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Inhibition of Topoisomerase II by ICRF-193 Prevents Efficient Replication of Herpes Simplex Virus Type 1

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Cellular topoisomerase II is specifically inactivated by the drug ICRF-193. This compound turns topoisomerase II into a closed clamp that is unable to cleave DNA. We have investigated the effects of this inhibitor on the replication of herpes simplex virus type 1. We show that ICRF-193 at low multiplicities of infection dramatically inhibits viral DNA synthesis and the production of infectious virus. The inhibition is less efficient at high multiplicities of infection. In addition, inhibition of viral DNA synthesis was observed only when ICRF-193 was present during the first 4 h of the infectious cycle. The transient replication of plasmids containing a herpes simplex virus type 1 origin of DNA replication, oriS, was affected by ICRF-193 in the same way. In contrast, neither cellular DNA synthesis nor replication of plasmids containing a simian virus 40 origin of DNA replication was inhibited. The observed effect on herpes simplex virus DNA replication was not caused by a decreased transcription of replication genes inasmuch as the levels of UL8, UL9, UL29, and UL30 mRNAs were unaffected by the drug. These results suggest that topoisomerase II plays a vital role during the replication of herpes simplex virus type 1 DNA. We speculate that topoisomerase II is involved in the decatenation of newly synthesized daughter molecules.

The replication of herpes simplex virus type 1 (HSV-1) depends on seven viral gene products (34). The origin-binding protein, UL9 or OBP, is an ATP-dependent DNA helicase that most probably is responsible for the initial activation of the viral origins of replication, oriL and oriS (5, 15, 16). The remaining six viral gene products, UL5, UL8, UL29, UL30, UL42, and UL52, seem to be integral members of a multienzyme complex, a replisome, capable of sustaining origin-independent DNA synthesis (37). Little is known about the role of cellular proteins during the replication cycle of the virus. The recently discovered interaction between the HSV-1 origin-binding protein and DNA polymerase α might play an important role during the infectious cycle (30). Since HSV-1 does not encode its own topoisomerase, it is reasonable to assume that a cellular topoisomerase will play an active role during the synthesis of viral DNA (14). We have investigated this matter by using the bisdioxopiperazine derivative ICRF-193 (24, 25, 40). In the presence of ATP, this compound converts topoisomerase II to a closed clamp unable to cleave DNA (36). Cells treated with ICRF-193 can readily traverse the cell cycle but are unable to pass through mitosis. The end result will be entangled chromosomes and an accumulation of polyploid cells (18, 25, 26). This observation is in complete agreement with the observed role of topoisomerases in yeasts. In yeasts, topoisomerases I and II play complementary roles in controlling the topological state of DNA during all parts of the cell cycle except for mitosis (43). At this stage, topoisomerase II is uniquely required for chromosome condensation and segregation (41). It is also of interest that topoisomerase II is a major component of the chromosome scaffold and has been proposed to be involved in the organization of cellular chromosomes (12, 13, 29).

We have now seen that ICRF-193 dramatically inhibits the synthesis of HSV DNA, resulting in a decreased yield of infectious virus. This effect was observed only if the drug was present during the first 4 h of the infectious cycle. Furthermore, the effect was specific for HSV-1, since neither cellular DNA synthesis nor simian virus 40 (SV40) origin-dependent DNA replication was affected by ICRF-193. The role of topoisomerase II during the replication of HSV-1 will be further discussed below.

MATERIALS AND METHODS

Cells, viruses, and plasmids. HSV-1, Glasgow strain 17 syn+, was propagated on BHK 21 cells (clone 13; ATCC CCL10). The cells were grown in Glasgow modified Eagle's medium (GIBCO) supplemented with 10% tryptose phosphate broth and 10% newborn calf serum (GIBCO) (4). The virus was grown at 37°C, and viral stocks were prepared as described previously (39). The virus titer was measured by following standard protocols (32). HSV-1 tsK was obtained from Nigel Stow, MRC Virology Unit, Institute of Virology, Glasgow, United Kingdom.

COS-1 cells were grown in Iscove's medium supplemented with 10% fetal calf serum, 2 mM glutamine (GIBCO), 50 U of penicillin per ml, and 50 μg of streptomycin per ml. A cell line of human skin fibroblasts referred to as DO4 cells was a kind gift from Göran Bondjers, Sahlgrenska Hospital, Gothenburg, Sweden. The cells were grown in Earle minimal essential medium (Seromed)–10% fetal calf serum–1× nonessential amino acids (Seromed)–2 mM glutamine–2 mM sodium pyruvate–50 U of penicillin per ml–50 μg of streptomycin per ml.

Plasmid replication experiments were performed with either plasmids containing the HSV-1 oriS, pORI(wt) and pOS822, or a plasmid harboring an SV40 origin of DNA replication, pELAM (3, 19, 44).

The series of plasmids pE5, pE8, pE9, pE29, pE30, pE42, and pE52 was a generous gift from Nigel Stow. These plasmids contain the genes for the essential HSV-1 replication proteins under the control of the major human cytomegalovirus immediate-early promoter (38). Studies on gene expression from a synthetic promoter were done with plasmid pOTL. This plasmid was derived from pUC19 and contained HSV-1 oriS surrounded by divergent T7 RNA polymerase promoters cloned in the polylinker. The luciferase gene was cloned immediately downstream of one of these promoters.

