Vol. 168, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 16, 1990 Pages 135-140

EXPOSURE TO CAMPTOTHECIN BREAKS LEADING AND LAGGING STRAND SIMIAN VIRUS 40 DNA REPLICATION FORKS

Cha-Gyun Shin and Robert M. Snapka*

Departments of Radiology and Medical Microbiology & Immunology
The Ohio State University, Columbus, OH 43210

Received March 7, 1990

SUMMARY: To better understand aberrant simian virus 40 DNA replication intermediates produced by exposure of infected cells to the anticancer drug camptothecin, we compared them to forms produced by S1 nuclease digestion of normal viral replication intermediates. All of the major forms were identical in both cases. Thus the aberrant viral replicating forms in camptothecin-treated cells result from DNA strand breaks at replication forks. Linear simian virus 40 forms which are produced by camptothecin exposure during viral replication were identified as detached DNA replication bubbles. This indicates that double strand DNA breaks caused by camptothecin-topoisomerase I complexes occur at both leading and lagging strand replication forks in vivo. © 1990 Academic Press, Inc.

Camptothecin is the prototype of a new family of drugs which have shown promise for treatment of human colon cancer (1). As a specific topoisomerase I poison, camptothecin traps eukaryotic topoisomerase I in an intermediate step of the DNA breakage-reunion cycle in which the enzyme is covalently attached to the DNA at the site of a DNA strand break (2-4). These covalent linkages of the enzyme to DNA are reversed when the drug is removed, so it is not clear why they should be cytotoxic. The fact that camptothecin is most cytotoxic in the S phase of the cell cycle (5,6), suggests that some interaction with the DNA replication machinery is critical to the mechanism of cytotoxicity. We have approached this problem by studying camptothecin effects on SV40 DNA replication (7,8). The SV40 "minichromosome" is often considered a model for the eukaryotic chromosome because of its extensive use of host cell DNA replication and packaging machinery (9). Both normal and abnormal viral DNA replication intermediates can be separated and identified by high resolution gel analysis (7,10). The reproducibility and logic of this system has contributed to an improved understanding of topoisomerase function in DNA replication and has led to insights into the mechanisms of anticancer topoisomerase inhibitors (7,8,11,12).

Type II topoisomerase inhibitors only interfere with the latest stages of SV40 DNA replication: completion of the late Cairns structure (the last 200 base pairs replicated) and separation of newly replicated daughter chromosomes (7,8,11). This is consistent with evidence that topoisomerase II is required only for separation of cellular chromosomes

* To whom correspondence should be addressed at Department of Radiology, Division of Radiobiology, 103 Wiseman Hall, 400 West 12th Avenue, Columbus, OH 43210.

ABBREVIATIONS: SV40, simian virus 40; $ZnSO_4$, zinc sulfate.

0006-291X/90 $1.50
Copyright © 1990 by Academic Press, Inc.
All rights of reproduction in any form reserved.

during eukaryotic DNA replication (13-15). In contrast, the type I topoisomerase poison camptothecin causes breakage of DNA replication forks at all stages of SV40 DNA replication (8,12). The broken DNA replication fork may be the lesion responsible for the S phase cytotoxicity and anticancer activity of this drug.

Although two of the aberrant SV40 DNA replication intermediates produced by camptothecin treatment have been previously identified as being due to DNA fork breakage, it has not been clear if the breakage occurs at leading or lagging strand forks exclusively or if it occurs at both. In addition, the origin of linear SV40 forms has not been completely understood. In this study, the camptothecin-induced linear forms were identified as detached replication bubbles by comparison with the forms produced from normal SV40 replication intermediates during S1 nuclease digestion. From this we conclude that camptothecin-trapped topoisomerase I-DNA complexes break replication forks efficiently on both the leading and lagging strand sides.

MATERIALS AND METHODS

Cells and Virus. CV1 cells were grown and infected with plaque-purified SV40 strain 777 as described (8).

