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Topoisomerase II Cleavage of Herpes Simplex Virus Type 1 DNA In Vivo Is Replication Dependent

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The genome of herpes simplex virus type 1 contains a large number of recognition sites for eucaryotic DNA type II topoisomerase. Topoisomerase II sites were identified by means of the consensus sequence described previously (J. R. Spitzner and M. T. Muller, Nucleic Acids Res. 16:5553–5556, 1988) and then confirmed by sequencing DNA cleavages introduced by purified topoisomerase II. In vivo, host topoisomerase II also introduced double-stranded DNA breaks in the viral genome at sites predicted by the consensus sequence. Host topoisomerase II acted on all immediate-early genes as well as on genes from other temporal classes; however, cleavages were not detected until 4 to 5 h postinfection and were most intense at 10 h postinfection. Topoisomerase II cleavages were not detected when viral DNA replication was prevented with phosphonoacetic acid. These data indicate that, although progeny viral genomes are acted upon by host topoisomerase II, this enzyme either does not act on parental viral genomes before DNA replication or acts on them with such low efficiency that cleavages are beyond our limit of detection. The findings suggest that host topoisomerase II is involved in aspects of viral replication at late times in the infectious cycle.

Herpes simplex virus type 1 (HSV-1) relies on host proteins to replicate efficiently, yet the nature and significance of many of the interactions between cellular proteins and viral DNA are not well understood. An essential cellular protein, DNA topoisomerase II (EC 5.99.1.3), has been implicated in the replicative process of simian virus 40 (43, 56), adenovirus (4, 42), and human cytomegalovirus (1, 2). Topoisomerase II, a structural component of the nuclear matrix (3, 9, 10, 12), acts as a decatenating enzyme that separates newly replicated daughter chromosomes (7, 17, 51–53). Decatenation is possible because the active form of the enzyme, a homodimer of 170-kilodalton subunits (26, 49), makes transient double-stranded breaks in DNA. There have been intensive studies on topoisomerase II, since it is the primary intracellular target for several antineoplastic compounds such as epipodophyllotoxins (etoposide [VP16], teniposide [VM26]), acridines [4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA)], anthracyclines (adriamycin), and some fluoroquinolones (M. Muller, unpublished data) (for recent reviews, see references 8, 23, and 37).

These inhibitors arrest the reaction at the cleavage stage, most likely by prolonging the half-life of the cleaved intermediate. Consequently, upon addition of a protein denaturant such as sodium dodecyl sulfate, the DNA is severed at the site of attachment to the enzyme and topoisomerase II is covalently linked to each 5′ end (30, 54). The inhibitors are useful for tracking the activity of topoisomerase II in vivo and in vitro at the DNA sequence level; although they appear to alter the specificity of cleavage sites to a degree, inhibitors such as *m*-AMSA and VM26 accurately reflect the correct local vicinity of topoisomerase II access and activity (41, 44, 48).

Recently, we analyzed a large number of DNA sequences known to be cleaved by topoisomerase II and derived a consensus recognition sequence (44). The consensus sequence is degenerate but has value in predicting the strongest recognition sites, although the predictive value quickly falls off with lower levels of homology (45). A computer-assisted search of the HSV-1 DNA sequence revealed a number of strong matches throughout the genome. In this report we show that endogenous topoisomerase II recognizes replicating or postreplicative DNA in vivo at specific locations within the HSV-1 genome. These findings demonstrate that host topoisomerase II can act catalytically on viral DNA in vivo and imply that inhibitors of type II topoisomerases may be effective antiviral agents (22).

MATERIALS AND METHODS

Materials. Restriction enzymes and tRNA were from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). T4 polynucleotide kinase and Sequenase were obtained from U.S. Biochemical Corp. (Cleveland, Ohio). All radioactive nucleotides were from New England Nuclear Corp. (Boston, Mass.). Electrophoresis reagents were purchased from Bio-Rad Laboratories (Richmond, Calif.). Oligo-labeling kits for the generation of internally labeled probes for Southern hybridizations were from Amersham Corp. (Arlington Heights, Ill.). Phosphonoacetic acid (PAA) was purchased from Sigma Chemical Co. (St. Louis, Mo.). VM26 (NSC-122819) and *m*-AMSA (NSC-249992) were generously donated by the natural and synthetic products divisions of the National Cancer Institute (Bethesda, Md.), respectively.

Cells and virus. All cells were grown in Dulbecco modified Eagle medium supplemented with 50 μg of gentamicin per ml and either 5% bovine calf serum (HeLa and Vero cells) from Hyclone Laboratories or 5% fetal bovine serum (BHKtk$^-$ cells) from Flow Laboratories, Inc. (McLean, Va.). Cultures were incubated in 0.225% $NaHCO_3$ in open vessels in a humidified atmosphere (95% air, 5% CO_2). Stocks of HSV-1 (KOS strain) were prepared from low-multiplicity passages as described previously (28); plaque titers were determined and virus stocks were prepared as described previously (28).

