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Effect of ICRF-193, a Novel DNA Topoisomerase II Inhibitor, on Simian Virus 40 DNA and Chromosome Replication In Vitro

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The effect of ICRF-193, a noncleavable-complex-forming topoisomerase II inhibitor, on simian virus 40 (SV40) DNA and SV40 chromosome replication was examined by using an in vitro replication system composed of HeLa cell extracts and SV40 T antigen. Unlike the topoisomerase inhibitors VP-16 and camptothecin, ICRF-193 had little effect on DNA chain elongation during SV40 DNA replication, but high-molecular-weight DNAs instead of segregated monomer DNAs accumulated as major products. Analysis of the high-molecular-weight DNAs by two-dimensional gel electrophoresis revealed that they consisted of catenated dimers and late Cairns-type DNAs. Incubation of the replicated DNA with topoisomerase II resulted in conversion of the catenated dimers to monomer DNAs. These results indicate that ICRF-193 induces accumulation of catenated dimers and late Cairns-type DNAs by blocking the decatenating and relaxing activities of topoisomerase II in the late stage of SV40 DNA replication. In contrast, DNA replication of SV40 chromosomes was severely blocked by ICRF-193 at the late stage, and no catenated dimers were synthesized. These results are consistent with the finding that topoisomerase II is required for unwinding of the final duplex DNA in the late stage of SV40 chromosome replication in vitro.

Eukaryotic DNA topoisomerases I and II relax both positive and negative supercoils by transiently producing single- and double-stranded DNA breaks, respectively, and only topoisomerase II decatenates intertwined dimer DNA (33). During replication of a circular duplex DNA, the positive supercoils should accumulate ahead of the replication fork, and they must be resolved with topoisomerases for fork movement to progress further (2, 3). Genetic evidence suggest that either topoisomerase I or topoisomerase II is sufficient as a swivelase for relief of the torsional strain generated by DNA replication (1, 17), and topoisomerase II but not topoisomerase I is essential for segregation of replicated daughter molecules in yeast cells (6, 10, 30, 31). In simian virus 40 (SV40) DNA replication in vitro, catenated dimers accumulated when topoisomerase II was depleted from cell extracts (37). They were also synthesized in a replication system containing purified proteins in the absence of topoisomerase II (13). These results suggest that the specific role of topoisomerase II is to separate catenated intertwines formed after the two daughter molecules have been replicated. This mechanism was first suggested by the observation that newly synthesized SV40 DNA accumulated as catenated dimers when SV40-infected CV-1 cells were placed into hypertonic medium (28). However, Weaver et al. (35) reported that in addition to the catenated dimers, late Cairns-type DNAs, which are intermediates of bidirectional DNA replication, accumulated in similar conditions. Although the effect of hypertonic medium seems to be mediated by inhibition of topoisomerase II, this effect has not been proven since the treatment also inhibits initiation of protein synthesis (22).

Topoisomerase II inhibitors have also been shown to induce the accumulation of catenated dimers and late replication intermediates in SV40 DNA replication in vitro (20) and in vivo (21, 24, 25). Snapka et al. (25) reported that topoisomerase II inhibitors had different effects on the decatenation of SV40 DNA. Intercalating-type topoisomerase II inhibitors such as 9-aminoacridine and proflavine were more effective than the nonintercalating agents VP-16 and VM-26. The inhibitors examined so far are all of the cleavable-complex-forming type of topoisomerase II inhibitors. Different from the cleavable-type inhibitors, ICRF-193, which is a derivative of the antitumor agent bis(2,6-dioxopiperazine), was recently found not to stimulate cleavable-complex formation; furthermore, it prevented the DNA strand breaks induced by VP-16 in vitro (29) as well as in vivo (12), suggesting that ICRF-193 interferes with topoisomerase II at some step(s) before the formation of the intermediate cleavable complex in the catalytic cycle. ICRF-193 affected cell cycle progression differently from VP-16, and the biological effects of ICRF-193 were more consistent than those of VP-16 with the phenotype observed in topoisomerase II mutants of yeast cells at a nonpermissive temperature (12, 30, 31).

In this study, we analyzed the effect of ICRF-193 on SV40 DNA and chromosome replication in vitro. The results indicate ICRF-193 blocks decatenation of intertwined daughter dimers and retards unwinding of the terminal region of parental molecules in the late stage of SV40 DNA replication. In contrast, in the chromosome replication system, it completely inhibits DNA synthesis in the late stage of the replication. We also discuss the different effects of two types of topoisomerase II inhibitors on SV40 DNA replication.

MATERIALS AND METHODS

Drugs. ICRF-193 was provided by Zenyaku Kogyo Co. Ltd., Tokyo, Japan. VP-16 and camptothecin were provided by Bristol-Meyers Co., Wallingford, Conn., and Yakult

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Honsha Co., Ltd., Tokyo, Japan, respectively. These drugs were dissolved with dimethyl sulfoxide.