Reagents. ICRF-193 was a generous gift from Zenyaku Kogyo Co., Ltd., Tokyo, Japan. It was dissolved and stored at −20°C in dimethyl sulfoxide. Cycloheximide, L-mimosine, and phosphonoacetate were purchased from Sigma and dissolved in water. Liposomes for the transfection experiments were prepared by mixing 0.24 ml of a 10-mg/ml solution of dimethyldioctadecylammonium bromide (Sigma) in chloroform with 0.6 ml of a 10-mg/ml solution of L-α-phosphatidylethanolamine dioleyl (Sigma), also in chloroform. The mixture was dried under vacuum in a Savant Speedvac. Then, the mixture of lipids was suspended in 6 ml of double-distilled water and sonicated with a probe sonicator until the solution became clear. The preparation of liposomes was stored at 4°C.

Measurements of viral DNA synthesis. Confluent monolayers containing 4.5×10^5 BHK cells were infected with virus at a multiplicity of infection of 1 PFU per cell, unless otherwise indicated, and incubated at 37°C. The cells were harvested at the times indicated. The medium was removed, and the cells were solubilized in 550 μl of 0.6% sodium dodecyl sulfate–10 mM Tris-HCl (pH 7.5)–1 mM

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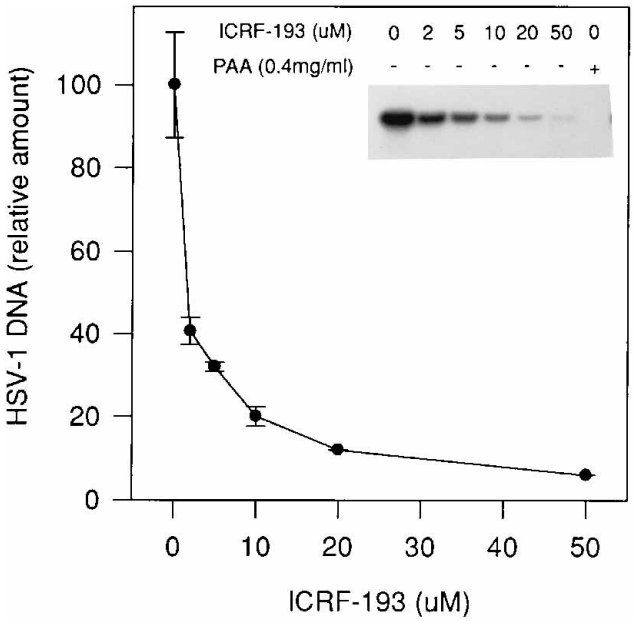

FIG. 1. ICRF-193 inhibits the synthesis of HSV-1 DNA. ICRF-193 was added to BHK cells at 0, 2, 5, 10, 20, and 50 μM before the addition of HSV-1 at a multiplicity of infection of 1 PFU per cell. In one experiment, phosphonoacetic acid (PAA) was added to a final concentration of 400 μg/ml. The cells were harvested at 16 h p.i. DNA was digested with *Bam*HI and analyzed on agarose gels followed by Southern blotting. The amount of radioactivity in the *Bam*HI fragment used to monitor replicated viral DNA was measured with a Phosphor Imager. The insert shows an autoradiograph of the Southern blot of replicated viral DNA.

EDTA–0.2 mg of proteinase K per ml–60 μg of glycogen per ml for 4 h at 37°C. Total DNA was prepared by phenol extraction and ethanol precipitation. The DNA was dissolved in a total volume of 100 μl and subsequently cleaved overnight with *Bam*HI (100 U/ml). The cleaved DNA was separated on 1% agarose gels and transferred to Hybond-N^+ membranes by alkali transfer as suggested by the supplier (Amersham). Prehybridization, hybridization, and washing of the membranes were performed as described by the supplier. The radioactive probe was a 1.5-kb *Bam*HI restriction fragment from the UL9 gene (nucleotides 21655 to 23232 as defined in the EMBL database) labelled with the Megaprime labelling system (Amersham). The amount of radioactivity of individual bands of the Southern blots was measured by a Phosphor Imager (Molecular Dynamics).

Measurements of viral RNAs. BHK cells were infected at a multiplicity of infection of 1 PFU per cell as above, and the infection was allowed to proceed for the times indicated. Total cellular RNA was prepared essentially as described previously (6). RNA from approximately 5×10^5 cells was analyzed on 1% agarose gels in 2.2 M formaldehyde–20 mM MOPS (3-*N*-morpholinopropanesulfonic acid [pH 7.0])–0.5 mM sodium acetate–1 mM EDTA. All the samples contained equal amounts of total RNA as determined spectrophotometrically. The RNA was transferred to Hybond-N+ membranes with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) as described by the manufacturer (Amersham). Fixation was carried out for 5 min in 0.05 M NaOH. Hybridization with DNA probes labelled with the Megaprime system was performed as described above. The amount of UL29 mRNA was measured with a 1.5-kb *Pst*I restriction fragment (nucleotides 58806 to 60288) as a probe. The amount of UL10 mRNA was measured with a *Nar*I fragment containing nucleotides 23546 to 24885. Similarly, a DNA probe was used to quantify UL30 mRNA. Riboprobes were used to measure UL8 and UL9 RNA essentially as previously described (21). The amount of radioactivity in the corresponding bands on Northern (RNA) blots was measured with a Phosphor Imager.

