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Inhibition of SV40 DNA Synthesis by Camptothecin and Neocarzinostatin

EDWARD A. SAUSVILLE[1] AND SUSAN B. HORWITZ[2]

Departments of Molecular Pharmacology and Cell Biology, Albert Einstein College of Medicine, Bronx, New York 10461

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SUMMARY

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Both camptothecin and neocarzinostatin cause 50% inhibition of simian virus 40 DNA synthesis in BSC-1 cells at a drug concentration of 1-0.1 μM. Uniformly prelabeled Form I simian virus 40 DNA is not converted to Form II by 100 μM camptothecin or 10 μM (10 μg/ml) neocarzinostatin, despite extensive drug-induced breakage of cellular DNA at these concentrations. Kinetic experiments examined the fate of replicative intermediates at the onset of inhibition of DNA synthesis. In the presence of 100 μg/ml neocarzinostatin, label proceeds through replicative intermediate molecules and is found largely (>75%) as Form I simian virus 40 DNA. At 100 μM camptothecin, up to 50% of newly made simian virus 40 DNA is found as a Form II-like species.

INTRODUCTION

Camptothecin, an alkaloid isolated from *Camptotheca acuminata* (Nyssaceae), is a potent inhibitor of nucleic acid synthesis in eucaryotic cells (1-3). Neocarzinostatin is a protein that is obtained from culture filtrates of *Streptomyces carzinostaticus*. The latter is also an effective inhibitor of DNA synthesis in eukaryotes, but RNA synthesis is not affected by the drug (4, 5). A common feature in the action of these inhibitors of DNA synthesis is that both drugs induce single-strand breaks in the nuclear DNA of treated cells (1, 5-8). This breakage may also be observed when sedimentation of DNA from drug-treated cells is conducted using neutral formamide sucrose gradients (8),[3] and therefore does not represent the exclusive induction of alkali-labile DNA damage as had been previously claimed (9). Camptothecin has also been shown to induce breakage of adenovirus-2 DNA and vaccinia DNA in infected HeLa cells (2, 10). Camptothecin does not cause breakage of purified DNA *in vitro* or inhibit DNA synthesis *in vitro* under any presently known conditions (1). In contrast, neocarzinostatin causes considerable degradation of isolated DNA when the drug and DNA are incubated in the presence of thiol compounds (7, 11, 12), and neocarzinostatin is an effective inhibitor of the incorporation of thymidine 5'-triphosphate into DNA by *E. coli* DNA polymerase I (13). Recently, evidence has been presented that neocarzinostatin may inhibit cell growth and DNA synthesis while it is attached to agarose beads (14), thus raising the possibility that drug action limited to the cell surface may be sufficient to cause cell death in certain cell types.

The experiments in this paper attempt to determine whether the breakage of tem-

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plate DNA is an effect that can be separated from the inhibition of DNA synthesis observed when cells are treated with camptothecin or neocarzinostatin. The system we have employed for this study is the productive infection with SV40[4] of an African green monkey derived cell line, BSC-1.

The DNA of SV40 is a closed circular supercoiled molecule (Form I) which sediments at 21S at neutral pH, and at 54S under alkaline conditions. On introduction of a single break in its 5,000 base pairs, a species (Form II) is produced which sediments at 16S under neutral conditions. In alkali, Form II SV40 DNA sediments as an 18S single-stranded circle plus a 16S single-stranded linear molecule. The structure of this DNA and the intermediates in its synthesis in infected cells are known in detail (for reviews, see refs. 15, 16). Labeled precursor added in a brief pulse enters an RI molecule, which is closed circular and sediments at neutral pH between 21S and 25S. Denaturation in alkali demonstrates that label in these RI molecules sediments first as short Okazaki-like pieces which are elongated up to almost unit length (16S) SV40 DNA strands. The amount of label that accumulates in Okazaki-like pieces is greatly enhanced by the presence of hydroxyurea or 5-fluorodeoxyuridine, suggesting that these drugs are selective inhibitors of elongation (17, 18). By a mechanism not yet clear, completed RI molecules are converted into Form I SV40 DNA.

It is therefore apparent that SV40 DNA replication can be used to determine at which step a drug acts to inhibit DNA replication (initiation of new rounds of DNA synthesis or elongation of previously initiated strands) and whether it alters the physical integrity of parental template DNA molecules. There is evidence that only one viral function, the A gene, is concerned with viral DNA replication. Since this function is involved only in the initiation of new rounds of viral DNA synthesis (19), subsequent events in the replicative process must involve enzymatic mechanisms of the host. Thus, effects of drugs on SV40 DNA synthesis may be of interest with respect to eukaryotic DNA replication.

[4] The abbreviations used are: SV40, simian virus 40; RI, replicative intermediate; PFU, placque-forming unit; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl, 0.015 M Na_3 citrate; p.i., post-infection.