Preparation of DNA and enzymes. Plasmid DNA containing the SV40 DNA replication origin (pSV01ΔEP; 2.79 kbp), plasmid DNA without the origin (pBR322ΔEP; 2.48 kbp), and cytosolic extracts of HeLa cells were prepared by a published method (36). SV40 chromosomes were prepared from SV40-infected CV-1 cells (26). Naked SV40 DNA was purchased from Sigma Chemical Co., and form I SV40 DNA was purified by agarose gel electrophoresis (23). SV40 T antigen was purified from *Spodoptera frugiperda* insect cells infected with recombinant baculovirus as reported elsewhere (15). Topoisomerase II was purified from HeLa cells according to the procedure of Miller et al. (19) through step 3 and had a specific activity of 1.2×10^5 U/mg of protein. A knotted P4 phage DNA was kindly provided by A. Kikuchi of the Mitsubishi Kasei Institute of Life Sciences. For internal labeling of DNA, the pSV01ΔEP DNA was linearized by digestion with *Nde*I. The 5′ termini were labeled with ^{32}P by using calf intestine alkaline phosphatase and T4 polynucleotide kinase (23). Supercoiled form I DNA was obtained by ligating the labeled DNA, using a DNA ligation kit (Takara, Kyoto, Japan), in the presence of 1 μg of ethidium bromide per ml. Purified form I DNA was digested with DNase I (0.07 U/20 μl) for 30 s in the replication reaction buffer to give form II DNA.

Replication assay. Essentially the conditions published previously (13) were used for the replication of pSV01ΔEP DNA. Reaction mixtures (40 μl) contained 40 mM creatine phosphate (di-Tris salt, pH 7.7), 7 mM $MgCl_2$, 0.5 mM dithiothreitol, 4 mM ATP, 200 μM each CTP, UTP, and GTP, 100 μM dATP, dGTP, and dTTP, 20 μM [α-^{32}P]dCTP (1×10^4 to 2×10^4 cpm/pmol), 0.1 μg of pSV01ΔEP DNA, 0.8 μg of creatine phosphokinase, 0.4 μg of SV40 T antigen, and 8 μl (160 μg of protein) of HeLa cell extracts. For replication of SV40 DNA and SV40 chromosomes, concentrations of $MgCl_2$ and ATP were decreased to 3.5 and 2 mM, respectively (15). Reactions were carried out at 37°C for the time indicated in the absence or presence of drugs, and acid-insoluble radioactivity was measured. To analyze the products by gel electrophoresis, the reactions were terminated by addition of 20 mM EDTA, 1% sodium dodecyl sulfate, and 40 μg of yeast tRNA as a carrier, and the mixture was digested with proteinase K (100 μg/ml) at 37°C for 30 min. The products were extracted with phenol-chloroform, precipitated with ethanol, and electrophoresed at 1 V/cm in 1.0% agarose gels (0.5 by 20 by 24 cm) in 80 mM Tris-HCl (pH 7.5)–5 mM sodium acetate–1 mM EDTA–0.1% sodium dodecyl sulfate for 40 h, with the buffer circulated (27). For analysis of denatured DNA products, electrophoresis was carried out by using a 1.0 or 1.5% agarose gel containing 30 mM NaOH and 1 mM EDTA. DNA was then fixed with 7% trichloroacetic acid, and the dried gels were subjected to autoradiography. For two-dimensional gel electrophoresis, DNA was first separated in a neutral agarose gel as described above. The lane of the gel was cut out, and the slice was rotated by 90°. After it was placed at the top of the electrophoresis apparatus, 1% agarose gel in 50 mM NaCl–1 mM EDTA was poured around it, and second-dimension gel electrophoresis in alkaline conditions was carried out.

Topoisomerase assay. Topoisomerase II activity was measured by unknotting of P4 phage DNA. The reaction mixtures (16 μl) consisted of 50 mM Tris-HCl (pH 7.9), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA, 30 μg of bovine serum albumin per ml, 0.5 mM ATP, 0.1 μg of knotted P4 DNA, and diluted HeLa cell extracts, and they were incubated for 15 min at 37°C. After the reactions were stopped by addition of sodium dodecyl sulfate (final concentration, 0.3%), they were electrophoresed in 1% agarose gel in 100 mM Tris-borate containing 25 mM EDTA at 80 V for 2.5 h. DNA was visualized by staining with ethidium bromide. P4 DNA was completely unknotted by incubation at 75°C for 15 min.