Labeling and Extraction of DNA. At 36 hr post-infection, old media was removed and serum-free medium containing [^{3}H]dThd (250 μCi/ml) was added. Hirt extraction (16) was carried out after 30 minutes of labeling. The Hirt supernatant was digested with proteinase K (1 mg/ml) at 37°C overnight). DNA was extracted with chloroform-isopropanol (24:1), then ethanol precipitated before being taken up in electrophoresis loading buffer.

Drug Treatment. Camptothecin (NSC 94600) was obtained from the National Cancer Institute, Division of Cancer Treatment, Natural Products Branch. The drug was added to labeling media 15 minutes after the start of label.

Electrophoresis. The high resolution one and two dimensional electrophoretic analysis of SV40 replication intermediates was done by the method of Sundin and Varshavsky (10).

S1 Nuclease Digestion. S1 nuclease was purchased from Sigma. Digestion of purified SV40 DNA (10 μg/ml) was carried out at 37 °C in 50 mM sodium acetate, pH 4.5, 10 mM $ZnSO_4$ and 200 mM NaCl. Reactions were stopped after 15 seconds by addition of 0.5 M NaEDTA to a final concentration of 2.0 mM.

RESULTS AND DISCUSSION

When pulse-labeled SV40 DNA replication intermediates were treated with S1 nuclease to digest single strand gaps at replication forks, distinct altered replication forms were produced (fig. 1). The distribution of altered forms changed continuously with the extent of nuclease digestion. We noted that the patterns of altered forms in the samples treated with 3-5 units/ml of S1 nuclease resembled those seen in camptothecin-treated cells (7,8) and figure 1, lane C. Normal SV40 DNA replication intermediates are seen in the far left lane in figure 1, (0.0 units of S1 nuclease). Replication structures (known as Cairns

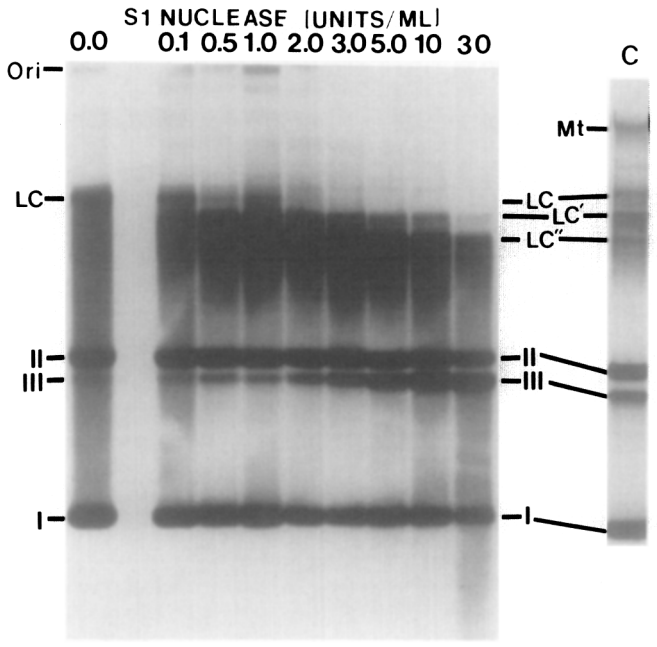

Figure 1. One dimensional gel analysis of S1 nuclease digested SV40 DNA replication intermediates. Ori; origin of electrophoresis, LC; late Cairns structure, II; form II (relaxed circular) full SV40 genomes, III; form III (full length linear) SV40 genomes, I; form I (superhelical) SV40 genomes, LC'; family of altered Cairns structures with one broken replication fork (sigma structures), LC''; family of altered Cairns replicating structures with two broken replication forks, Mt; mitochondrial DNA. Unaltered SV40 replication intermediates were not treated with S1 nuclease (0.0 units/ml). Lane C; pulse-labeled SV40 DNA replication intermediates extracted from camptothecin-treated cells. Other samples were treated with the concentrations shown above each lane.