Trapping topoisomerase II cleavage complexes. In vitro reactions were carried out as described previously (44). Topoisomerase was purified from two sources, chicken blood and human placenta, by existing methods (30, 44). Topoisomerase sites in HSV-1 DNA were determined on DNA sequencing gels (12% acrylamide, 8.3 M urea) with

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both sources of purified enzyme. In vivo topoisomerase II cleavages were detected as follows. The medium from infected cells was removed and replaced with 1 ml (per 100-mm dish) of serum-free medium containing 100 μM VM26 or 20 μM *m*-AMSA (43). The cultures were incubated for 30 min at 37°C, the medium was removed, and Hirt extractions were carried out (16). The DNA in the Hirt supernatant was purified by phenol extractions and ethanol precipitation and then digested with the designated restriction enzymes. The DNA samples were loaded onto agarose gels, along with molecular weight markers (5′-end-labeled lambda DNA restriction fragments); after electrophoresis, they were blotted by capillary action onto GeneScreen Plus membranes (DuPont, NEN Research Products, Boston, Mass.). The blots were then probed with a specific DNA fragment as described previously (29). Hybridization conditions and washing procedures of the blots were those recommended by the manufacturer (DuPont).

Plasmids and DNA probes. A 245-base-pair *Sst*II-*Sst*II fragment of p111 (36) extending from positions −111 to −356 relative to the transcriptional start site of immediate-early gene 1 (IE-1) was subcloned into the *Sst*II site of Bluescript Plus (Stratagene) to create pObs2a. This plasmid was digested at the *Bam*HI site (in the vector) and subjected to 5′ end labeling as follows. The linearized plasmid DNA was incubated with [γ-^{32}P]ATP and T4 polynucleotide kinase followed by *Sst*I restriction and isolation of the labeled DNA from a preparative polyacrylamide gel. Topoisomerase II cleavages were mapped on this fragment by using purified topoisomerases and DNA sequencing as described previously (30, 44). The following hybridization probes were used to map topoisomerase II cleavages in vivo by indirect end labeling (also see Fig. 8): IE-1, *Kpn*I-*Xho*I 321-base-pair fragment from p111 (36); IE-2, *Sst*I-*Sma*I 271-base-pair fragment from pMC150 (55); IE-3 and IE-4/5, *Hin*dIII-*Acc*I 371-base-pair fragment from pJD1 (27). Unlabeled DNA used for in vitro cleavages consisted of the 2.4-kilobase *Sst*I-*Bam*HI IE-2 fragment from pMC150 (55).

RESULTS

Distribution of the topoisomerase II recognition sites in HSV-1 DNA. The topoisomerase II consensus sequence spans 18 nucleotides, among which 8 can be any nucleotide. The consensus sequence is as follows (44):

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5′ R  N  Y  N  N  C  N  N  G  Y N G K T N Y N Y 3′,
−10 −9 −8 −7 −6 −5 −4 −3 −2 −1 1 2 3 4 5 6 7 8
```

where R is purine, Y is pyrimidine, K is G or T, N is any base, and cleavage is between −1 and +1. A perfect match to the above consensus sequence corresponds to a 10-out-of-10 (100%) match to the non-N base positions (i.e., −10, −8, −5, etc.) and a 90% match would be a 9-out-of-10 homology score. Both 100 and 90% homology scores on one strand predict strong topoisomerase II cleavages when the opposite strand possesses a 50% or better match. Note that opposite strand cleavage is 4 bases 3′ to top-strand cleavage; thus, cleavage products contain a 4-base-pair 5′ extension, and the terminal base possesses a 5′ phosphate.

There are 39 100% matches (about half of which are predicted to be strong sites based on opposite strand homology) and 593 90% matches to the consensus sequence in the HSV-1 genome (the pattern for 90% matches closely reflects that of the 100% matches). This corresponds to an average of one 90% or better match site every 241 base pairs. These values approximate the expected frequency of sites based on genome size and G+C content of HSV-1 (558 90% or better matches for an average of one site every 272 base pairs). Figure 1A shows the distribution of 100% matches to the topoisomerase II consensus sequence across the HSV-1 genome. One of these 100% match sites is located in the upstream regulatory region of IE-1 (ICP0), and there are two additional strong consensus matches nearby (Fig. 1B). These sites flank the alpha-*trans*-inducing factor binding sequence (AT site, Fig. 1B) upstream of IE-1 (13, 33). The 100% site (60% homology on the opposite strand) at position −131 of IE-1 was the predominant cleavage site detected in this fragment (Fig. 3C). This fragment ran slightly slower than the closest guanine, indicating that the cleavage site was to the 5′ side of the guanine. Note that, because the topoisomerase II cleavage fragment has a 3′ hydroxyl, its electrophoretic mobility is slightly different than the chemical sequence marker that contains a 3′-phosphoryl terminus. Note also that cleavages were not detected in the absence of drug (Fig. 1C, lane 4), because of the low amounts of purified human topoisomerase II that were employed. A similar situation occurs when topoisomerase II cleavages are mapped in vivo (see below) (29); thus, the experiment in vitro (Fig. 1C) reflects this. Additional cleavage sites that correspond to lower homology sites can be seen in this fragment (above the site marked in Fig. 1C). These sites map as a cluster in the immediate vicinity of the AT site (TAATGARAT). Since this cluster of cleavages occurs at weak homology sites, the consensus sequence may not be sufficient by itself to predict all sites, but clearly the strongest site, at least in this fragment of DNA (Fig. 1B and C), does cut at the 100% match site. This region of IE-1 has enhancer activity (S. Ebert and M. Muller, unpublished observations); therefore the presence of strong topoisomerase II sites is not surprising, since we previously showed that enhancer regions contain topoisomerase II sites at above-average frequencies (44).