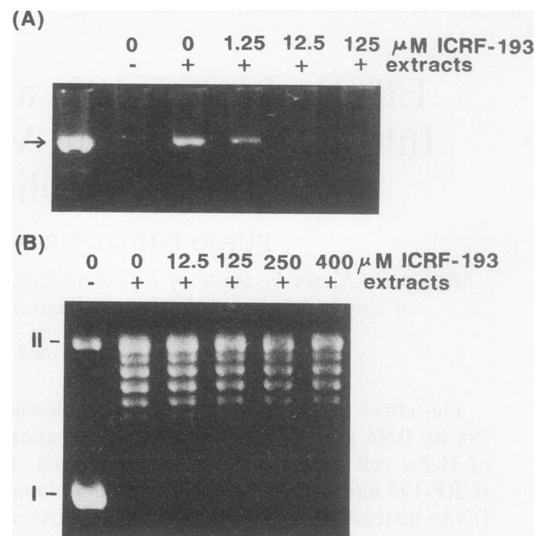

FIG. 1. Inhibition by ICRF-193 of activity of topoisomerase II but not topoisomerase I in HeLa cell extracts. (A) Knotted P4 DNA incubated with 1 μl of 30-fold-diluted HeLa cell extracts in the absence or presence of ICRF-193 in topoisomerase II assay conditions. (B) Supercoiled pBR322 DNA incubated with 1 μl of 100-fold-diluted HeLa cell extracts in topoisomerase I assay conditions. DNA was analyzed by 1% agarose gel electrophoresis. In panel A, the leftmost lane contained heat-treated P4 phage DNA, and the position of unknotted DNA is indicated by an arrow. The presence (+) or absence (−) of diluted cell extracts and concentrations of ICRF-193 used for the reactions are indicated above the lanes.

Topoisomerase I activity was measured by relaxation of supercoiled pBR322 DNA. Assay conditions were the same as for the topoisomerase II assay except that ATP was omitted and P4 DNA was replaced by negatively supercoiled pBR322 DNA.

RESULTS

ICRF-193 inhibits decatenation of replicated SV40 DNA. The effects of ICRF-193 on the activities of topoisomerases I and II in HeLa cell extracts were examined (Fig. 1). Topoisomerase II in the extracts was assayed by the ability to resolve knotted P4 phage DNA, and topoisomerase I was detected by the activity that relaxes negatively supercoiled DNA. ICRF-193 at a concentration of 12.5 μM almost completely inhibited topoisomerase II activity but did not affect the activity of topoisomerase I at concentrations up to 400 μM. These results are consistent with previous findings that ICRF-193 inhibits purified topoisomerase II but not topoisomerase I (29).

Circular duplex DNA containing the SV40 replication origin (pSV01ΔEP DNA; 2.79 kbp) (36) was used as a template for replication. The purified DNA was homogeneous and consisted of form I DNA (Fig. 2A). The DNA replicated with HeLa cell extracts and SV40 T antigen was analyzed by neutral agarose gel electrophoresis according to

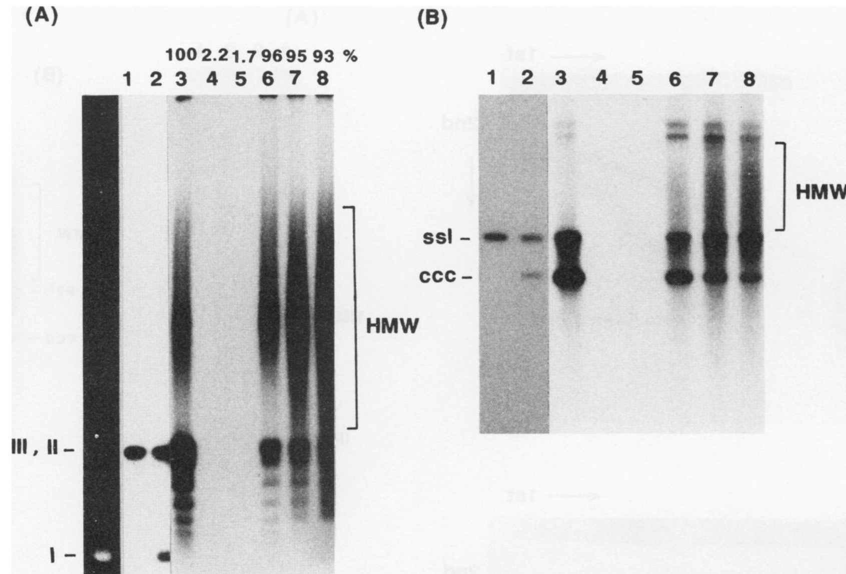

FIG. 2. Effect of ICRF-193 on SV40 DNA replication in vitro. Plasmid DNA with the SV40 replication origin (pSV01ΔEP DNA) or without the origin (pBR322ΔEP DNA) was replicated with HeLa cell extracts and SV40 T antigen for 1 h in the absence or presence of ICRF-193 as described in Materials and Methods. DNA was purified and analyzed by neutral (A) or alkaline (B) agarose gel electrophoresis. The gels were analyzed by autoradiography. The pSV01ΔEP DNA was electrophoresed in leftmost lane of panel A and stained with ethidium bromide. In lanes 1 and 2 of both panels contained labeled marker DNAs. Lanes: 1, form III DNA; 2, mixture of form II and form I DNAs; 3 to 8, replicated DNAs; 3, control reaction; 4, reaction without T antigen; 5, reaction containing pBR322ΔEP in place of pSV01ΔEP; 6, reaction with 1.25 μM ICRF-193; 7, reaction with 12.5 μM ICRF-193; 8, reaction with 125 μM ICRF-193. Relative DNA synthesis is indicated above the lanes in panel A. The amount of DNA synthesis in each reaction is expressed as a percentage of the amount in the reaction without ICRF-193, in which 113 pmol of total deoxyribonucleotides was incorporated in 1 h. Positions of migration of monomer and high-molecular-weight (HMW) DNAs are indicated in panel A, and those of ccc, ssl, and high-molecular-weight DNAs are indicated in panel B.