structures or theta structures), appear as a continuous smear between the form I band and the late Cairns structure in this undigested control. The late Cairns structure is a theta form with about 200 base pairs unreplicated. On one-dimensional gels of pulse-labeled SV40 DNA replication intermediates the late Cairns appears as a dark band due to a pause at this point in replication (17,18). As the extent of nuclease digestion was increased, the smear of intermediate Cairns structures was replaced by two shorter smears, LC' and LC'', . These short smears have been previously identified in camptothecin-treated infected cells as SV40 replication intermediates with one and two broken replication forks respectively (8). Further digestion weakened the LC' smear while intensifying the LC'' smear and the form III band. At the highest levels of digestion, a new smear could be seen to extend from the form III band beyond the form I band (fig.1 10 and 30 units of S1).

To confirm these observations and to investigate the structure of the altered forms, second dimension alkaline gels were run after first dimension neutral gel electrophoresis (fig. 2). In the untreated control (fig. 2, A), the form III band from the first dimension gel gives rise to a single spot which marks the level of full length linear single strand SV40 DNA in the alkaline second dimension. Form II gives rise to two spots, one a single strand linear and one a single strand circle. Form I (superhelical) SV40 DNA migrates as a single spot since the labeled strand is still topologically linked to the parental strand. An arc of

