Topoisomerase I and II Cleavage of Adenovirus DNA In Vivo: Both Topoisomerase Activities Appear To Be Required for Adenovirus DNA Replication

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Sites of topoisomerase I and II cleavage across large portions of the adenovirus type 5 genome were mapped by using the drugs camptothecin and VM26, respectively. These drugs prolong the half-lives of the covalent DNA-protein intermediates in which the DNA is transiently cleaved, and so treatment with protein denaturants after exposure to the drugs leads to DNA strand scission at the site of topoisomerase cleavage. Strong topoisomerase II cleavage sites occurred in clusters throughout the regions examined, including both transcribed regions and transcriptional control regions. The efficiency of topoisomerase II cleavage increased as the rate of adenovirus DNA replication increased and then decreased with the decreasing rate of replication late in the infection cycle. The increase was not dependent on expression of the E1A gene, whose products activate transcription of the early viral genes. Positions of topoisomerase II cleavage sites did not vary during the infection. Topoisomerase I cleavage sites were also found throughout the examined regions, with the strongest sites occurring near the ends of the transcription units. Topoisomerase I cleavage in the E1 region occurred much more frequently than topoisomerase II cleavage, was not dependent on E1A gene expression, and remained at a similar level from the early viral phase into the late viral phase. Treatment of infected cells with either drug prevented efficient replication of adenovirus DNA. Inhibition of topoisomerase I activity led to an immediate cessation of adenovirus DNA replication, while inhibition of topoisomerase II blocked replication only after completion of approximately one additional round.

DNA topology is regulated by two enzyme activities. Topoisomerase I in eucaryotes occurs as a monomeric protein of approximately 110 kilodaltons, does not require cofactors, and relaxes supercoiled DNA in a process involving transient cleavage of one DNA strand. Strand cleavage occurs with concerted transfer of the DNA phosphoester bond to the protein. Eubacterial topoisomerase I relaxes only negatively supercoiled DNA, while eucaryotic topoisomerase I relaxes both positively and negatively supercoiled DNA. Topoisomerase II occurs as an α_2/β_2 tetramer in *Escherichia coli* (DNA gyrase) and as a homodimer of 170-kilodalton subunits in eucaryotes. The eucaryotic subunit appears to have evolved through fusion of the genes encoding the α and β subunits from eubacteria. Topoisomerase II exhibits an absolute requirement for Mg^{2+} and ATP. The enzyme from *E. coli* and from other eubacteria can introduce negative supercoils into DNA, while the eucaryotic enzyme has been shown to relax only supercoiled DNA. Enzymes from both types of organism transiently cleave both strands of the DNA, with concerted transfer of the phosphoester bonds to the protein (for reviews, see references 21, 41, and 42).

Topoisomerases I and II both have the potential to play roles in DNA replication. Studies of simian virus 40 (SV40) replication in vivo (26, 33) and in vitro (25, 32, 43, 46) and genetic studies of *Saccharomyces cerevisiae* (6, 11, 14, 35) and *Schizosaccharomyces pombe* (38–40) have demonstrated that the presence of either topoisomerase I or topo-

isomerase II activity permits elongation. However, only topoisomerase II can function at the terminal steps of replication, because of the requirement for double strand passage in the condensation and separation of daughter molecules.

Genetic analysis of topoisomerase functions is not readily feasible in mammalian cells. However, these functions can be analyzed by using drugs which specifically inhibit topoisomerases. Camptothecin inhibits topoisomerase I (15), and VM26 inhibits topoisomerase II (3, 20, 23, 44, 45), in both cases by prolonging the half-life of the covalent DNA-protein cleavage intermediate. As a result, these drugs can be used to probe topoisomerase functions and to identify sites of cleavage by the topoisomerases through denaturation of the proteins after drug treatment, followed by analysis of the sites of strand scission. This method has permitted the mapping of topoisomerase II sites in vitro and in vivo in SV40 (19, 45), *Drosophila* heat shock and histone genes (12, 29, 30, 36, 37), *c-myc* (27, 28), and *c-fos* (5) and the in vivo mapping of topoisomerase I sites in *Drosophila* heat shock genes (9), the rat tyrosine amino transferase gene (34), human rRNA genes (47), and SV40 DNA (16).