DNA synthesis dependent on SV40 origin of replication. COS-1 and BHK cells were transfected with liposomes as follows. For each subconfluent monolayer containing 4.5×10^5 cells, 0.2 μg of plasmid pELAM was diluted in 30 μl of 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)–NaOH (pH 8.0)–150 mM KCl–1 mM EDTA and mixed with 5 μl of the liposome preparation described above. The mixture was incubated for 10 min at room temperature and subsequently diluted in serum-free medium (Optimem [GIBCO]) to a final volume of 200 μl. The monolayers were washed with Optimem and overlaid with 200 μl of the liposome-DNA mixture for 4 h at 37°C. Then, 200 μl of the growth medium containing the inhibitors was added as indicated. The liposome-containing medium was removed and replaced with fresh growth medium plus inhibitors 24 h after the start of transfection. At 28 h posttransfection, the cells were lysed and total cellular DNA was prepared as described above and cleaved overnight with *Eco*RI (100 U/ml) and *Dpn*I (20 U/ml). The DNA samples were subsequently analyzed on Southern blots prepared from 1% agarose gels as described above. Plasmid pELAM was labelled with the Megaprime system and used as a probe.

DNA synthesis dependent on plasmids containing an HSV-1 origin of replication. DNA replication was measured essentially as described previously (19). Subconfluent monolayers of BHK cells were transfected with liposomes containing either 0.05 μg of plasmid pOS822 or 0.05 μg of pORI(wt). Each monolayer contained 4.5×10^5 cells. The monolayers were incubated with the liposomes for 4 h. Then, 200 μl of growth medium was added. At 3 h later, the cells were infected with virus (1 PFU per cell). The cells were harvested at 16 h postinfection (p.i.), and total cellular DNA was prepared as described above. The DNA was cleaved overnight with *Eco*RI and *Dpn*I. The samples were subjected to agarose gel electrophoresis. The gels were stained with ethidium bromide to make sure that the samples contained equal amounts of DNA. After electrophoresis, the gels were subjected to a Southern blot analysis. The probe was pTZ19 plasmid DNA labelled with the Megaprime system.

In one experiment, the transacting factors were provided by the pE plasmid series rather than a superinfecting virus (38). In this instance, liposomes were made from a mixture of 0.1 μg of pORI and equimolar amounts of the pE plasmids as indicated in the appropriate figure. The cells were harvested after incubation for 30 h. Replicated oriS-containing plasmid DNA was measured as described above.

Measurements of cellular DNA and protein synthesis. The effects of ICRF-193 on cellular protein and DNA synthesis were measured by [^{35}S]methionine and [^{3}H]thymidine incorporation, respectively. Protein synthesis in BHK cells was measured after an incubation with ICRF-193 or cycloheximide (48 μM) for 3 h in the growth medium. The medium was then replaced with Dulbecco's modified Eagle's medium without methionine but supplemented with 10% dialyzed newborn calf serum, 29 μCi of [^{35}S]Pro-mix (Amersham) (1,000 Ci/mmol), and inhibitors. After 1 h at 37°C, the cells were washed and trypsinized. The cells were thoroughly suspended in 1 ml of 10% trichloroacetic acid. The suspension was filtered through a Sartorious nitrocellulose membrane filter. The filters were then washed with 1 ml of 10% trichloroacetic acid, followed by 1 ml of 95% ethanol. The dried filters were counted in a liquid scintillation counter.

DNA synthesis was monitored in asynchronously growing cells. After 3 h of preincubation with inhibitors in growth medium as indicated, the medium was supplemented with 6 μCi of [^{3}H]thymidine (79 Ci/mmol; Amersham) per ml. Incubation was continued for an additional 3 h. The cells were harvested as described above. DNA was precipitated with 10% trichloroacetic acid as described above. The precipitate was collected on nitrocellulose filters. The filters were washed and processed as described above.

Measurements of luciferase activity. BHK cells (5×10^5) in 15-mm wells were transfected with 0.5 μg of pOTL as described above. Inhibitors were added 4 h later as indicated. The cells were infected either with HSV-1 strain 17 syn+ or HSV-1 tsK, using 1 PFU per cell, at 6 h after lipofection. Incubation was carried out at 38.5°C. The cells were harvested by trypsinization 12 h p.i. Luciferase activity was measured with a LUMAT LB 9501 luminometer essentially as described in the Promega technical bulletin.

RESULTS

ICRF-193 inhibits HSV-1 DNA replication. We have looked at the effects of ICRF-193 on the synthesis of HSV-1 DNA as well as on the production of infectious virus (Fig. 1; Table 1). The synthesis of viral DNA in cells treated with ICRF-193 at different concentrations was measured. BHK cells were infected with 1 PFU per cell for 16 h in the presence of 0, 2, 5, 10, 20, and 50 μM ICRF-193. The use of 2 μM ICRF-193 reduced viral DNA synthesis to 40%; 20 μM reduced replication to 10%. At elevated concentrations of ICRF-193, we could still detect approximately 5% of the normal amount of viral DNA in the infected cells, and it was never possible to completely prevent viral DNA synthesis. In contrast, phosphonoacetic acid at 0.4 mg/ml caused more than a 1,000-fold reduction of the synthesis of viral DNA (Fig. 1). The concentrations of ICRF-193 used in the experiments described above are similar to the concentrations that result in an inhibition of cellular proliferation (20, 26).