MATERIALS AND METHODS

Cells and virus. BSC-1 cells were obtained from Dr. J. Maio and were grown in minimal essential medium (Grand Island Biological Co.), supplemented with 10% fetal calf serum and 2 mM glutamine containing 80 U/ml benzylpenicillin and 40 μg/ml streptomycin (complete medium). CV-1 cells and SV40 were a gift of Dr. S. Baum. Stock virus for these experiments was prepared by infection of confluent cultures of CV-1 cells at 0.002 PFU/cell. At the appearance of substantial (>80%) cytopathic effect, cells were scraped into the medium, frozen and thawed twice, and this stock lysate stored at −20°.

Drugs and reagents. Sodium camptothecin and neocarzinostatin, lots T29 and T46, were obtained from the National Cancer Institute. Both lots of neocarzinostatin were examined by SDS-polyacrylamide gel electrophoresis and found to be >90% pure. We have therefore used a molecular weight of 10,700 (20) to compare the molar potency of neocarzinostatin with that of camptothecin. Bleomycin A_2 (Lot 71L 489) was a gift of Bristol Laboratories. [^{3}H]methyl-thymidine (6.7 Ci/mmole) and 2-[^{14}C]thymidine (50 mCi/mmole) were obtained from New England Nuclear. High specific activity [^{3}H]methyl-thymidine was obtained from ICN, and used at the specific activities indicated. Unlabeled thymidine was from Sigma and hydroxyurea from Aldrich. All other reagents were of the highest commercially obtainable grade.

Infection of cells and analysis of viral DNA. BSC-1 cells were plated at 1.8–3 × 10^6 cells in 60 mm dishes. At confluence, they were infected with 0.5–10 PFU/cell of SV40 in 1–3 ml of stock lysate for 3 hr with intermittent rocking. The virus solution was removed, and complete medium added. On completion of the experimental protocols described in figure legends, cells were lysed and the viral DNA selectively extracted by the method of Hirt (21). In the Hirt extraction procedure, greater than 95%

of cellular DNA is recovered in a precipitate in 1 M NaCl; low molecular weight viral DNA (about 80%) is found in the supernatant (21). In our protocols wherein viral DNA has been labeled with radioactive precursor and then chased with unlabeled precursor to dilute nucleotide pools, the Hirt supernatant fraction contains labeled Form I and Form II SV40 DNA. This Form II SV40 DNA arises from damage to the closed circular supercoiled molecule during the extraction procedure. The fraction of DNA which is Form II varies among different series of experiments from 5 to 25% of the total amount of SV40 DNA present. In protocols where a chase period has not been employed, the Hirt supernatant fraction contains labeled Form I, Form II, and RI molecules at various stages of synthesis. Where distinction between Form II (16 + 18S in alkali) and RI (>16S in alkali) is necessary, this can be accomplished by longer sedimentation periods. An aliquot of the Hirt supernatant fractions from indicated experiments was sedimented through 5–20% alkaline sucrose in 0.7 M NaCl, 0.3 M NaOH and 0.01 M EDTA. Sedimentation was at 22° for 80–100 min at 40,000 rpm in an SW 50.1 rotor or for 140 min at 39,000 rpm in an SW 41 rotor, unless otherwise noted. Trichloroacetic acid-insoluble radioactivity in gradient fractions was collected on Whatman GF/C filters for counting in 8 ml of Yorktown TT21 scintillant.

Alternatively, after completion of labeling and drug treatment, cells were treated with 0.1% trypsin in SSC, washed once with SSC, and a portion of the cells (not more than 3×10^5) layered onto an equal volume of 2% SDS in SSC on top of a neutral 15–30% sucrose gradient, prepared as described by Yu et al. (22). Cellular DNA was collected on a 60% sucrose cushion and viral DNA resolved in the gradient. These neutral sucrose gradients were centrifuged in polyallomer tubes at 20° for 15 hr at 22,000 rpm in an SW 27 rotor. Fractions were collected from just above the cushion using a peristaltic pump, and acid-insoluble radioactivity determined. The cells remaining after sampling for sedimentation were pelleted at 500 *g* for 4 min, and a Hirt supernatant fraction prepared.

Analysis of cellular DNA. SV40 infected BSC-1 cells containing [^{14}C]thymidine cellular DNA were treated with trypsin after drug treatment and washed in 0.15 M NaCl. An aliquot of the cell suspension was layered onto 5–20% alkaline sucrose gradients prepared and fractionated as described previously (1).

RESULTS

Inhibition of SV40 DNA synthesis by camptothecin and neocarzinostatin. The experiments in Fig. 1 demonstrate that both camptothecin and neocarzinostatin are potent inhibitors of SV40 DNA synthesis. In this experiment, infected cells received labeled thymidine after a 20 min incubation with either drug. Incubation with label was followed by chase with unlabeled thymidine in the presence of drug. Both camptothecin and neocarzinostatin cause 50% inhibition of Form I SV40 DNA synthesis at about 0.1 μM of drug. It should also be noted from Fig. 1 that the DNA made in the presence of low concentrations of either drug sediments as covalently closed circular Form I. Broken Form II DNA does not occur in drug-treated cells to a significantly greater extent that in non-drug-treated cells. Also, the inset to Fig. 1A demonstrates that under our conditions, uninfected cells do not incorporate significant quantities of thymidine into acid-insoluble material in the Hirt supernatant.