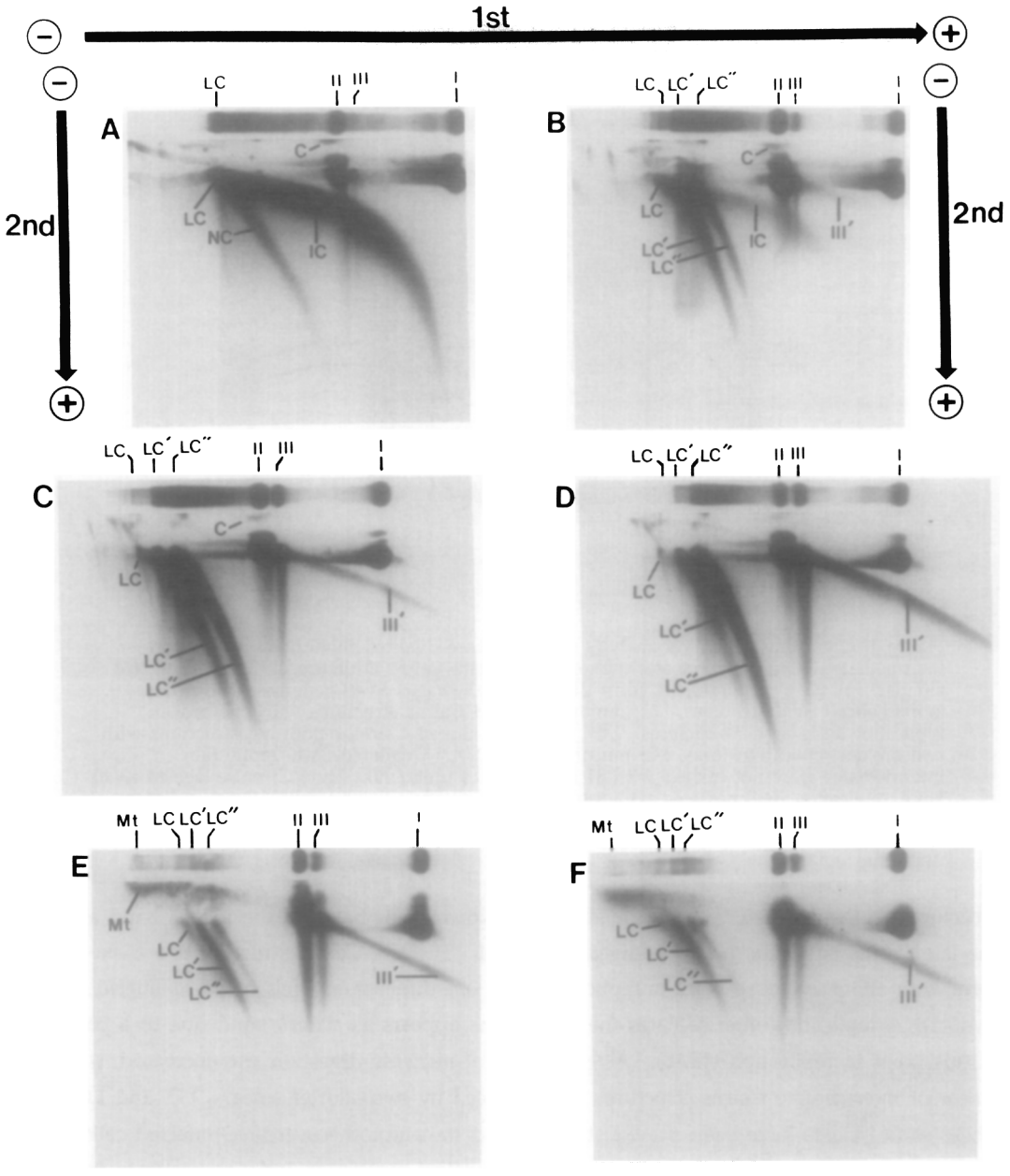

Figure 2. Two dimensional gel analysis of S1 digested SV40 replication intermediates. First dimension (neutral) gel electrophoresis is shown from left to right, and the separation in the alkaline second dimension is shown from top to bottom for each sample. IC; intermediate Cairns replication intermediates, NC; nicked Cairns replication intermediates, III' arc of detached replication bubbles ending at the position of full length linear (form III) genomes. All other intermediates same as in fig. 1.
A; Normal pulse-labeled SV40 DNA replication intermediates, B; same as A but treated with 0.5 units of S1 nuclease, C; same but treated with 3 units of S1 nuclease, D; same as A but treated with 10 units of S1 nuclease, E and F; SV40 DNA replication intermediates from cells treated with 10 μM camptothecin (duplicate experiments).

density which is asymptotic to form I and extends up to the level of full length linear at the late Cairns structure is composed of nascent strands from early and intermediate Cairns structures. Replication intermediates in which the unreplicated region is relaxed due to a

single strand nick give rise to a separate arc of nicked Cairns structures. Families of replication intermediates and structures derived from replication intermediates typically appear as arcs in two dimensional neutral-alkaline gel electrophoretic patterns (7,8,18).

The arc of intermediate Cairns structures was diminished by light S1 nuclease digestion as LC' and LC'' arcs appeared (fig. 2, B). A faint arc (III') extending from form III could also be seen at this stage. More extensive digestion lead to intensification of the LC'' and III' arcs and disappearance of normal intermediate Cairns structures (fig. 2, C). Finally the LC' arc weakened as the form III band and its associated arc (III') intensified. The structures giving rise to the III' arc contain nascent DNA strands (since they are labeled) and are derived from normal replication intermediates by digestion of the single strand regions at replication forks. One family of structures expected to be produced by such treatment would include detached replication bubbles. Detachment of replication bubbles from normal Cairns replication structures would give a family of labeled linear DNAs varying continuously in length from a few nucleotides up to full length SV40 genomes. Such a family of linear DNAs would be expected to give an arc identical to the III' arc. The late Cairns structure itself would yield a detached replication bubble 200 base pairs less than full length. This is only about 4 % shorter than full length and would not be resolved from full length linear SV40. Since the late Cairns structure appears as a distinct band in pulse-labeled normal SV40 replication intermediates, it will appear as a band in the family of detached replication bubbles and will not be resolved from form III. From these considerations, we conclude that detached replication bubbles give rise to the III' arc and the intensification of the form III band during S1 digestion of normal viral replication intermediates.