The effects of ICRF-193 on the time course of accumulation of viral DNA were also examined. In this experiment, the drug was added at the time of infection and viral DNA was measured at various times after infection. The results showed that DNA synthesis was not delayed in the presence of the drug and

TABLE 1. Effects of ICRF-193 on the production of infectious virus

Concn of ICRF-193 (μM)	Yield of virus (PFU/ml)	% Yield[a]
Expt 1[b]		
0	2.2×10^7	100
2	2.5×10^6	11
10	3.7×10^5	2
50	2.4×10^5	1
Expt 2[c]		
10	9.5×10^6	43

[a] Percentage relative to absence of ICRF-193 (set at 100%).
[b] ICRF-193 was added 15 min before the infection. HSV-1 at 1 PFU per cell was used for the infection.
[c] ICRF-193 was added 4 h p.i. HSV-1 at 1 PFU per cell was used for the infection. Addition of phosphonoacetic acid (0.4 mg/ml) at 4 h p.i. produced less than 10^3 PFU of HSV-1 per cell.

that severely reduced amounts of viral DNA were also observed at 25 h after infection (results not shown).

We then performed experiments in which 10 μM ICRF-193 was added at different times after infection. We found that efficient inhibition of viral DNA synthesis in BHK cells occurred only when the drug was present during the first 4 h of the infectious cycle (Fig. 2a and b).

We have also examined the effects of ICRF-193 on the viral replication cycle in a different cell line. This was important, since transformed cells generally show aberrations in the regulation of their DNA metabolism. It is therefore possible that different cell lines would react differently to inhibitors of these metabolic pathways. An experiment was therefore performed with nontransformed human fibroblasts (referred to as DO4 cells in this paper). The results were similar to those described above. Viral DNA replication was efficiently inhibited when the drug was supplied at the time of infection (Fig. 2c and d). In this instance, less than 2% of the normal amount of viral DNA was synthesized. When ICRF-193 was added at 6 h p.i., substantial amounts of DNA were synthesized after the time of drug addition (Fig. 2c and d). We do not know the reason for the more pronounced inhibition observed in DO4 cells. However, it has been noted that the topoisomerase II activity in cells depends on cellular growth conditions and may also vary between different cell lines (22, 33). We speculate that such differences could account for the different sensitivity toward ICRF-193 shown by BHK and DO4 cells.

We have also examined how the inhibitory effect of ICRF-193 depended on the protocol used for infection of the cells. Interestingly, we noted that the extent of inhibition of viral DNA synthesis was influenced by the multiplicity of infection (Fig. 3). Using 10 μM drug, we found an 85% inhibition of viral DNA synthesis in BHK cells infected at 0.2 PFU per cell. The inhibitory effect decreased to 50% at 10 PFU per cell.