Breakage of cellular DNA by camptothecin. If cells containing [^{14}C]thymidine labeled cellular DNA are infected with SV40, and then treated with 0.1 mM camptothecin or 10 μg/ml of neocarzinostatin, one can observe (Fig. 2) that a substantial fraction of the cellular DNA sediments as a broad peak of 40–60S. In contrast, cellular DNA not treated with drugs sediments to the pellet. Therefore, infection of this cell line with SV40 does not alter the sensitivity of cellular DNA to breakage by these drugs. This effect has been extensively studied in uninfected cell cultures (1, 5–8).

The relation of SV40 DNA synthesis inhibition to strand breakage. If strand scission of template DNA is directly related to DNA synthesis inhibition, then parental SV40 DNA molecules might experience

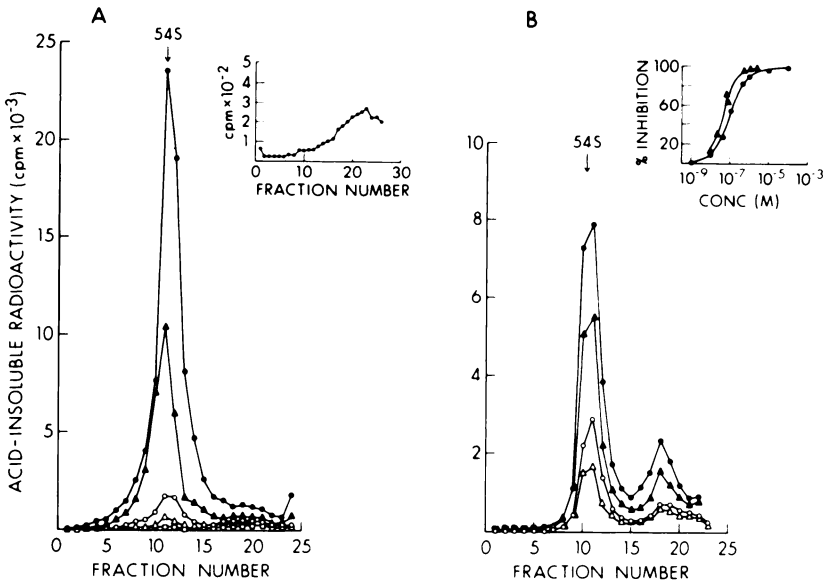

FIG. 1. *Inhibition of SV40 DNA synthesis by camptothecin and neocarzinostatin.*

A. Effect of camptothecin. Replicate cultures of BSC-1 cells were infected as described in METHODS. At 36 h p.i., camptothecin was added to a final concentration of (μM): 0 (●), 0.1 (▲), 1 (○), 10 (△), and 100 (□). After 20 min, [^{3}H]thymidine (6.7 Ci/mmole) was added to a final concentration of 20 μCi/ml and the cells incubated at 37° for 3 h, at which time labeled medium was removed. Cells were incubated for 1 h in complete medium which contained 0.2 mM unlabeled thymidine plus drug as appropriate. Hirt extracts were prepared, and an aliquot sedimented through alkaline sucrose. The *inset* shows an uninfected culture which was treated analogously in the absence of drug. B. Effect of neocarzinostatin. Procedure as in A, except that neocarzinostatin was used at the following final concentrations (μg/ml): 0 (●), 0.5 (▲), 1 (○), and 5 (△). The *inset* is a comparison of the potency of camptothecin and neocarzinostatin in inhibition of Form I SV40 DNA synthesis. Camptothecin (●); neocarzinostatin (▲).

breakage in association with inhibition of DNA synthesis by these drugs. The experiments illustrated in Figs. 3 and 4 address this question. In this experiment, SV40 infected cells were labeled with [^{14}C]thymidine in the absence of drug from 36–48 hr after infection. After removal and chase of this label from cellular pools, viral DNA was pulse-labeled with [^{3}H]thymidine in the presence of drug and then chased with unlabeled precursor. Figure 3 reveals that when the viral DNA from whole cells is examined under neutral conditions, both camptothecin (0.1 mM) and neocarzinostatin (10 μg/ml) efficiently inhibit viral DNA synthesis, whereas uniformly prelabeled viral DNA molecules are not broken. Identical results were obtained with 100 μg/ml neocarzinostatin. In contrast, bleomycin causes extensive breakage of SV40 DNA as virtually all of the viral DNA sediments as Form II SV40 DNA. Bleomycin is known to produce breakage of vaccinia viral DNA in infected HeLa cells (23). Figure 4 examined the viral DNA in Hirt supernatant fractions, prepared from the cultures of the experiment of Fig. 3, on alkaline sucrose gradients. Parental viral DNA molecules sediment as Form I SV40, and neither neocarzinostatin nor camptothecin induced single strand breaks or alkali-labile bonds in the great majority of prelabeled DNA molecules, although the drugs cause efficient inhibition of SV40 DNA synthesis. As expected, the broken viral DNA from bleomycin-treated cultures sediments as Form II SV40 DNA under these conditions. The results in Figs. 3 and 4 may be taken as evidence that inhibition of SV40 DNA synthesis by camptothecin and neocarzinostatin is not obligatorily coupled to breakage of most SV40 DNA molecules in infected cells.