Mapping double-strand DNA cleavages in vivo. The presence of topoisomerase II recognition sites in HSV-1 suggests that endogenous cellular topoisomerase II may be catalytically active on the viral genome. To test this idea, experiments were carried out to determine whether topoisomerase II could be trapped in a covalent complex with HSV-1 DNA in vivo. Inhibitors that arrest the DNA-topoisomerase II cleavage intermediate were then used to map these sites in infected cells by the indirect end-labeling procedure (29). This method allows mapping of cleavage sites outside of diploid regions of the viral genome; additionally, from this type of analysis it is not possible to distinguish between cleavages in one as opposed to both repeat regions. A series of experiments was first performed to optimize the conditions for detecting endogenous topoisomerase II cleavages on viral DNA. Variables such as cell types, cellular growth state, type and concentration of drug, length of treatment, and time postinfection were evaluated (data not shown). Two inhibitors, *m*-AMSA (an intercalator) and VM26 (a nonintercalator) were shown to be effective at 20 and 100 μM, respectively; however, VM26 consistently yielded more intense cleavage products compared with *m*-AMSA. Thus, VM26 was judged to be superior to *m*-AMSA for trapping endogenous topoisomerase II cleavages on viral DNA in infected cells. Finally, our ability to detect topoisomerase II cleavage bands was significantly enhanced by using viral DNA recovered from the Hirt supernatant; however, we confirmed that the same results were seen with total infected cell DNA as well as DNA isolated from the Hirt pellet (data not shown).

The cleavage pattern produced by endogenous topoisom-

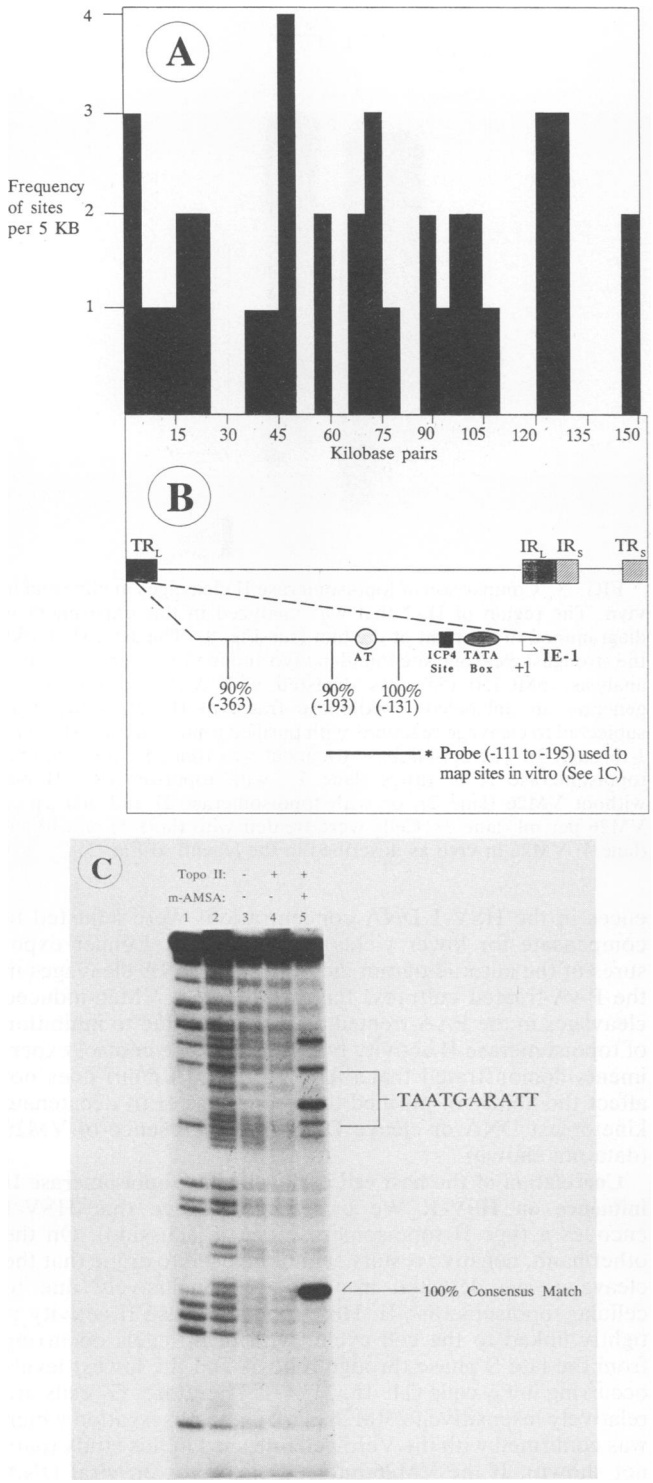

FIG. 1. Distribution of topoisomerase II consensus sites in the HSV-1 genome. (A) Consensus sequence matches were determined at each nucleotide. The histogram represents the total number of perfect match sites per 5 kilobases (KB). (B) Schematic representation of the HSV-1 genome with an expanded view of the regulatory-promoter region of the IE-1 gene. The vertical lines with percentages represent the percent match at those positions to the consensus sequence and the AT element represents a TAATGA RAT binding site for the alpha-*trans*-inducing factor (13, 33). (C) Results of cleavage reactions in vitro with purified avian topoisomerase II. The end-labeled fragment extending from positions −111 to −195 (B) was used for the cleavages in vitro. The reaction products were analyzed on a 12% sequencing gel. Lanes: 1 and 2, Maxam and Gilbert G and G+A sequencing ladders of the fragment; 3, control, no topoisomerase and no drug; 4 and 5, reactions with topoisomerase II, without and with topoisomerase II inhibitor, respectively.