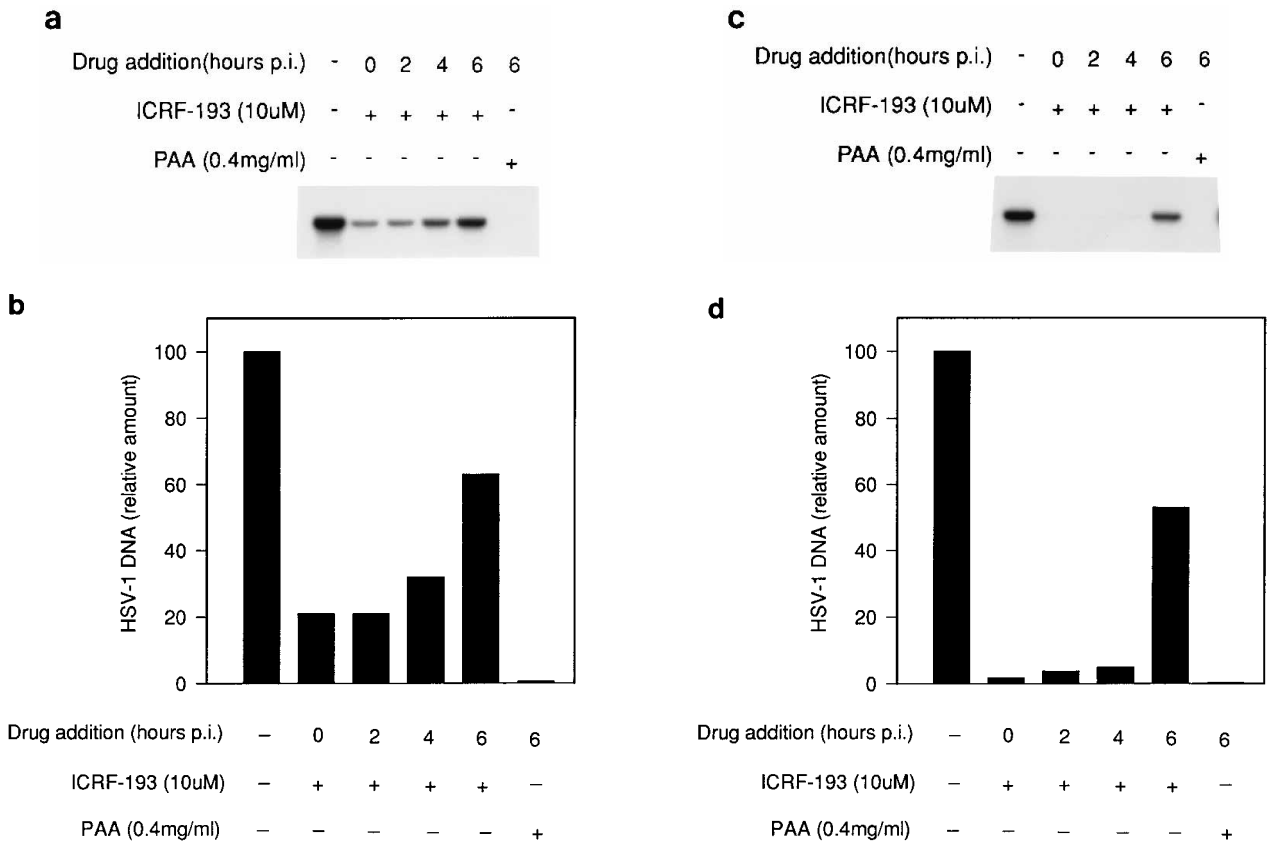

FIG. 2. Inhibition of HSV-1 replication depends on the time of addition of ICRF-193. ICRF-193 was added at 0, 2, 4, and 6 h p.i. The cells, BHK or DO4 cells, were infected with HSV-1 at 1 PFU per cell. The amount of replicated HSV-1 DNA was measured by the Southern blot procedure described in Materials and Methods. (a) Autoradiograph of replicated viral DNA obtained from BHK cells. (b) Histogram showing the values obtained by Phosphor Imager analysis of the experiment in panel a. (c) Autoradiograph of replicated viral DNA from DO4 cells. (d) Histogram showing the values obtained by Phosphor Imager analysis of the experiment in panel c. PAA, phosphonoacetic acid.

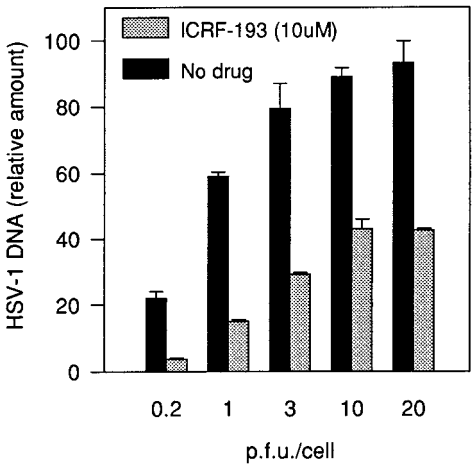

FIG. 3. The inhibition of viral DNA synthesis by ICRF-193 is affected by the multiplicity of infection. BHK cells were infected with virus at 0.2, 1, 3, 10, and 20 PFU per cell in the presence or absence of 10 μM ICRF-193. The amount of viral DNA in cells at 16 h p.i. was determined by Southern blot and Phosphor Imager analyses.

Similar results were obtained with DO4 cells. Here, the inhibitory effect ranged from 95% at 0.5 PFU per cell to 50% at 15 PFU per cell (results not shown).

To assess the antiviral effect of ICRF-193, we measured the yield of virus from infected subconfluent BHK monolayers treated with ICRF-193. We noted that ICRF-193 caused a pronounced decrease in the production of infectious virus (Table 1). The effects on DNA synthesis and production of infectious virus were of a similar magnitude. For example, 10 μM ICRF reduced the synthesis of DNA to 20% and production of virus to 2% of the values in untreated cells.

ICRF-193 does not inhibit transcription of the UL8, UL9, UL29, and UL30 genes. We wanted to make sure that the inhibition of HSV-1 replication was not caused by an altered transcription of the viral genes required for DNA synthesis. We therefore examined the amount of RNA corresponding to a set of early genes, UL8, UL9, UL29, and UL30, and one late gene, UL10 (1, 2). The transcription of early genes is largely independent of DNA replication, whereas the transcription of late genes requires DNA synthesis (42). In this context, it is of interest that the time course of accumulation of UL29 RNA depends on the multiplicity of infection. Under our experimental conditions, maximal levels of UL29 RNA were already seen at 5 h p.i. when 10 PFU per cell was used. In contrast, when 1 PFU per cell was used in the experiment, maximal levels of UL29 RNA were observed after 12 h or later (results not shown). In the experiment, the cells were either treated with 50 μM ICRF-193 or left untreated (Fig. 4). The cells were infected with 1 PFU of HSV-1 per cell, and RNA was isolated at 5 and 13 h p.i. to allow measurements of early and late transcription. The experiment showed that the levels of the UL29 transcript were not reduced in the presence of ICRF-193. Substantial amounts of UL29 RNA were observed at 5 and 13 h p.i. under these experimental conditions. To allow a comparison, we defined the amount of UL29 RNA seen at 13 h p.i. in the absence of ICRF-193 as 100%. Quantitation then revealed that at 5 h p.i., 18% of UL29 RNA was observed in the absence of ICRF-193 and 33% was observed in the presence of the drug. At 13 h p.i., 91% of UL29 RNA was seen in the presence of ICRF-193. Similar results were obtained when the transcription of other early genes such as UL8, UL9, and UL30 was studied (results not shown). When the late UL10 transcript was measured at 5 h p.i., only very small amounts were detected. Also, in the absence of ICRF-193, large amounts (100%) of transcripts were observed at 13 h p.i. In contrast, in the presence of the compound, the amount of UL10 RNA was reduced to 7%. Under the same experimental conditions, total viral DNA synthesis as measured at 16 h p.i. was also reduced to 7% (Fig. 4). These results indicate that the inhibition of DNA replication caused by ICRF-193 was not caused by a decreased transcription of early genes, since the levels of UL8, UL9, UL29, and UL30 mRNAs were not affected by the drug. Furthermore, they also show that the inhibition of viral DNA synthesis caused a severe reduction in the amount of the late UL10 transcript observed at 13 h p.i.