In experiments such as those illustrated

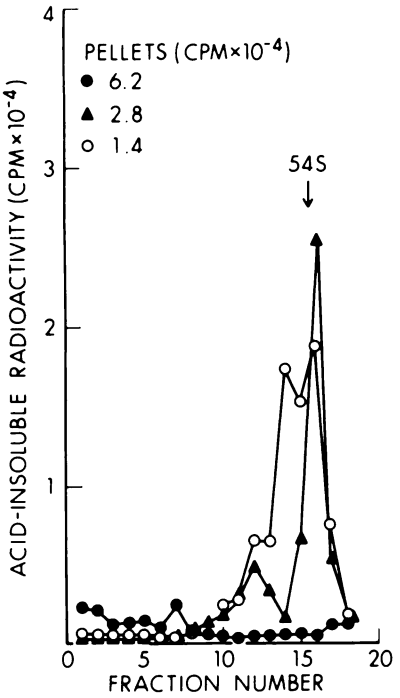

FIG. 2. *Effect of camptothecin and neocarzinostatin on cellular DNA from SV40-infected cells.*

BSC-1 cells were plated in medium which contained 2 μCi/ml [^{14}C]thymidine. After growth for 24 h, labeled medium was removed and complete medium containing unlabeled thymidine (0.2 mM) was added for 4 h followed by incubation in complete medium without added thymidine for 12 h. At that time, replicate cultures were infected with SV40 as described in METHODS. At 36 h p.i., 0.1 mM camptothecin (▲), 10 μg/ml neocarzinostatin (○), or no drug (●) was added for 45 min, after which the cells were treated with trypsin and washed in the presence of drug as appropriate. The resuspended cells (9×10^5) were layered on alkaline sucrose gradients for whole cells as described in METHODS. The radioactivity present in the pellet fraction of this sedimentation technique is indicated.

in Figs. 3 and 4 in which 0.5–10 mM camptothecin were employed, some (up to 40%) conversion of prelabeled Form I to Form II was observed. However, the concentration of camptothecin required for this effect clearly differed from that associated with the onset of DNA synthesis inhibition. It should be noted that the experiments of Figs. 3 and 4 were conducted with drugs present in the trypsin and SSC used to disperse and wash cell cultures. This precaution was taken because the breakage of cellular DNA by camptothecin is rapidly reversible on removal of drug from treated cells (1).

Effect of camptothecin and neocarzinostatin on newly made SV40 DNA. A possible objection to the interpretation given in the experiments of Figs. 3 and 4 is that the prelabel could not be used to indicate those molecules involved in DNA synthesis at the time of drug addition. To examine this question in greater detail, experiments were conducted in which drug and high specific activity label were added simultaneously. At various times after this treatment, cultures were lysed and a Hirt supernatant fraction prepared. Sedimentation on alkaline gradients separated the newly made Form I SV40 DNA from Form II SV40 DNA (18 + 16S) and RI molecules (<16S). Figure 5A demonstrates that in non-drug-treated cultures, after brief periods of labeling, most radioactive precursor is found in the RI and Form II fraction; with increasing time, label enters Form I SV40 DNA while label in the RI and Form II pools remains relatively constant. This pattern of labeling, including the lag in appearance of Form I, has been described by others (24).

In contrast, it will be observed from Fig. 5B that 50 μM camptothecin causes a cessation of viral DNA synthesis. The onset of this effect occurs between 15 and 30 min after addition of the drug, and in this particular experiment is apparent after 30 min. It can be seen that the Form I SV40 DNA, which is labeled up to the time of onset of DNA synthesis-inhibition, is apparently stable: no decrement is observed in Form I between 30 and 60 min after addition of drug. Similarly, label that migrates with Form II and RI also undergoes no change during this period. This type of behavior could be consistent with either an efficient inhibition of strand elongation alone, or an inhibition of elongation and initiation after the initial lag period. Replicative intermediates do not proceed into Form I after the onset of DNA synthesis-inhibition, nor does RI continue to accumulate. Alternatively, a limited fraction of newly made Form I could undergo strand scission in association with the onset of DNA synthesis inhibition;