erase II on HSV-1 DNA was essentially the same in three different cell types (Fig. 2), indicating that these events are not unique to any of these cell types. HeLa cells did, however, consistently produce more efficient cleavages than the other cell lines tested. Treatment with *m*-AMSA did not induce double-strand cleavages in vivo in this experiment; however, this inhibitor tends to stimulate single-strand cleavages with topoisomerase II (30) which are not detectable by the indirect end-labeling assay. Double-strand breaks were clearly observed in cells treated with VM26. The predominant cleavage site represents a maximum of 2% of the total DNA loaded per lane (as quantified by liquid scintillation counting of the Southern blots) and maps close to one or more of the strong consensus match sites shown in Fig. 1B. Due to the lower resolution of agarose gels, tightly clustered sites detected on sequencing gels appear as a single intense band on Southern blots (44).

We next made direct comparisons between DNA cleavages mapped in vivo (Fig. 2) and cleavages mapped in vitro with purified enzyme. All IE genes were analyzed in several different experiments as follows. The DNA substrates used for the topoisomerase II cleavage reactions in vitro were purified by restriction digestion followed by polyacrylamide gel electrophoresis and electroelution of the desired fragment. For the in vivo samples, viral DNA from VM26-treated cells was restricted with the desired enzyme. Southern blotting was performed, and indirect end labeling was used to detect cleavage patterns. Since the same sequence is represented in vivo and in vitro, cleavage bands can be compared directly in the same gel with the same probe. With all of the IE genes, the most prominent sites in vitro show identity with the strongest sites detected in vivo. A representative experiment comparing sites in IE-2 (ICP27) is shown in Fig. 3. Note that the sites mapped in vivo appear to be a subset of the more plentiful in vitro sites. The endogenous sites in IE-1 and IE-2 flanked protein-coding regions. The sites in IE-3 (ICP4), however, tended to be clustered in intragenic regions as well as regions 5′ and 3′ of coding sequence (Fig. 4).

Cleavages are dependent on viral DNA replication. A time-course analysis was performed to determine the relationship between topoisomerase II cleavages and DNA replication. A probe was selected that would identify sites in the regulatory regions of IE-3 and IE-4/5 as well as at an origin of DNA replication (Ori_S) (Fig. 4). Cleavages in this region were not detectable at 3.25 h postinfection (lanes 1 and 2, Fig. 4) and were barely detectable at 5.5 h (lanes 3 and 4); however, the cleavages were clearly evident at later times (lanes 5 through 8) in the infectious cycle.

The data in Fig. 4 suggest that topoisomerase II may not act on viral DNA before the onset of viral DNA synthesis; however, the lack of detectable cleavages at early times might have been the result of reduced DNA concentrations. To resolve this issue, more DNA was loaded per lane at the early time points relative to that loaded at later times (Fig. 5: 3.25 h, 50 μg per lane; 5.5 h, 20 μg per lane; 10 h, 2 μg per lane; 19 h, 0.5 μg per lane). This adjustment in DNA concentration was judged to be sufficient based on amounts

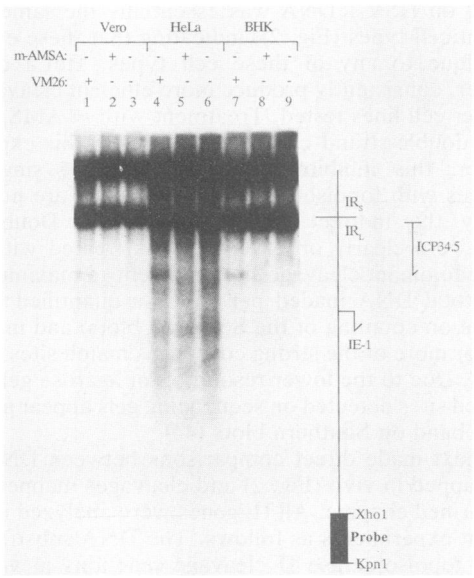

FIG. 2. Topoisomerase II cleavages of HSV-1 DNA in three cell lines. Cells were infected at an input multiplicity of 25 and were treated with 20 μM *m*-AMSA or 100 μM VM26 in 1 ml of serum-free medium per 100-mm petri dish from 10 to 10.5 h postinfection. Viral DNA was harvested by Hirt extraction, purified, digested with *Kpn*I, loaded onto a 1.5% agarose gel (25 μg per lane), and analyzed on a Southern blot (25). The blot was probed with the 321-base-pair *Kpn*I-to-*Xho*I fragment from the IE-1 gene as designated in the schematic representation shown to the right of the autoradiogram (see Fig. 8). The map positions are given for the genes that encode ICP34.5 (5) and ICP0 (IE-1) (36) as well as the boundary between the long (IR_L) and short (IR_S) inverted repeat regions. Lanes: 3, 6, and 9, control lanes where the cells were not exposed to any drugs; 2, 5, and 8, cells treated with 20 μM *m*-AMSA; 1, 4 and 7, cells treated with 100 μM VM26; 1 through 3, Vero cells; 4 through 6, HeLa cells; 7 through 9, BHKtk$^-$ cells.