To further investigate the effects of ICRF-193 on gene expression, we used a reporter plasmid, pOTL, containing HSV-1 oriS and a luciferase gene under the control of a T7 RNA polymerase promoter. This promoter can be used by the cellular RNA polymerase II (31). In addition, the activity of minimal promoters can be significantly stimulated by HSV-1 in an ICP4-dependent way (23). Initial experiments revealed that luciferase expression was increased approximately 10-fold following infection with HSV-1. Furthermore, this effect was not caused primarily by template amplification, since luciferase expression was also activated in the presence of phosphonoacetic acid and when the HSV-1 tsS strain, harboring a temperature-sensitive mutation in the UL9 gene, was used at the nonpermissive temperature (results not shown). We then examined the influence of ICRF-193 on luciferase expression (Table 2). The results show that HSV-1 increased the luciferase expression approximately 10-fold. This effect was dependent on ICP4, since an infection with HSV-1 tsK failed to activate luciferase expression. Finally, when ICRF-193 was added at 10 or 25 μM, the expression of luciferase was not reduced, indicating that transcription and translation were not affected by ICRF-193.

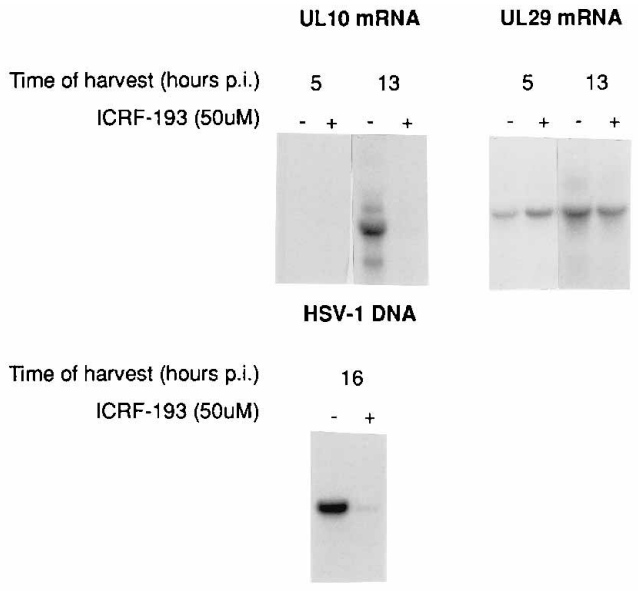

FIG. 4. Effect of ICRF-193 on the synthesis of UL29 and UL10 mRNA. BHK cells were infected with HSV-1 at 1 PFU per cell in the presence of 50 μM ICRF-193. Total cellular RNA was prepared at 5 and 13 h p.i. Northern blotting was done with UL10- and UL29-specific probes as described in Materials and Methods. DNA replication was measured at 16 h p.i. as described in the legend to Fig. 1. Quantitation was done with a Phosphor Imager, and the values are given in the text.

TABLE 2. Effects of ICRF-193 on the expression of luciferase from a minimal promoter[a]

Virus[b]	Concn of ICRF-193 (μM)[c]	Luciferase activity[d]		
		Mean (10^3 U)[e]	Range (10^3 U)[e]	%
		1.4[f]	ND[g]	
No HSV-1		130	100–159	11
HSV-1 17syn+		1,153	1,093–1,240	100
HSV-1 17syn+	10	1,081	893–1231	94
HSV-1 17syn+	25	1,178	1,066–1,432	102
HSV-1 tsK		179	169–189	15

[a] The reporter plasmid, pOTL, contained HSV-1 oriS and in addition the luciferase gene under the control of a T7 RNA polymerase promoter (see Materials and Methods and references 23 and 31).
[b] At 6 h after lipofection, 1 PFU of virus per cell was added at 38.5°C.
[c] ICRF-193 was added 4 h after lipofection.
[d] Luciferase activity from plasmid pOTL is expressed as light units.
[e] Mean of three experiments.
[f] Background value of the luminometer.
[g] ND, not determined.

ICRF-193 does not inhibit cellular DNA synthesis and counteracts the inhibition caused by etoposide. To examine the effects of ICRF-193 on cellular metabolism under our experimental conditions, we looked at protein synthesis as well as DNA replication. We noted that ICRF-193 had no effect on total cellular protein synthesis as measured by [^{35}S]methionine incorporation into trichloroacetic acid-precipitable material (results not shown). Similarly, ICRF-193 did not reduce the extent of cellular DNA synthesis in asynchronous cell cultures (Fig. 5). In contrast, both inhibition of topoisomerase I with camptothecin and inhibition of topoisomerase II with etoposide caused a significant reduction in the incorporation of [^{3}H]thymidine into DNA (Fig. 5). L-Mimosine, which inhibits DNA synthesis by interfering with ribonucleotide reductase (8), completely inhibited DNA synthesis (Fig. 5). The remarkable specificity of the drugs was illustrated by the observation that the reduction in cellular DNA synthesis caused by the inhibition of topoisomerase II by etoposide was abolished by ICRF-193 (Fig. 5). The most likely explanation of this result is that ICRF-193 turns topoisomerase II into an inactive form that is unable to cleave DNA in the presence of etoposide. The inhibition of topoisomerase I by camptothecin was not affected. Our results agree perfectly with previously published reports and support the notion that topoisomerase II is also the prime target for ICRF-193 in BHK cells (24, 40).