Camptothecin stabilizes a reaction intermediate in which type I topoisomerase is covalently attached to DNA at the site of a DNA strand break (2,4). Exposure of replicating SV40 chromosomes to camptothecin breaks replication forks and produces forms identical to those produced by S1 digestion of normal viral replication intermediates (figure 2, E), including the intensified form III band and the III' arc. These aberrant forms are not produced from completely replicated viral genomes by in vivo camptothecin exposure (Shin, C.-G. and Snapka, R.M. unpublished data). The progressive shift from LC to LC' to LC'' with intensification of form III is seen with both increasing extent of S1 digestion and with increasing time of exposure to camptothecin in vivo (7). This pattern is easily explained as being due to progressive breakage of replication forks either by S1 nuclease or by camptothecin stabilized topoisomerase I-DNA complexes.

It has previously been unclear if the replication fork breaks caused by camptothecin exposure occur exclusively at either the leading or lagging strand side of the replication forks. The identification of the camptothecin-induced linear forms as detached replication bubbles indicates that the breaks occur efficiently on both the leading and lagging stand sides of replication forks since one break of each type is required to detach a replication bubble.

ACKNOWLEDGMENT: This work was supported by Public Health Service Grant # CA 45208 to RMS.

REFERENCES

1. Giovanella, B.C., Stehlin, J.S., Wall, M.E., Wani, M.C., Nicholas, A.W., Liu, L.F., Silber,R., and Potmesil, M. (1989) Science 246, 1046-1048.
2. Hsiang, Y.-H., Hertzberg, R., Hecht, S., and Liu, L.F. (1985) J. Biol. Chem. 260, 14873-14878.
3. Jaxel, C., Kohn, K.W., and Pommier, Y. (1988) Nucl. Acid Res. 23, 11157-11171.
4. Eng, W.-K., Faucette, L., Johnson, R.K., and Sternglanz, R. (1988) Mol. Pharmacol. 34, 755-760.
5. Covey, J.M., Jaxel, C., Kohn, K.W., and Pommier, Y. (1989) Cancer Res. 49, 5016-5022.
6. Mattern, M.R., Mong, S.M., Bartus, H.F., Mirabelli, C.K., Crooke, S.T., and Johnson, R.K. (1987) Cancer Res. 47, 1793-1798.
7. Snapka, R.M., Powelson, M.A., and Strayer, J.M. (1988) Mol. Cell. Biol. 8, 515-521.
8. Snapka, R.M. (1986) Mol. Cell. Biol. 6, 4221-4227.
9. DePamphilis, M.L., Chalifour, L.E., Charette, M.F., Cusick, M.E., Hay, R.T., Henderson, E.A., Pritchard, C.G., Tack, L.C., Wassarman, P.M., Weaver, D.T., and Wirak, D.O. (1983) Papovavirus chromosomes as a model for mammalian DNA replication (pp.423-447). Alan R. Liss, Inc., New York.
10. Sundin, O., and Varshavsky, A. (1981) Cell 25, 659-669.
11. Richter, A., and Strausfeld, U. (1988) Nucl. Acid Res. 16, 10119-10129.
12. Avemann, K., Knippers, R., Koller, T., and Sogo, J.M. (1988) Mol. Cell. Biol. 8, 3026-3034.
13. DiNardo, S., Voekel, K., and Sternglanz, R. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 2616-2620.
14. Holm, C., Goto, T., Wang, J.C., and Botstein, D. (1985) Cell 41, 553-565.
15. Uemura, T., and Yanagida, M. (1986) EMBO J. 5, 1003-1010.
16. Hirt, B. (1967) J. Mol. Biol. 26, 365-369.
17. Tapper, D.P., and DePamphilis, M.L. (1978) Mol. Biol. 120, 401-422.
18. Sundin, O., and Varshavsky, A. (1980) Cell 21, 103-114.