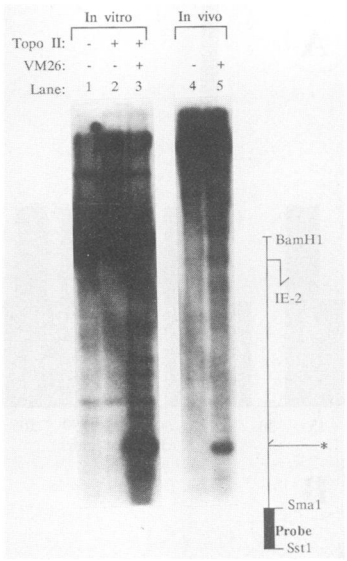

FIG. 3. Comparison of topoisomerase II cleavages in vitro and in vivo. The region of IE-2 that was analyzed in this experiment is diagrammed to the right of the blot (see Fig. 8). The asterisk marks the strongest cleavage site found in vivo and in vitro. For the in vitro analysis, pMC150 (55) was digested with *Sst*I and *Bam*HI to generate an unlabeled 2.4-kilobase fragment (IE-2), which was subjected to cleavage reactions with purified topoisomerase II (lanes 1 through 3). The 2.4-kilobase fragment was treated either without topoisomerase II or drugs (lane 1), with topoisomerase II but without VM26 (lane 2), or with topoisomerase II and 500 μg of VM26 per ml (lane 3). Cells were treated with (lane 5) or without (lane 4) VM26 in vivo as described in the legend to Fig. 2.

of HSV-1 DNA detected per lane on the Southern blot at the early times as compared with those at the later times (Fig. 5). The first detectable topoisomerase II cleavages appeared between 4 and 5 h postinfection, but the efficiency of cleavage at this time was less than approximately 0.1% of the total DNA loaded per lane as measured by liquid scintillation counting of the bands (Fig. 4 and 5; unpublished observations). Even when input DNA was concentrated under these conditions, VM26-dependent cleavage bands were not observed at 3.25 h postinfection (Fig. 5), thereby indicating that the cleavage efficiency was below our limit of detection (i.e., less than approximately 0.04% of total when 50 μg of DNA was loaded per lane). Topoisomerase II-mediated cleavages were easily seen at later times, even though there was significantly less total HSV-1 DNA loaded per lane. The cleavage efficiency increased at least 50- to 100-fold from 3 to 10 h postinfection. Hence, topoisomerase II cleavages closely parallel the onset of viral DNA replication (39, 40).

We next addressed whether endogenous topoisomerase II was able to recognize and cleave parental viral DNA molecules. Infected cells were treated with PAA to prevent the onset of viral DNA synthesis (34). The cells were then treated with VM26 from 10 to 10.5 h postinfection. Inhibition of DNA replication by PAA totally prevented detectable topoisomerase II cleavage of HSV-1 DNA in the IE-1 region (Fig. 6). This same region contained one major and several minor topoisomerase II sites in non-PAA-treated cells (lane 2, Fig. 6). As in the time-course experiment (Fig. 5), differences in the HSV-1 DNA concentrations were adjusted to compensate for lower yields of viral DNA. Longer exposures of the autoradiogram did not reveal DNA cleavages in the PAA-treated cultures. Failure to detect VM26-induced cleavages in the PAA-treated cells was not due to inhibition of topoisomerase II activity by PAA, because control experiments demonstrated that PAA (up to 600 μg/ml) does not affect the ability of purified topoisomerase II to decatenate kinetoplast DNA or cleave DNA in the presence of VM26 (data not shown).