ICRF-193 has different effects on transiently replicating plasmids containing either an HSV-1 or a SV40 origin of DNA replication. We have examined if the transient replication of plasmid molecules in BHK cells was affected by ICRF-193. Two plasmids containing either a large, very efficient version of oriS, pOS822, or a minimal oriS, pORI(wt), were used. We noted that the replication of both plasmids was inhibited by ICRF-193 (Fig. 6a). DNA replication decreased from a relative value of 100% in the absence of ICRF-193 to 12 and 16% for pORI(wt) and pOS822, respectively. Plasmid DNA replication and viral DNA synthesis were thus apparently affected to a similar extent (compare Fig. 1 and 6a). In striking contrast, we observed that the replication of a plasmid of similar size containing a SV40 origin, pELAM, in T-antigen-producing COS-1 cells was not reduced (Fig. 6b). Quantitation revealed no difference in the amounts of DNA synthesized. Our results strongly suggest that the T-antigen-dependent synthesis of plasmid DNA in COS-1 cells was unaffected by ICRF-193. This conclusion is in good agreement with previously published results (27, 28, 35).

The different effects of ICRF-193 on DNA replication from an HSV-1 origin of replication and on DNA replication from an SV40 origin of replication were not due to different properties of the cells used. In fact, HSV-1 DNA replication in COS-1 cells was found to be somewhat more sensitive to ICRF-193 than was DNA replication in BHK cells (results not shown).

DNA replication dependent on oriS is inhibited by ICRF-193 also in the absence of a viral infection. In the transient-replication assay described in the previous paragraph, all the transacting factors required for DNA synthesis were provided by the viral genome. It could be argued that the effects of ICRF-193 on plasmid replication were secondary to an effect on viral replication and transcription. We therefore performed an experiment in which the seven viral gene products were provided by a collection of expression plasmids (38). The results showed that ICRF-193 caused a strong inhibition of DNA synthesis under these conditions also (Fig. 7). Quantitation demonstrated that in the presence of the drug, the amount of DNA synthesized was reduced to 25%. If plasmid pE9, encoding the HSV-1 origin-binding protein, was excluded from the experiment, no oriS-dependent DNA synthesis was seen (Fig. 7). These results seem to indicate that inhibition of topoisomerase II by ICRF-193 directly affects the ability of plasmid molecules to participate in efficient DNA replication.

DISCUSSION

In this communication, we have presented evidence indicating that at least one cellular protein, topoisomerase II, is required for efficient replication of HSV-1. This conclusion was based on experiments with a specific inhibitor, ICRF-193. This compound converts topoisomerase II into a closed clamp that is unable to cleave DNA (36). Cells treated with ICRF-193 are able to pass through S phase in a seemingly normal way, but they cannot divide (26). The effect of the drug on cells seems to be rapid, since cells that enters mitosis 90 min after the addition of the drug show definite signs of defects in chromosome condensation and segregation (18).

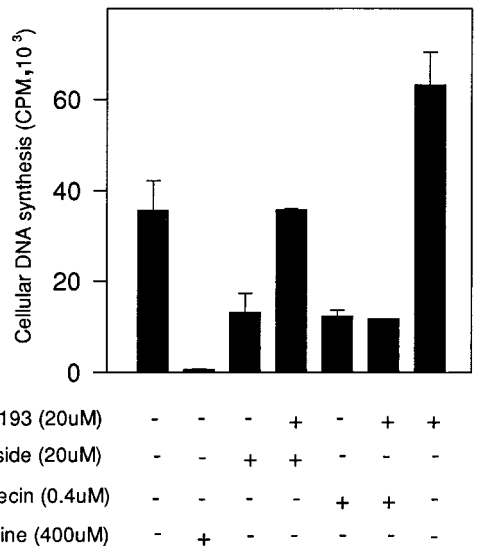

FIG. 5. ICRF-193 counteracts the effect of etoposide but not camptothecin in BHK cells. Subconfluent BHK cells were incubated for 3 h with the inhibitors as indicated in the figure. ICRF-193 was added 15 min before the other substances. Cellular DNA synthesis was measured after labelling with [^{3}H]thymidine as described in Materials and Methods.