the method of Sundin and Varshavsky (27) (Fig. 2A). In this system, segregated monomer DNAs with different linking numbers and a broad band of high-molecular-weight DNAs were synthesized. DNA replication was dependent on the presence of T antigen and the SV40 replication origin. When the products were analyzed in denaturing conditions, a covalently closed circular (ccc) duplex DNA, which was derived from the monomer DNAs, was detected as a major product in addition to single-stranded linear (ssl) DNA (Fig. 2B). ICRF-193 did not greatly affect total deoxyribonucleotide incorporation. When the concentration of the drug was increased, however, synthesis of the monomer DNA as well as the ccc molecule decreased and the high-molecular-weight DNAs accumulated as a major product, as judged by neutral and alkaline gel electrophoresis (Fig. 2). A smeary band from the high-molecular-weight DNA to the middle position between forms I and II appeared in a neutral gel upon the addition of ICRF-193 (Fig. 2A).

The structure of the replicated DNA was analyzed by two-dimensional gel electrophoresis (Fig. 3). The DNA was first electrophoresed in a neutral agarose gel and subsequently was separated in an alkaline agarose gel after 90° rotation. When DNA replicated in the absence of ICRF-193 was analyzed, a large portion of monomer DNAs migrated to the position of ccc DNA, and high-molecular-weight DNAs synthesized were split into two arcs (Fig. 3A). The lower arc most likely consisted of late Cairns-type DNAs and rolling-circle DNAs, and the upper one may have contained circular dimers (24). Upon the addition of ICRF-193, a new arc from monomer DNA to the position crossing the upper arc seen in the control, forming a composite arc, as well as a horizontal line of ssl DNA from monomer DNA to late Cairns-type DNA appeared, and the amount of late Cairns-type DNAs increased (Fig. 3B). Most probably, the new arc consisted mainly of catenated dimers of the C family, in which two covalently closed monomer DNAs were intertwined, and the ssl line came from dimers of the A and B families, in which at least one of the monomer DNAs was nicked (28). A spot on the ssl line probably derived from form III DNA. Rolling-circle DNA replication may occur in the presence of ICRF-193, since some of the late Cairns-type DNA has nascent DNA longer than ssl DNA (Fig. 3B).

DNA replicated in the presence of ICRF-193 was purified and then incubated with topoisomerase II prepared from HeLa cells. In a neutral gel (Fig. 4A), a smeary band from the high-molecular-weight DNA to the middle position between forms I and II disappeared, and monomer DNAs increased upon the addition of topoisomerase II. The treatment resulted in an increased amount of ccc molecules and a decreased amount of high-molecular-weight DNAs in an alkaline agarose gel (Fig. 4B). When the products were analyzed by two-dimensional gel electrophoresis, the two lines from catenated dimers disappeared and the amount of monomers DNAs increased (Fig. 4C). The amounts of monomer DNAs and of late Cairns-type DNAs and/or rolling-circle DNAs were determined to be almost equal in this gel. These results show that in the presence of ICRF-193, catenated dimers and late Cairns-type DNAs accumulated during SV40 DNA replication in vitro.

ICRF-193 neutralizes the effect of VP-16 but not of camptothecin. The effects of camptothecin and VP-16, which are specific inhibitors of topoisomerases I and II, respectively, on SV40 DNA replication were examined. It has been shown that both inhibitors stimulate formation of cleavable com-

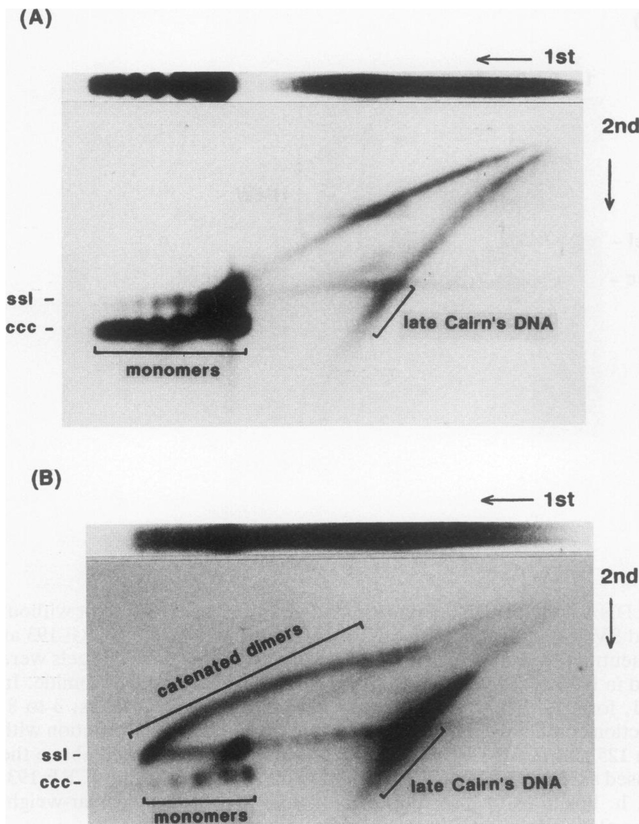

FIG. 3. Two-dimensional gel electrophoresis of DNA replicated in the presence of ICRF-193. pSV01ΔEP DNA replicated in the absence (A) or presence (B) of ICRF-193 (125 μM) was first separated by neutral agarose gel electrophoresis as shown. After rotation by 90°, the DNA was electrophoresed in an alkaline agarose gel. An autoradiogram of the first-dimension gel is shown at the top. The various DNA structures separated are indicated.