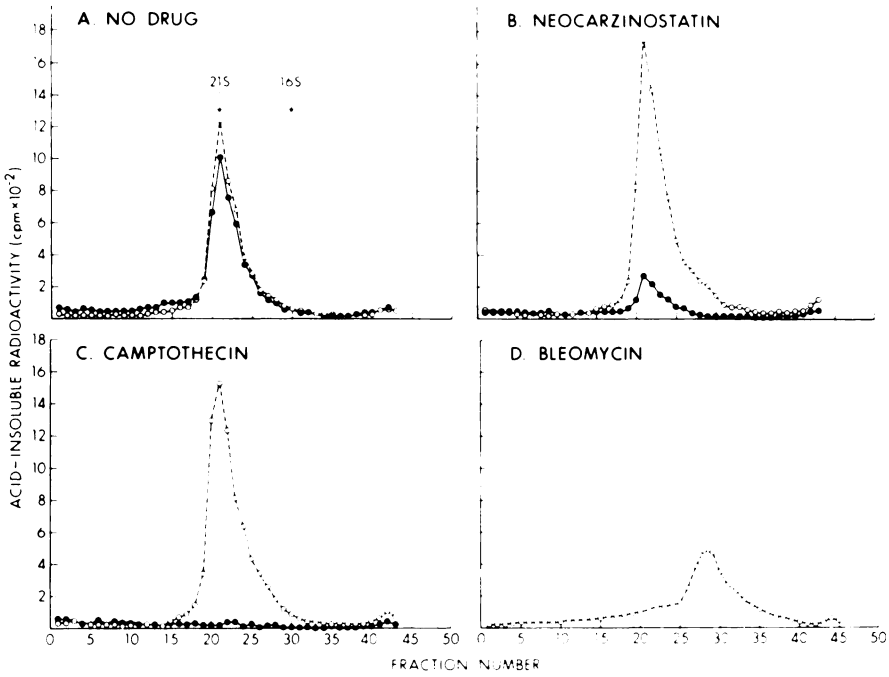

FIG. 3. *Effect of camptothecin and neocarzinostatin on the synthesis of SV40 DNA and on pre-labeled SV40 DNA.*

Replicate cultures of BSC-1 cells were plated and infected with SV40 as described in METHODS. At 36 h p.i., cells received complete medium containing 5 μCi/ml of [^{14}C]thymidine for 12 hr. They were then incubated with 50 μM thymidine for 1 hr and in complete medium for 2 hr. At 51 hr p.i., cells received no drug (A); 10 μg/ml neocarzinostatin (B); 0.1 mM camptothecin (C), or 500 μg/ml bleomycin (D) for 15 min. 20 μCi/ml of [^{3}H]thymidine was added to cultures A, B, and C and incubation continued for 45 min. The medium was removed and cultures A, B, C, and D received complete medium plus drug as appropriate containing 0.2 mM of unlabeled thymidine for 20 min. All cultures were then washed with SSC, trypsinized, and resuspended with SSC in the presence of drug as appropriate. An aliquot of cells from each culture was layered on neutral sucrose gradients as described in METHODS. [^{14}C]-prelabeled DNA (--O--); [^{3}H]pulse-labeled (——●——). Cultures which received bleomycin contained only [^{14}C]-prelabeled DNA.

these molecules would sediment in the same region of the sucrose gradients (18–16S). This point will be discussed below.

The behavior of neocarzinostatin in this type of experiment (Fig. 5C) differs from that of camptothecin. The onset of DNA synthesis inhibition by neocarzinostatin occurs between 10 and 20 min after addition of drug. As with camptothecin, newly made Form I is stable and does not decrease in amount after complete inhibition of DNA synthesis. In contrast to camptothecin, molecules in the RI and Form II pool decrease after the onset of DNA synthesis inhibition by neocarzinostatin, and Form I increases by approximately the same amount. This type of behavior is consistent with a primary action of neocarzinostatin to inhibit initiation of new rounds of SV40 DNA synthesis, with little or no effect on the elongation, termination, and segregation of SV40 DNA molecules in the RI pool.

SV40 DNA made in the presence of camptothecin. The experiments of Fig. 5 suggest that Form II SV40 DNA derived from newly made SV40 DNA or unfinished RI molecules may accumulate just as the inhibition of SV40 DNA synthesis by camptothecin starts. This is in contrast to the lack of effect of the drug on the great majority of intracellular SV40 DNA molecules described in Fig. 3. The size of the SV40 DNA labeled as DNA synthesis inhibition

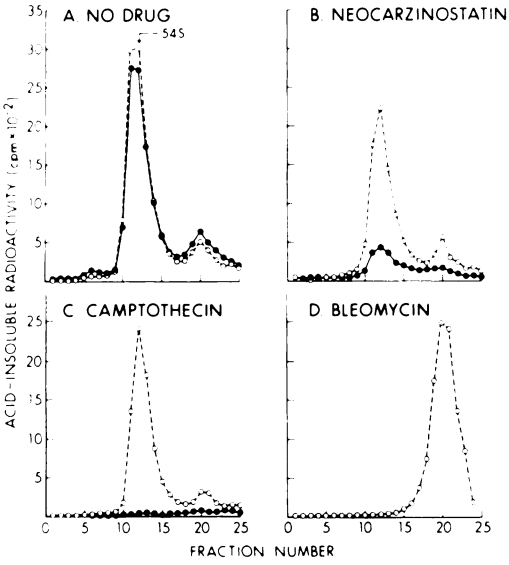

FIG. 4. *Analysis of Hirt extracts from the experiment of Fig. 3.*

The cells remaining from the cultures of Fig. 3 after sampling for neutral non-DNA-denaturing gradients were extracted as described in METHODS to give a Hirt supernatant fraction. An aliquot of this solution from each culture was analyzed by sedimentation in alkaline sucrose. No drug (A); 10 μg/ml neocarzinostatin (B); 0.1 mM camptothecin (C); and 500 μg/ml bleomycin (D-prelabeled DNA only), [^{14}C]-prelabeled DNA (--O--); [^{3}H]-pulse labeled DNA (—●—).

by camptothecin takes place is examined in Fig. 6, and is compared with DNA labeled in the presence of hydroxyurea, a known inhibitor of elongation of nascent SV40 DNA strands (17). Alkaline sucrose sedimentation was conducted to examine DNA of less than 20S in size; Form I SV40 DNA is not retained on these gradients. The experiments of Fig. 6 demonstrate that the slowly sedimenting DNA which accumulates under these labeling conditions in camptothecin-treated cultures sediments at 18–16S: shorter pieces of DNA do not occur to a greater extent than in non-drug-treated cultures. We conclude from the experiment illustrated in Fig. 6 that camptothecin does not prevent formation of full length (16S) SV40 DNA strands. This is in marked contrast to cultures treated with hydroxyurea in which most of the newly made DNA sediments at about 4–6S.