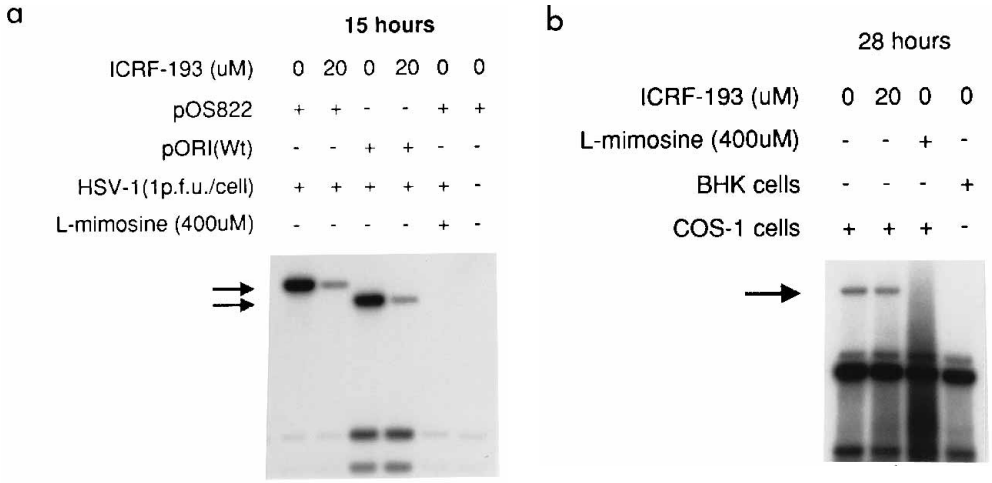

FIG. 6. Effects of ICRF-193 on transient replication of plasmids containing HSV-1 or SV40 origins of DNA replication. (a) BHK cells were subjected to lipofection with plasmid pORI(wt) or pOS822. The cells were superinfected with HSV-1 at 1 PFU per cell and incubated at 37°C for 15 h. *Dpn*I-resistant replicated plasmid DNA was analyzed by a Southern blot procedure as described in Materials and Methods. The arrows show the position of linearized replicated DNA. (b) COS-1 or BHK cells were subjected to lipofection with plasmid pELAM. The inhibitors were added as indicated. The cells were harvested at 28 h after the start of lipofection. Replicated DNA was measured as described in Materials and Methods. The arrow shows the position of linearized replicated DNA.

Our experiments revealed that ICRF-193 had to be present during the first 4 h of the infectious cycle to cause a 20- to 50-fold reduction of the amount of viral DNA produced. We also found that the inhibitory effect was most pronounced at low multiplicities of infection. The effect is apparently specific for HSV-1, since it has been demonstrated that ICRF-193 does not cause a reduced synthesis of SV40 DNA (27, 28, 35). In this context, an earlier report demonstrated that the DNA gyrase inhibitor novobiocin was able to inhibit HSV-1 DNA synthesis but only marginally affected residual cellular DNA synthesis (17).

Several explanations for our results on the role of topoisomerase II during the infectious cycle of HSV-1 should be considered. It could, for example, be argued that the inhibitor might turn topoisomerase II into a form that prevents the progression of a replication fork. We find this unlikely, since studies on the effect of ICRF-193 on the synthesis of cellular DNA as well as SV40 DNA have shown that DNA synthesis per se is not affected by the drug (26). An alternative view is that the inhibition of topoisomerase II might have unexpected effects on the execution of viral activities other than DNA replication. A related possibility is that ICRF-193 could directly interact in an unknown way with viral gene products. In this context, it should be emphasized that ICRF-193 is a very specific inhibitor that is active at low concentrations. We should point out that an inhibition of topoisomerase II caused by ICRF-193 can explain all the known effects of this compound on eukaryotic cells (11, 24–26, 40). Furthermore, we have also demonstrated that the transcription of the early genes UL8, UL9, UL29, and UL30 was not affected by the drug. Additional experiments with a reporter plasmid containing the luciferase gene under the control of an ICP4-inducible promoter showed that the drug had no effect on luciferase expression. These observations suggest that the drug affects primarily the DNA metabolism of the infected cell rather than transcription.

With the above information in mind, we will therefore limit ourselves to discussing three plausible roles for topoisomerase II during the lytic cycle of HSV-1. First, topoisomerase II might act as a swivel at the replication fork. Our experiments indicate, however, that this function can also be executed efficiently by topoisomerase I, since the addition of ICRF-193 at 6 h p.i. failed to inhibit DNA synthesis. A similar conclusion can be drawn from studies on the effect of ICRF-193 on the synthesis of cellular DNA as well as SV40 DNA. Second, topoisomerase II might be involved in the formation of viral replication compartments (9). This could perhaps be thought of as a function analogous to its putative role as a major constituent of the chromosome scaffold (13, 29). It has been demonstrated that the p170 species of topoisomerase II relocates to the replicative sites during an infectious cycle (14). The structure and function of the replicative compartments are not well understood, but it is tempting to speculate that topoisomerase II might interact directly with components of the viral replication apparatus. Finally, it has been convincingly demonstrated that topoisomerase II is essential for chromosome segregation in eukaryotic cells (10). A similar role could be required during the first part of the lytic cycle of HSV-1. It

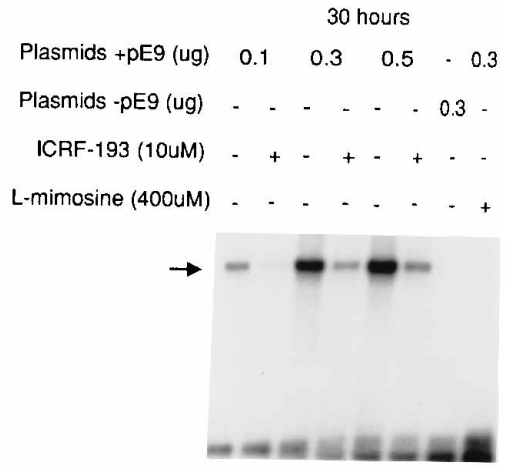

FIG. 7. Inhibition by ICRF-193 of oriS-dependent DNA replication supported by a series of plasmids encoding the HSV-1 replication proteins. BHK cells were subjected to lipofection with pORI(wt) and a mixture of the plasmids pE5, pE8, pE9, pE29, pE30, pE42, and pE52. In one experiment, pE9 was excluded as indicated. The cells were incubated for 30 h, and *Dpn*I-resistant replicated DNA was measured as described in Materials and Methods.

is conceivable that the initial replication of the viral genome during this phase will produce circular intertwined molecules. Topoisomerase II could then carry out a decatenation of the daughter molecules and allow a later phase dominated by rolling-circle replication to take place. At high multiplicities of infection, other means to provide substrates for rolling-circle replication could exist. Perhaps an increased initial copy number of viral genomes might suffice. One could also imagine that homologous recombination could convert linear molecules to efficient templates for replication.

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