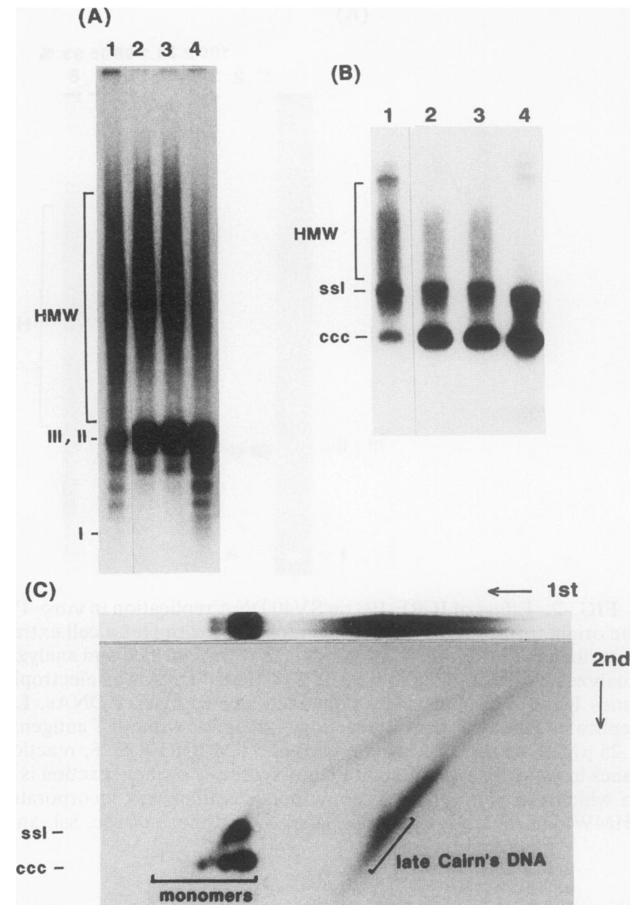

FIG. 4. Conversion by topoisomerase II of catenated dimer DNAs to monomer DNAs. DNA replicated in the presence of ICRF-193 (125 μM) as described in the legend to Fig. 2 was purified and incubated for 10 or 30 min with 40 U of topoisomerase II purified from HeLa cells. The DNA was analyzed by neutral (A), alkaline (B), or two-dimensional (C) gel electrophoresis. (A and B) Lanes: 1, DNA replicated in the presence of ICRF-193; 2, DNA incubated with topoisomerase II for 10 min; 3, DNA incubated for 30 min; 4; DNA replicated in the absence of ICRF-193. A sample like that in lane 3 was analyzed in panel C. HMW, high-molecular-weight DNA.

plexes between DNA and topoisomerases (4, 11). DNA synthesis was partially inhibited by the addition of either camptothecin or VP-16. Synthesis of the ccc DNA decreased, and nascent DNAs of immature size were generated as replication products in the presence of camptothecin or VP-16, as judged by alkaline agarose gel electrophoresis (Fig. 5A). These results are in contrast with those obtained with ICRF-193 (Fig. 2B). Two of these three drugs were added to the replication reaction, and the products were analyzed in an alkaline agarose gel (Fig. 5B). SV40 DNA replication was severely inhibited in the reaction containing both camptothecin and VP-16, and short nascent DNAs from 900 to 1,700 nucleotides (nt) were detected. When ICRF-193 was added to the replication reaction containing camptothecin, it did not affect the replication products. In contrast, ICRF-193 reversed the inhibitory effect of VP-16 on DNA chain elongation, and the replication products became similar to those synthesized in the presence of ICRF-193 alone. These results suggest that ICRF-193 interferes with the action of VP-16, which induces DNA strand breaks by stabilizing topoisomerase II-DNA complexes, during SV40 DNA replication in vitro. This is the result expected from the mode of action of ICRF-193 in antagonizing VP-16 in vitro (29) and in vivo (12).