Correlation of the host cell cycle with the topoisomerase II influence on HSV-1. We have no evidence that HSV-1 encodes a type II topoisomerase (see Discussion). On the other hand, negative results cannot be used to argue that the cleavages we detected in vivo are conclusively due to cellular topoisomerase II. Host topoisomerase II activity is tightly linked to the cell cycle, with peak levels occurring from the late S phase through mitosis and the lowest levels occurring in G_1 cells (11, 15, 31, 47). Therefore, G_1 cells are relatively insensitive to VM26 (47, 57), an observation which was confirmed with the Vero cell line used in this study (data not shown). If the VM26-induced cleavages on viral DNA were due to host topoisomerase II, we would predict that cleavage efficiency in vivo would be reflected by the amount of topoisomerase II present in the cells. We consistently observed that the efficiency of VM26-dependent cleavage of viral DNA was dependent upon the cell cycle status of the host. G_1-arrested cells have low levels of topoisomerase II activity and display higher tolerance to VM26 inhibition relative to exponentially growing Vero cells (data not shown). Since cleavages are dependent upon viral DNA replication (see above), decreased replicative activity of

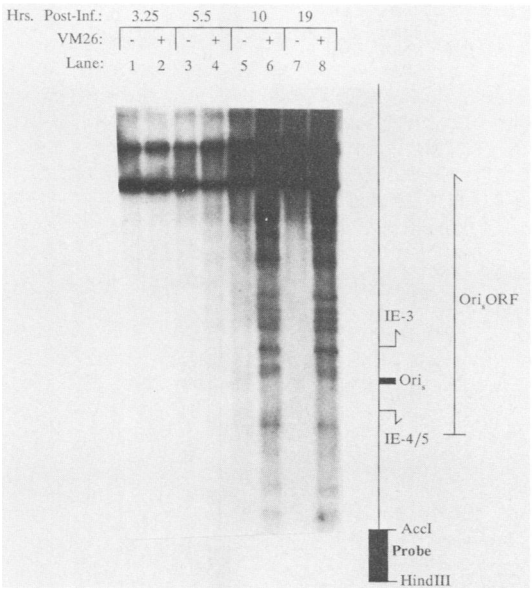

FIG. 4. Analysis of VM26-enhanced topoisomerase II cleavages at various times after infection. Cleavages in infected cells were mapped as described in the legend to Fig. 2, except that viral DNA was harvested at the designated times postinfection, digested with *Hin*dIII, Southern blotted, and probed with a 371-base-pair *Hin*dIII-*Acc*I fragment from plasmid pJD1 (27). To the right is shown a partial restriction map of the region tested, including the probe used in the experiment, the regulatory-promoter regions of IE-3, -4, and -5, and the positions of Ori$_S$ and the Ori$_S$ open reading frame (Ori$_S$ ORF) (19).

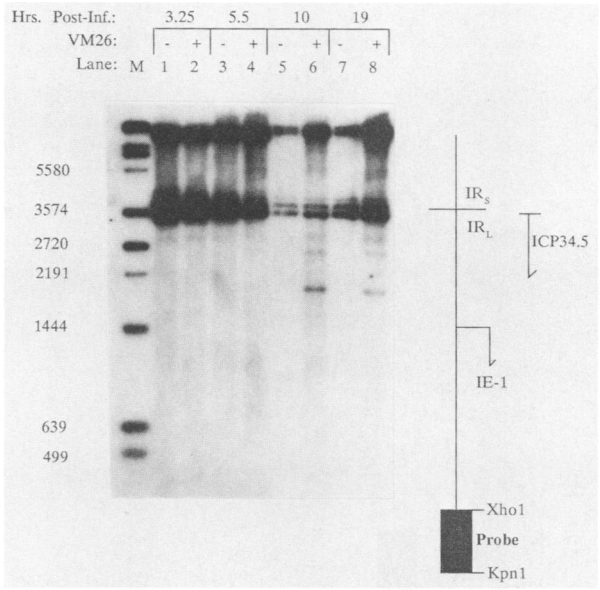

FIG. 5. Time-course analysis with adjustment for differential template concentrations. Cleavages in vivo were mapped as described in the legend to Fig. 2, except harvests were carried out at the times designated. The amount of DNA loaded at early time points was concentrated relative to the amount loaded per lane at late times as follows (lanes): 1 and 2, 50 μg; 3 and 4, 20 μg; 5 and 6, 2 μg; 7 and 8, 0.5 μg. Lane M contains size marker DNA consisting of end-labeled lambda *Acc*I fragments.

HSV-1 in G_1 cells should produce a proportional decrease in topoisomerase II cleavages; however, viral DNA replication was reduced by no more than a factor of 5- to 10-fold in G_1-arrested Vero cells, whereas topoisomerase II cleavages were suppressed by 100-fold or more (Fig. 7). Thus, G_1 cells exhibit a cleavage efficiency that is estimated to be 10-fold down from that of exponentially growing cells.