At the concentration of drug used in Fig. 6, camptothecin causes a significant fraction of newly made SV40 DNA to sediment as a Form II-like species. This action occurs

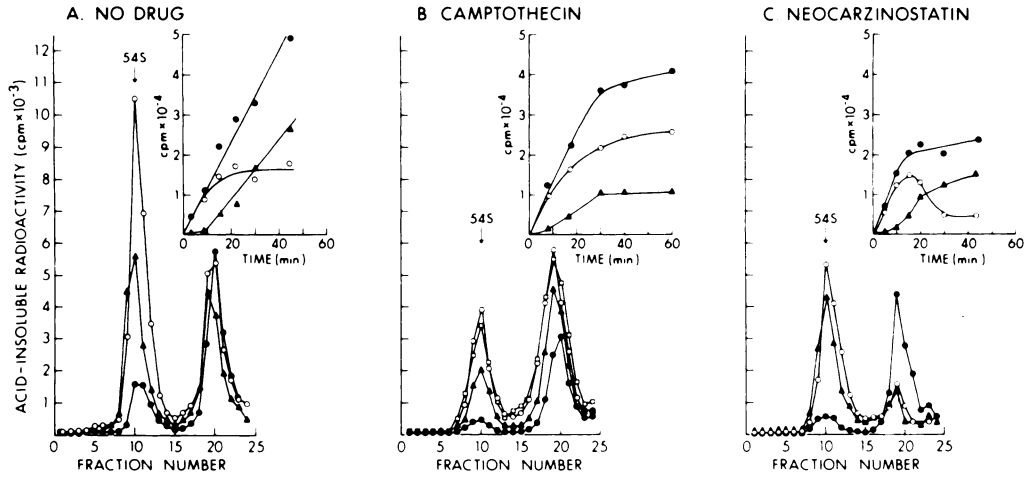

FIG. 5. *Kinetics of SV40 DNA synthesis inhibition by camptothecin and neocarzinostatin.*

Replicate cultures of BSC-1 cells were plated and infected with SV40 as described in METHODS. At 36 hr p.i., cells received 100 μCi/ml of [^{3}H]thymidine (40–70 Ci/mmole) plus drugs as indicated. At various times after addition, Hirt extracts of cultures were prepared and analyzed by alkaline sucrose density gradient centrifugation. A, no drug: 15 (●); 30 (▲); 45 (○) min; B, 50 μM camptothecin: 8 (●); 18 (▲); 30 (○); 40 (□) min; C, 100 μg/ml neocarzinostatin: 10 (●); 30 (▲); 45 (○) min. The *inset* in each panel plots the distribution of label in Form I (▲) and Form II plus RI (○), and also the total radioactivity (●) present on each gradient.

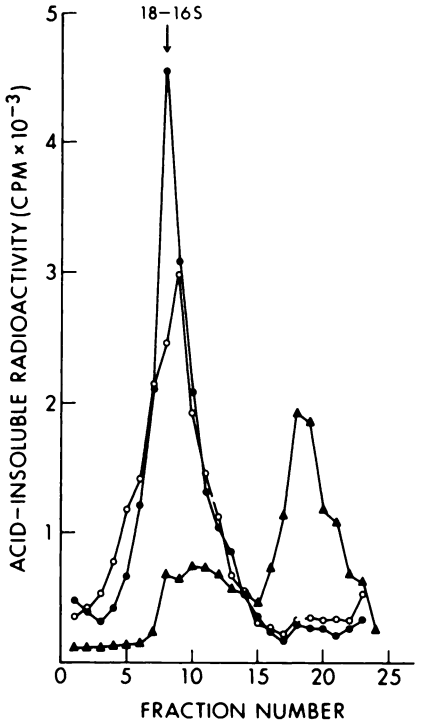

FIG. 6. *Slowly sedimenting DNA from camptothecin-treated cells.*

Replicate cultures of BSC-1 cells were plated and infected with SV40 as described in METHODS. At 36 hr p.i., cells received 100 μCi/ml of [^{3}H]thymidine (40–70 Ci/mmole) in the presence of no drug (●), 0.1 mM camptothecin (○), or 10 mM hydroxyurea (▲). After 45 min, Hirt extracts were prepared and an aliquot from each culture sedimented in a 10–30% alkaline sucrose gradient for 16.5 hr at 39,500 rpm in an SW 41 rotor at 22°. The position of 16–18S Form II SV40 DNA is indicated. Under these conditions, Form I SV40 DNA sediments to the bottom of the gradient.

in association with the onset of inhibition of SV40 DNA synthesis. In experiments not shown here, a maximum of about 50–60% of newly made SV40 DNA occurs as this Form II-like species in cultures treated with 10 and 100 μM camptothecin. At 1 μM camptothecin, 30–35% of the SV40 DNA accumulates in this form despite substantial (>50%) inhibition of SV40 DNA synthesis.