ICRF-193 blocks DNA synthesis in the late stage of SV40 chromosome replication. SV40 chromosomes prepared from infected CV-1 cells can be replicated with HeLa cell extracts supplemented with T antigen. DNA replication was initiated from the origin region and proceeded bidirectionally (26). Two major products of the ccc and ssl molecules were synthesized from SV40 chromosomes as well as SV40 DNA (5.2 kbp) (Fig. 6A). As the concentration of ICRF-193 increased, heterogeneous-size products from 3,700 to 5,400 nt instead of the ccc molecule were synthesized during the replication of SV40 chromosomes. These results are in contrast with those obtained for SV40 DNA replication (Fig. 6A), during which the high-molecular-weight DNAs were generated by addition of ICRF-193 to the system, similarly to the reaction containing pSV01ΔEP DNA (Fig. 2B). To linearize SV40 DNA, the replication products were digested with *Bgl*I, which cuts SV40 DNA in the origin region, and were analyzed by alkaline agarose gel electrophoresis (Fig. 6B). During SV40 chromosome replication, the replicated linear DNA drastically decreased and shorter nascent DNAs whose average sizes were about half that of the template DNA were produced as the concentration of ICRF-193

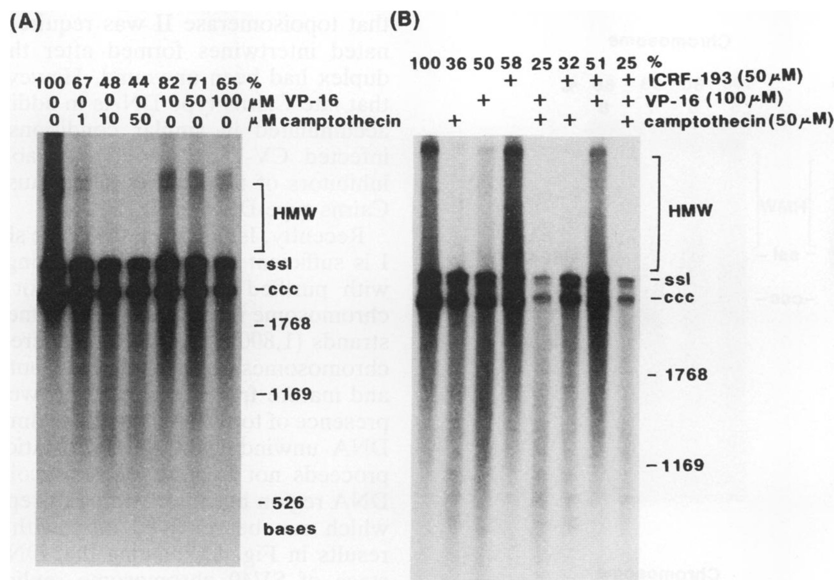

FIG. 5. Inhibition by ICRF-193 of the effect of VP-16 but not camptothecin. (A) Replication of pSV01ΔEP DNA in the presence of camptothecin (1, 10, or 50 μM) or VP-16 (10, 50, or 100 μM). Concentrations of the drugs and relative values for DNA synthesis are indicated above the lanes. (B) DNA replication in the presence of camptothecin (50 μM), VP-16 (100 μM), and ICRF-193 (50 μM) singly or in combination. Combinations of two or three drugs added to the reaction, and relative values for DNA synthesis are indicated above the lanes. Purified DNA was analyzed by alkaline agarose gel electrophoresis. SV40 DNA was digested with *Bam*HI or *Hin*dIII to produce marker DNAs.

increased. Short DNAs of similar sizes were also detected during SV40 DNA replication, but the accumulation of these DNAs was less evident compared with that in SV40 chromosome replication. Full-size DNAs which came from catenated dimers synthesized in the presence of ICRF-193 were almost exclusively detected in SV40 DNA replication. These results suggest that ICRF-193 blocks DNA replication in the terminal region of SV40 chromosomes.

DISCUSSION

Addition of ICRF-193 to an SV40 DNA replication reaction generated catenated dimers and late Cairns molecules as replication products. The catenated dimers were resolved into monomer DNAs by treatment with topoisomerase II. ICRF-193 antagonized the inhibitory effect of VP-16 but not of camptothecin on DNA chain elongation. A similar effect of ICRF-193 was observed in vitro when the agent was added to a reaction in which double-stranded DNA breaks of pBR322 DNA were generated with VP-16 (29). The same competition was observed in vivo when ICRF-193 was added to cells together with VP-16; in this case, the cleavage of DNA due to VP-16 was interfered with by ICRF-193 (12). These results suggest that ICRF-193 induces the synthesis of catenated dimers by blocking the action of topoisomerase II and that topoisomerase I functions as a swivelase up to the step of formation of the catenated dimers, as discussed below, and topoisomerase II is needed for the last step of replication, i.e., decatenation of intertwined catenated dimers.

As shown previously, SV40 DNA replication can proceed to produce catenated dimers different in linking number in a system consisting of purified proteins, including topoisomerase I (13). Although topoisomerase I may not recognize positive supercoils accumulated in the unreplicated DNA region in the final stage of bidirectional replication, replication proceeds to completion by unwinding of the unreplicated double helix without swiveling, which resulted in production of intertwined replicated daughter molecules (32). The number of intertwines of the dimers is proportional to the length of the unreplicated DNA region. These catenated dimers were also synthesized in a replication system composed of HeLa cell extracts and T antigen when topoisomerase II was depleted from the extracts (37). Addition of ICRF-193 to the replication system of cell extracts generated catenated dimers, and thus the drug has an effect similar to that observed upon the depletion of topoisomerase II. These results strongly suggest that ICRF-193 affects mainly topoisomerase II in this system.