DISCUSSION

Topoisomerase II consensus sequences are distributed throughout the HSV-1 genome, and some of these sites are recognized and cut by topoisomerase II both in vivo and in vitro. Endogenous cellular topoisomerase II can recognize and access viral DNA sequences during the course of the infection. The following pieces of evidence support our assertion that cellular topoisomerase II is responsible for the cleavages that we mapped by indirect end labeling. (i) The cleavages detected in vivo are dependent upon the addition of agents that stabilize the cleavage complex between topoisomerase II and DNA. The inhibitors are quite specific for topoisomerase II (8, 23, 37), and we found that the epipodophyllotoxins VP16 and VM26 were more effective than the intercalator *m*-AMSA at arresting the double-stranded cleavage complex. (ii) The cleavage sites detected in vivo aligned closely, if not identically, with cleavages generated by purified cellular topoisomerase II on HSV-1 DNA. (iii) The cleavages detected in infected cells correspond to sites predicted by the eucaryotic consensus sequence, at least within resolution of agarose gel electrophoresis. (iv) Topoisomerase II activity was partially purified from mock-infected and infected cell extracts by fractionation over BioRex 70, phenyl Sepharose, and Superose 6 columns. Novel activities were not detected from the infected extracts, and the partially purified topoisomerase was identical from both infected and uninfected sources (32; unpublished observations). (v) Concentrations of topoisomerase II inhibitors that reduce viral yields by 2 to 3 orders of magnitude are similarly cytotoxic to the host cells (data not shown). (vi) We observed a very similar cleavage pattern in vivo for three distinct cell types representing three separate species. This observation is consistent with the fact that the topoisomerase II consensus sequence is conserved among vertebrates (44). (vii) Cleavage efficiencies were higher in transformed (HeLa) cells, which are known to have more topoisomerase II activity than nontransformed cells (31, 46, 47). (viii) Finally, we observed that in exponentially growing cells (possessing high levels of topoisomerase II activity), VM26-dependent cleavages were at least 10-fold more efficient than those in G_1 cells. Additionally, in HSV-2, levels of topoisomerase I and II were unchanged up to 10 h postinfection, and attempts to isolate HSV-2 mutants that were resistant to the epipodophyllotoxin VP16 were uniformly negative (32). Nishiyama et al. concluded that HSV-2 utilizes host topoisomerase II (32).

Topoisomerase II access to binding sites in vivo is clearly different from the in vitro situation, in which DNA is not hindered by other DNA-protein interactions. Some sites, which are potentially strong sites on the basis of sequence comparison, may be occluded in vivo, and other weaker consensus matches may actually be strong sites when the local environment surrounding that sequence is taken into account (29). As noted in other systems (29, 50, 56), the sites observed in vivo appear to be a subset of the sites observed in vitro.

Figure 8 summarizes the cleavages mapped in vivo in this

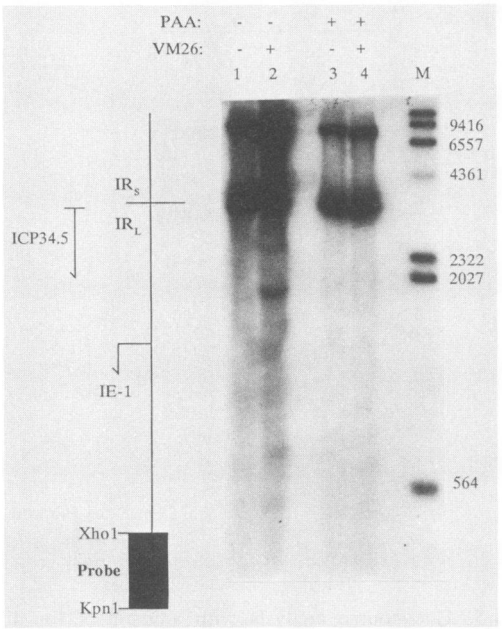

FIG. 6. Effect of inhibition of HSV-1 DNA replication on topoisomerase II cleavages in vivo. PAA (300 μg/ml) was added to infected HeLa cells after a 1-h adsorption period. The infection was allowed to proceed for 10 h in the presence of PAA, at which time the cultures were treated with VM26 and harvested for topoisomerase II cleavages in vivo as described in the legend to Fig. 2. Template concentration was compensated for by increasing the amount of DNA loaded per lane from PAA-treated cells (lanes 3 and 4, 25 μg per lane) relative to that in untreated cells (lanes 1 and 2, 0.25 μg per lane). Longer exposures of the autoradiogram did not reveal cleavages from cells treated with PAA. Lane M contains size markers consisting of end-labeled lambda *Hin*dIII fragments.

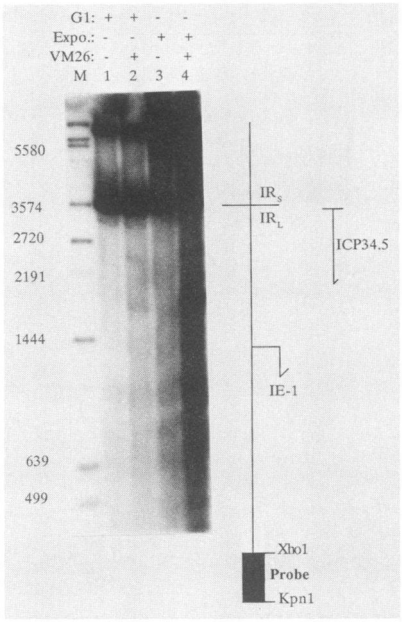

FIG. 7. Topoisomerase II cleavages in G_1 cells. G_1-arrested Vero cells (lanes 1 and 2) were obtained by decreasing the serum concentration on freshly confluent monolayers from 5 to 0.5% bovine calf serum for at least 3 days before infection. After the infection, the spent medium was reapplied. Exponentially growing Vero cells (lanes 3 and 4) were split 1:5 from freshly confluent cells in medium containing 5% bovine calf serum 20 h before infection. M contains size markers consisting of end-labeled lambda *Acc*I fragments. Cleavages were mapped in vivo as outlined in the legend to Fig. 2.