The experiments of Fig. 5 suggest that a substantial fraction of label added in the presence of drug may be found in Form I even as DNA synthesis inhibition by camptothecin or neocarzinostatin is beginning to occur. It becomes of interest to inquire whether Form I made in the presence of drugs has the same degree of supercoiling as Form I DNA labeled in the absence of drug. Form I SV40 DNA labeled in the presence of cycloheximide is known to possess an altered degree of supercoiling when examined by dye-buoyant density gradient centrifugation (25). Experiments to examine this point reveal that Form I DNA labeled after a 20 min pulse in the presence of camptothecin or neocarzinostatin (and chased for 1 hr in the presence of drug) does not display a banding pattern in ethidium bromide-CsCl that is appreciably different from that of [^{14}C]DNA labeled in the absence of drug (data not shown). It therefore appears that camptothecin and neocarzinostatin do not alter the degree of supercoiling of newly made SV40 DNA.

The fate of DNA labeled in the presence of camptothecin is examined after removal of the drug (Fig. 7). In nontreated cultures there is efficient chase of label from the RI and Form II fraction to Form I SV40 DNA, reflecting continued DNA synthesis in these cultures. In contrast, the small amount of DNA that is labeled in the presence of 0.1 mM camptothecin is unchanged during the 2 hr of drug-free incubation employed in this experiment.

DISCUSSION

The experiments in this paper demonstrate that both camptothecin and neocarzinostatin are effective inhibitors of SV40 DNA synthesis. Substantial inhibition (greater than 50%) occurs at a concentration between 0.1 and 1 μM of either drug. Neither camptothecin nor neocarzinostatin cause the breakage of uniformly prelabeled SV40 DNA molecules in infected cells at concentrations that inhibit viral DNA synthesis by greater than 90% (Figs. 3 and 4). When SV40 DNA molecules involved in DNA synthesis at the time of drug addition are examined, camptothecin and neocarzinostatin elicit different responses.

Labeled thymidine present in RI molecules shortly after addition of neocarzinostatin is subsequently found in Form I SV40 DNA. One may estimate from the data of Fig. 5C that no more than 20–25% of the SV40 DNA molecules labeled at the onset

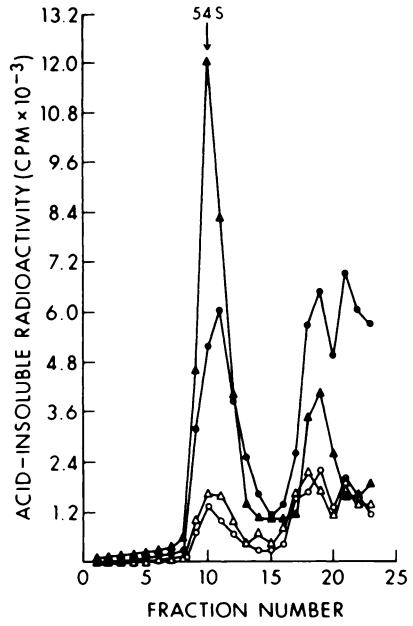

FIG. 7. *Fate of SV40 DNA made in the presence of camptothecin.*

At 36 hr p.i., two cultures received 100 μCi/ml of [^{3}H]thymidine (40 Ci/mmole) for 45 min in the absence of drug (controls), and two cultures received the same amount of label plus 0.1 mM camptothecin. Hirt extracts were prepared from one of the control cultures (●) and one of the drug-treated cultures (○) after removal of medium containing label. To the second control culture was added 0.2 mM thymidine for 135 min and a Hirt extract was prepared (▲). To the second drug-treated culture was added 0.2 mM thymidine and 0.1 mM camptothecin for 15 min after removal of the label. This culture was washed and incubated in medium with 0.2 mM thymidine in the absence of camptothecin for 2 hr and a Hirt extract was prepared (△). Aliquots of the Hirt supernatants were sedimented in alkaline sucrose gradients as described in METHODS.

of inhibition by neocarzinostatin sediments like Form II SV40 DNA. This level does not differ from that which can be obtained from untreated cultures chased with unlabeled precursor and then extracted (for example, in Fig. 1B), but greatly differs from prelabeled SV40 DNA isolated from cultures treated with bleomycin (Figs. 3D and 4D). Therefore, neocarzinostatin does not inhibit elongation of nascent SV40 DNA in RI molecules or their conversion to mature Form I DNA with the correct degree of supercoiling in association with inhibition of SV40 DNA synthesis. Rather, it would appear from Fig. 5C that neocarzinostatin inhibits the initiation of new rounds of SV40 DNA synthesis in infected cells. This result occurs without breakage of prelabeled SV40 DNA, although it is conceivable that a small fraction of SV40 DNA, not detected in these experiments, could be broken in the establishment of DNA-synthesis inhibition. A similar experimental design and rationale was employed by Manteuil and Girard (26) in experiments which suggested that chloroquine and Miracil D also act to inhibit the initiation of SV40 DNA synthesis, while agents including 5-fluorodeoxyuridine, cytosine arabinoside, and hydroxyurea act principally to inhibit elongation of nascent SV40 DNA strands. Yu *et al.* (22) have also used this type of experiment to demonstrate that cycloheximide causes inhibition of initiation of polyoma DNA synthesis.

