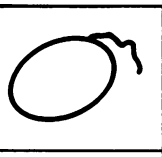
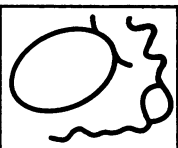
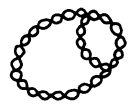
[a] See Table 1, footnote *a*.

in camptothecin-treated cells. However, this possibility can be excluded for at least two reasons: (i) we found that strand breakage preferentially occurred at the branch engaged in leading-strand synthesis (Table 2), where the size of the single-stranded region should be minimal; and (ii) fork breakage was not observed in aphidicolin-treated cells (Table 3). This is an important control, because the structure of the replication fork in aphidicolin-arrested replicative intermediates has been carefully analyzed before and shown to consist of unwound single-stranded regions of up to 100 base pairs (8). Thus, the absence of excess sigma forms in aphidicolin-treated cells excluded the possibility that exposed single strands may be an important reason for fork breakage.

Our [^{3}H]thymidine incorporation data suggest that DNA replication in camptothecin-treated cells is severely inhibited but not altogether blocked (see below). This conclusion is supported by the electron microscopic examination of the length distribution of broken replication branches. The observed distribution was very similar to the length distribution of the replicated branches in intact replicative intermediates and never exceeded the unit viral DNA length of ca. 5,200 base pairs. This finding excludes the possibility that DNA replication continued on broken molecules, for example, in the form of a rolling circle-type mechanism, as has been proposed before by others (23). Together, our findings give strong support to the notion that camptothecin inhibits DNA synthesis because it induces single-strand breaks at replication forks.

It has been shown that camptothecin is a specific inhibitor of type I topoisomerase (17). In the presence of the drug, mammalian type I topoisomerase induces a single-stranded break and becomes covalently attached to the 3′ phosphate end of the broken strand but fails to complete the cycle, being unable to reseal the nick. Given the high specificity and the proposed mechanism of camptothecin inhibition, it may be concluded that the sites of strand breakage are identical with the location of type I topoisomerase. Based on the data presented above, we propose that type I topoisomerase is localized in the vicinity of replicative growth points. We considered the possibility that the type I topoisomerase introduced a single-strand break in the parental DNA at some distance ahead of the replication fork. The advancing replication fork would disintegrate only when passing this discontinuity in the template strand. Our experiments with a double aphidicolin-camptothecin block appeared to exclude this possibility. Pretreatment with aphidicolin, leading to an almost immediate halt in fork movement (8), did not prevent the breakage of forks induced by camptothecin added 5 min after the addition of aphidicolin. Thus, our results are consistent with the proposition that type I topoisomerases are functioning as a swivel at the forks of replicating SV40 DNA. In fact, type I topoisomerase was identified on SV40 minichromosomes some time ago (14).

TABLE 4. Analysis of replicative SV40 DNA intermediates from VM26-treated and DMSO-treated cells[a]

				Total no. of analyzed molecules		
VM26	105 (32%)	30 (9%)	192 (59%)	327	136 (71%)	56 (29%)
DMSO	24 (10%)	2 (1%)	204 (89%)	230	151 (74 %)	53 (26 %)

[a] DMSO was the solvent for all three drugs used in this study. See Table 1, footnote *a*, for details.

This does not necessarily mean that all replication forks carry a type I topoisomerase(s). Even in the presence of the relatively high doses of camptothecin that we used in our study, some DNA replication continued, as demonstrated by the [^{3}H]thymidine incorporation data (Fig. 2) and by the pulse-chase experiments, showing some conversion to mature SV40 DNA forms (data not shown) (23), in contrast to aphidicolin-arrested cells, in which DNA synthesis was completely blocked (Fig. 2) (8). It is conceivable that those replicons which continue DNA replication in the presence of high camptothecin levels have engaged a type II topoisomerase as a swivel. This possibility is supported by the fact that some fork breakage was indeed observed in cells treated with VM26, a specific inhibitor of type II topoisomerase (Table 4).

It has been shown previously (5, 7, 11, 36) that topoisomerase I can be replaced by topoisomerase II in a chain elongation reaction (see Introduction). However, after camptothecin treatment, the replication template may be irreversibly damaged and unable to be used for further DNA replication even in the presence of sufficient amounts of functional type II topoisomerase.

Finally, we want to emphasize that not all type I DNA topoisomerases on replicating minichromosomes must be localized in the vicinity of replication forks. We found (Table 1) that a substantial fraction of replicating intermediates (with unbroken branches) in camptothecin-treated cells were relaxed and not in the normal superhelical conformation of replicating SV40 DNA (19, 21). This means that single-strand breaks may have occurred at sites other than replicating growth points. However, this is not necessarily the case. If topoisomerase I cuts ahead of the replication fork, detachment of the daughter DNA branches must not necessarily occur, since psoralen cross-linking is not inhibited to a noticeable extent by the replication machinery (25). Therefore, small duplex regions immediately ahead of the replication fork may be stabilized against denaturation. Such molecules would appear as unbroken replicative intermediates with relaxed parental strands. On the other hand, we can speculate that topoisomerase I recognizes gene segments involved in transcriptional regulation, since camptothecin is known to induce strand breakage in regions of transcriptionally active genes (9, 29).

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