Two-dimensional gel electrophoresis showed that catenated dimers synthesized in the presence of ICRF-193 consisted mainly of C-family dimers in which two covalently closed monomer DNAs were intertwined (28). They are heterogeneous in mobility in the first-dimension gel because of the difference in number of intertwines of the two DNAs and in number of nucleosomes assembled onto replicated DNA. Snapka (24) reported that ellipticine, a DNA-intercalating drug, blocked decatenation of replicated SV40 DNA in vivo, resulting in the appearance of a smeary band from the position of form II to the middle position between forms I and form II. Similar products, probably highly catenated dimers, were synthesized during SV40 DNA replication in the presence of ICRF-193 (Fig. 2A).

In the presence of ICRF-193, synthesis of late Cairns-type DNAs as well as of catenated dimers was enhanced (Fig. 3B). Furthermore, short nascent DNAs whose average sizes were about half that of the template DNA were detected after *Bgl*I digestion during SV40 DNA replication (Fig. 6B, left). These results suggest that ICRF-193 partially interferes with DNA synthesis in the final stage of SV40 DNA replication. Two-dimensional gel electrophoresis also shows that some of the late Cairns-type DNAs synthesized in the

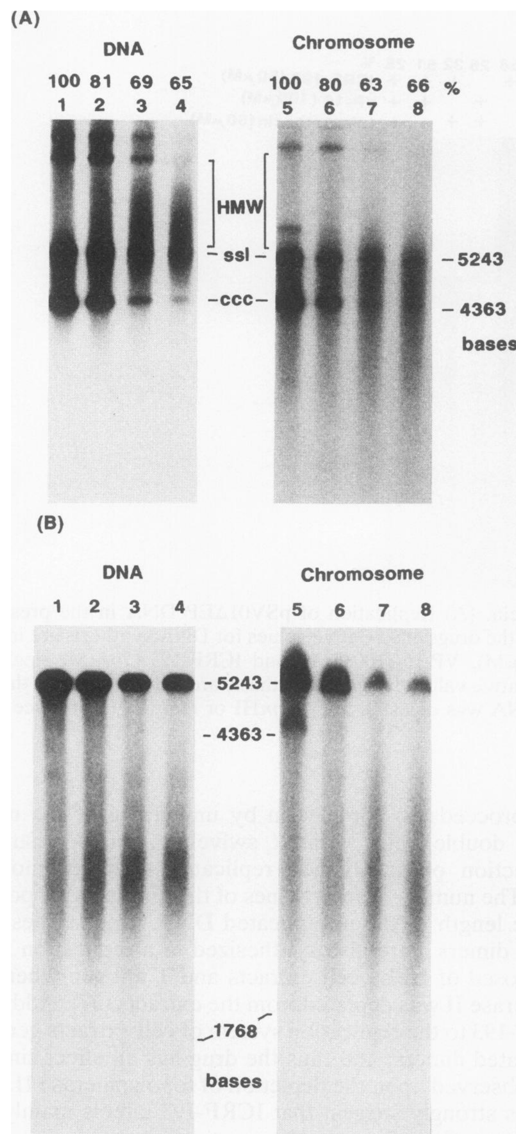

FIG. 6. Inhibition by ICRF-193 of DNA synthesis in the late stage of SV40 chromosome replication. SV40 DNA (5.2 kbp) (lane 1 to 4) and SV40 chromosomes (lane 5 to 8) were replicated with HeLa cell extracts and SV40 T antigen for 1.5 h in the absence or presence of ICRF-193. DNA was purified and analyzed by alkaline agarose gel electrophoresis before (A) or after (B) digestion with *BglI*. Concentrations of ICRF-193 were 0 μM (lanes 1 and 5), 1.25 μM (lanes 2 and 6), 12.5 μM (lanes 3 and 7), and 125 μM (lanes 4 and 8). Relative values for DNA synthesis are indicated above the lanes in panel A. Total incorporation of deoxyribonucleotides was 344 pmol during SV40 DNA replication and 166 pmol during SV40 chromosome replication. SV40 DNA digested with *BamHI* or *HindIII* and pBR322 DNA digested with *BamHI* were used as marker DNAs.

presence of ICRF-193 have nascent DNA longer than the monomer size of the template DNA, which was revealed by second-dimension gel electrophoresis (Fig. 3B). These products may be synthesized by a rolling-circle mechanism after the replication fork of late Cairns molecules has been broken.

Sundin and Varshavsky (28) observed that catenated dimers of SV40 DNA accumulated when infected CV-1 cells were placed in hypertonic conditions, and they suggested that topoisomerase II was required for separation of catenated intertwines formed after the unreplicated parental duplex had been unwound. However, it was also reported that late Cairns-type DNAs in addition to catenated dimers accumulated in similar conditions (9, 35). Treatment of infected CV-1 cells with cleavable-complex-forming-type inhibitors of topoisomerase II caused accumulation of late Cairns-type DNAs (20, 21, 25).