Beerman and Goldberg (5) have studied the effects of neocarzinostatin on HeLa DNA and HeLa DNA synthesis. They find that the onset of DNA synthesis inhibition is preceded by the degradation of HeLa DNA, and they conclude that strand scission of cellular DNA may be an important aspect of the mechanism of action of neocarzinostatin. The results of our experiments suggest that inhibition of SV40 DNA synthesis by neocarzinostatin occurs with little discernible effect of the drug on the great majority of viral DNA molecules present in infected cells. Thus, if template DNA strand scission by neocarzinostatin is important for establishment of SV40 DNA synthesis inhibition, it must involve only a small fraction of potential templates present.

The interpretation of experiments with camptothecin is much less straightforward than that of studies with neocarzinostatin. The data in Figs. 3 and 4 indicate that uniformly prelabeled SV40 DNA is not appreciably affected by treatment with up to 0.1 mM camptothecin. However, the data from Fig. 5 and other experiments indicate that at 0.01 and 0.1 mM of drug, but not at 1 μM up to 50% of newly made SV40 DNA occurs as a Form II-like species. The Form I SV40 DNA that accumulates after the

onset of DNA synthesis inhibition is stable for up to 2 hr of continued incubation with the drug, as a time-dependent conversion of Form I to Form II SV40 DNA is not apparent in these experiments. When the slowly sedimenting DNA is examined in detail (Fig. 6), it is clear that it consists of largely 18–16S DNA, which would be expected if Form II SV40 DNA were produced. Significantly, short RI molecules of the type observed in cultures treated with hydroxyurea do not accumulate in camptothecin-treated cultures.

It is not posssible on the basis of these experiments to define the point at which SV40 DNA synthesis is inhibited by camptothecin. Although the breakage of prelabeled SV40 DNA may be clearly separated from SV40 DNA synthesis inhibition by camptothecin, this is not true for a fraction of newly made SV40 DNA labeled at relatively high (0.01–0.1 mM) concentrations of camptothecin. Rubinstein and Rein (27) have also observed the appearance of a Form II-like SV40 DNA species on treatment of SV40-infected cells with camptothecin, although the relationship of this species to prelabeled DNA breakage and the degree of DNA synthesis inhibition was not explored. Whether this Form II-like DNA derives from a pool of Form I molecules that is transiently susceptible to the DNA breaking action induced by the drug shortly after their synthesis, or whether this species represents a synthesis intermediate that has not been adequately characterized, is not revealed by the present experiments. At low concentrations of camptothecin (1 μM), the labeling of this Form II-like species is greatly diminished even though greater than 50% inhibition of DNA synthesis has occurred. Therefore, inhibition of SV40 DNA synthesis and limited breakage of SV40 DNA induced by camptothecin may be coupled; if this is true, only a small and perhaps specific fraction of available SV40 DNA molecules must be involved in this process. It is possible that these molecules may not be revealed to advantage by the pulse-chase techniques employed in these experiments.

An alternative point of view is that camptothecin and neocarzinostatin may interact with specific components of the SV40 DNA replicative apparatus, and thus what little breakage of SV40 DNA that may occur need not be specifically related to the onset of DNA synthesis inhibition. Nonetheless, it is of importance to point out that during these experiments, camptothecin and neocarzinostatin caused substantial damage to cellular DNA (Fig. 2; Ref. 1, 5). Thus inhibition of viral DNA synthesis by both of these drugs may be one expression of their demonstrated capacity to break cellular, as opposed to viral DNA. BSC-1 cellular DNA broken by camptothecin sediments at 40–60S. Therefore the average double stranded size of this DNA is between 3.1×10^7 and 1×10^8 daltons (28). If it is assumed that this size range adequately represents the effective target for the action of camptothecin, then prelabeled Form I SV40 DNA (3.3×10^6 daltons) might be expected to undergo little net conversion to Form II. That is, even if it is assumed that all the prelabeled viral DNA molecules present in the experiments of Figs. 3 and 4 were a potential "substrate" for drug-induced breakage, only 3–10% of the molecules would be affected, a level that would not be resolved in these experiments. An analogous argument could be made in the case of neocarzinostatin.

Recent experiments of Povirk (29) examine light-induced damage to cellular DNA labeled in a defined manner with 5-bromodeoxyuridine. There is evidence that inhibition of DNA synthesis after such treatment occurs at the level of replicon initiation, and does not affect replicon elongation. While the relationship of this phenomenon to drug-induced DNA damage and inhibition of DNA synthesis is not yet clear, it does underscore the possibility that even limited damage to viral or cellular DNA could have profound consequences for replication of DNA at a site removed from the DNA damage.

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