paper. Both IE and non-IE gene regions exhibited topoisomerase II cleavages. Although the significance of topoisomerase site distribution in HSV-1 is not clear at present, it is noteworthy that the strongest sites in infected cells are frequently near coding and noncoding junction regions. IE-3 was unique in exhibiting additional sites in its coding region. Topoisomerase II sites located within coding regions were also reported in adenovirus, and, as reported here for HSV-1, the intensity of endogenous topoisomerase II cleavages closely parallels viral DNA replication (42). The two viruses differ in that low levels of topoisomerase II cleavages were detected in adenovirus before viral DNA replication, whereas we were unable to detect sites before DNA replication in HSV-1. We conclude that host topoisomerase II is not active on parental (input) HSV-1 DNA either because it is shielded from topoisomerase II activity or because (for reasons unknown) the high-affinity binding sites are not recognized by topoisomerase II.

Due to the clustering of topoisomerase II sites in IE regulatory regions, there was reason to believe that interaction at these positions might be important in IE transcription. Although this possibility has not been completely eliminated, it seems doubtful, because topoisomerase II does not detectably interact with these sites until long after IE genes have been expressed at maximum levels (6, 18, 20, 24). Furthermore, the topoisomerase inhibitors do not block IE gene expression (S. Ebert and M. Muller, unpublished observations).

Recent reports have shown that topoisomerase II inhibitors block replication of human cytomegalovirus DNA (1, 2). In the present study, cytotoxic (but still reversible) concentrations of VM26 (4 μM) significantly reduced the quantity of infectious HSV-1 produced in culture, and preliminary data suggest that inhibition is at the level of DNA replication. The data imply a role for topoisomerase II in HSV-1 replication (possibly decatenation of daughter DNA molecules). One interpretation for the lack of topoisomerase II action on prereplicated HSV-1 DNA is that, during DNA replication, a massive reorganization of nuclear components occurs which releases topoisomerase II from matrix sites into the nucleoplasm, where it can react with a large number of viral genomes. Another possibility is that viral genomes undergo subnuclear relocalization during replication, which makes their DNA accessible to topoisomerase II. Support for the latter possibility comes from several reports, which suggest that nuclear relocalization of the viral DNA correlates with HSV-1 DNA replication (14, 21, 38). In any case, the interaction between topoisomerase II and DNA only becomes apparent when infected cells are exposed to agents that stabilize the cleavage intermediate. In the absence of agents like VM26, topoisomerase II may fleetingly bind viral DNA; however, this drug makes the enzyme-DNA interaction permanent, and the resulting DNA breaks are detrimental to the virus.

In summary, we have evidence that cellular topoisomerase II can induce double-strand breaks at specific locations in viral DNA under the influence of antitumor drugs, which can decrease the yield of infectious progeny. These findings justify further studies on use of topoisomerase II inhibitors not only as clinically useful antiviral agents but also as tools in the effort to develop an understanding of the interactions between viral DNA and cellular DNA-binding proteins.

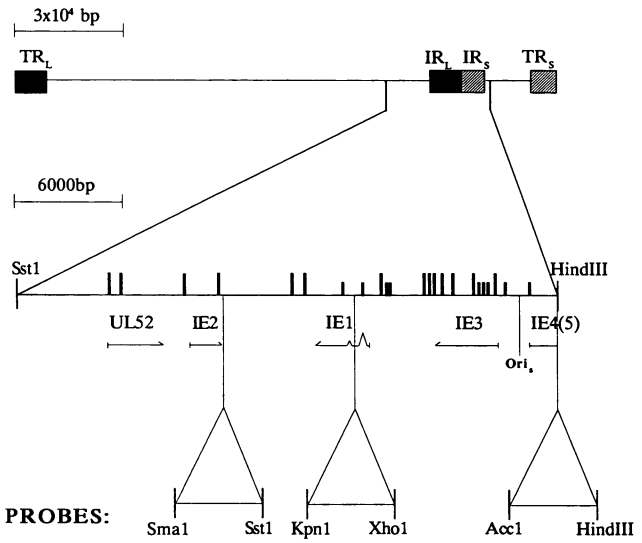

FIG. 8. Summary of topoisomerase II cleavage sites mapped in HSV-1 in vivo. The region of the genome between the *Sst*I site at position 103808 and the *Hin*dIII site at position 133466 (numbering system adopted from Perry and McGeoch [35]) is expanded, and the locations of strong cleavage sites (tall vertical lines) and weaker cleavage sites (shorter vertical lines) are shown. The probes used to map these sites are illustrated (for details about actual probe sizes and plasmids from which they were derived, see Materials and Methods). The map of the genes diagrammed here represents open reading frames.

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