Recently, Ishimi et al. (14) have shown that topoisomerase I is sufficient as a swivelase during SV40 DNA replication with purified proteins but is not sufficient during SV40 chromosome replication in the same system. Shorter leading strands (1,800 nt on average) were synthesized from SV40 chromosomes in the reaction containing topoisomerase I, and mature fragments (2700 nt) were produced only in the presence of topoisomerase II. Ishimi et al. (14) proposed that DNA unwinding of the termination region of replication proceeds not by positive supercoiling of the unreplicated DNA region but intertwining of replicated daughter DNAs, which can be resolved only with topoisomerase II. The results in Fig. 6, showing that DNA synthesis in the final stage of SV40 chromosome replication was inhibited by ICRF-193, are consistent with this model. The findings presented above strongly suggest that topoisomerase II is required for unwinding of final duplex DNA in a late Cairns structure as well as resolution of catenated dimers during SV40 chromosome replication in vivo and in vitro.

In the presence of ICRF-193, nascent DNAs from 3,700 to 5,200 nt were synthesized from SV40 chromosomes (Fig. 6A), suggesting that replication of the SV40 chromosome may be arrested before the step at which DNA unwinding of the unreplicated DNA region can proceed without topoisomerases. When VP-16, a cleavable-complex-forming inhibitor of topoisomerase II, was added to the SV40 chromosome replication reaction, nascent DNAs averaging 1,800 nt, shorter than those synthesized in the presence of ICRF-193, were synthesized (14). The results for VP-16 suggest that topoisomerase II works as a swivelase in the latter half of SV40 chromosome replication with crude extracts. The different inhibitory effects of the two topoisomerase II inhibitors on SV40 chromosome replication might be due to the different mode of inhibition of topoisomerase II. In the reaction containing VP-16, replication fork movements may be arrested by cleavable complexes which are generated ahead of the replication fork. Since ICRF-193 inhibits topoisomerase II without formation of the DNA cleavage complex, DNA replication proceeds with topoisomerase I, and as a result, longer nascent DNA can be synthesized in the replication reaction containing ICRF-193.

Drake et al. (8) reported a novel inhibitor of topoisomerase II, merbarone, whose function appears to be similar to that of ICRF-193. Merbarone did not cause topoisomerase II-induced DNA cleavage; furthermore, it inhibited the production by amsacrine or teniposide of topoisomerase II-associated DNA strand breaks. However, merbarone is not chemically similar to ICRF-193, and it inhibited topoisomerase I at high concentrations. Merbarone preferentially inhibited the catalytic activity of the P170 form of topoisomerase II compared with P180 form (7). The effect of ICRF-193 on each form of topoisomerase II remains to be determined.

Since chromosome DNA in eukaryotic cells is long and linear and has multiple replicons, during replication it faces topological problems similar to those encountered by SV40 DNA which must be resolved in the DNA replication system. Genetic evidence from *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* suggests that either topoisom-

erase I or topoisomerase II is required for DNA replication at S phase and that topoisomerase II is essential for condensation and segregation of daughter chromosomes at mitosis. When temperature-sensitive mutants of topoisomerase II are grown at a nonpermissive temperature, they can traverse S phase but became arrested at mitosis (10, 30). In the mutant cells, the nuclei never appear to undergo normal separation and show an unusual morphology. In another topoisomerase II mutant, catenated dimers of 2μm plasmid DNA were produced at a nonpermissive temperature (6), suggesting that topoisomerase II is not required for DNA synthesis. These results seem to contradict the data presented here, because 2μm DNA is not present as free DNA but forms a chromosome structure similar to SV40 chromosomes (18). Possibly this difference results from residual activity of topoisomerase II that may be present in the heat-treated mutant cells. It is also possible that some unknown topoisomerase may take over the role of topoisomerase II as a swivelase in the late stage of bidirectional DNA replication in *S. cerevisiae*. Still another possibility is that there are some basic differences in chromatin structure between the two species which cause the different action of topoisomerase II. In *Escherichia coli*, several topoisomerases might be involved in resolving replicated DNA. DNA topoisomerase I can decatenate DNA containing single-stranded regions in vitro (33), and DNA topoisomerase III has been purified as the enzyme that efficiently decatenates gapped plasmid DNA near the end of replication (5). Recently, a novel type II topoisomerase, DNA topoisomerase IV, has been shown to be required in addition to topoisomerase II, (DNA gyrase) for segregation of chromosomes (16). These topoisomerases might participate in different pathways of segregation of daughter molecules (34).

When human lymphoblastic leukemia RPMI 8402 cells were treated with ICRF-193, all mitotic cells exhibited early mitotic forms with less condensed and entangled chromosomes (12). Analysis of whether DNA synthesis is completed in ICRF-193-treated cells is required to determine the role of topoisomerase II in cellular DNA replication. In conclusion, ICRF-193, a noncleavable-complex-forming topoisomerase II inhibitor, blocks completion of DNA synthesis in SV40 chromosome replication in vitro, suggesting that topoisomerase II is required for DNA unwinding in a late Cairns structure as well as for resolving catenated dimers. ICRF-193 exhibits a biological effect different from that of the cleavable-complex-forming-type inhibitor VP-16, and it is more suitable than the cleavable-complex-forming inhibitor for in vivo study of topoisomerase II.

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