In this study, we have used camptothecin and VM26 to examine the sites of cleavage by the topoisomerases on the adenovirus type 5 (Ad5) chromosome, the relationship between the sites and the stage of the infection, and the role of topoisomerases in viral DNA replication. Topoisomerase I cleavage sites were found throughout the E1 region. Cleavage varied little as a function of the rate of replication and was not responsive to the E1A protein, which activates transcription. Topoisomerase II cleavage sites were found in clusters throughout the regions examined. Cleavage by this enzyme increased dramatically with increasing rate of viral

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DNA replication. Both camptothecin and VM26 inhibited Ad5 DNA replication.

MATERIALS AND METHODS

Cells and viruses. All assays were performed with HeLa suspended-cell cultures. HeLa cells were grown in medium containing 5% calf serum or 10% horse serum. Cells at a concentration of 5×10^6 /ml were infected with Ad5 at a multiplicity of 25 PFU per cell, except where noted, for 30 min at 37°C and then diluted 10-fold with medium containing 5% calf serum or 10% horse serum. The viruses used were *dl309*, a phenotypically wild-type Ad5 mutant (17), and *dl343*, an E1A⁻ derivative of *dl309* with a 2-base-pair deletion near the 5' end of the E1A coding region (13).

Topoisomerase inhibitors. Camptothecin, an antitumor drug which inhibits topoisomerase I (15), and the epipodophylotoxin VM26, an antitumor drug which inhibits topoisomerase II (3, 20, 23, 44, 45), were each dissolved in dimethyl sulfoxide and stored at -20°C. The drugs were added to cultures of Ad5-infected HeLa cells at the concentrations noted, and the cultures were incubated with stirring at 37°C for 15 min for in vivo studies. For in vitro studies, Ad5-infected cells were pelleted, washed with phosphate-buffered saline, and lysed in hypotonic buffer containing 0.5% Nonidet P-40. The nuclei were then suspended in buffer A* (60 mM KCl, 15 mM NaCl, 15 mM Tris hydrochloride, pH 7.5, 0.3 mM spermine, 1 mM spermidine, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 0.25 M sucrose [37]), with 5 mM MgCl₂ and 1.5 mM ATP added as noted, and treated with VM26 for 15 min at 37°C as noted.

Determination of sites of topoisomerase cleavage. Camptothecin and VM26 inhibit topoisomerase I and II, respectively, by prolonging the half-lives of the covalent DNA-protein intermediates. In these intermediates, the DNA is cleaved (a single strand by topoisomerase I and both strands by topoisomerase II). Thus, denaturation of the protein after treatment with the drugs results in DNA strand scission, with covalent attachment of a protein moiety at the cleavage site (3, 15, 20, 44, 45). To determine the site of cleavage, infected cells or nuclei were treated with the drugs and then lysed by the addition of sodium dodecyl sulfate (SDS) to 1%. DNA was purified by digestion with proteinase K (100 µg/ml) overnight at 37°C, followed by phenol extraction, three successive ethanol precipitations, and digestion with RNase A. The relative concentration of Ad5 DNA in each sample was determined by slot-blot analysis of appropriate dilutions of denatured DNA bound to nitrocellulose by using as a probe the entire Ad5 chromosome, which was labeled with ³²P (7). For analysis of double strand cleavage, the same quantity of Ad5 DNA from each sample was digested with a restriction endonuclease, resolved by electrophoresis on 20- or 40-cm agarose gels, denatured, transferred to nitrocellulose, and indirectly end labeled with specific Ad5 DNA fragments labeled with ³²P to high specific activity (7). To analyze the left end of the viral genome (from 0 to 7.8 map units [m.u.]) and the major late promoter region (7.8 to 16.9 m.u.), the DNA was digested with *Hind*III and probed with the ³²P-labeled fragments from 5.7 (*Kpn*I site) to 7.8 m.u. (*Hind*III site) and from 7.8 (*Hind*III site) to 9.2 m.u. (*Bgl*II site), respectively. To analyze the right end of the viral genome, the DNA was digested with *Eco*RI and probed with the ³²P-labeled fragment from 75.9 (*Eco*RI site) to 77.9 m.u. (*Bgl*II site). For analysis of single strand cleavage, the DNA was denatured after restriction endonuclease digestion and resolved by electrophoresis on 20-cm agarose gels

containing 40 mM NaOH and 5 mM EDTA. To probe individual strands, ³²P-labeled cRNA of high specific activity was synthesized by using SP6 or T7 RNA polymerase (22) to transcribe the appropriate Ad5 DNA fragment for indirect end labeling.

RESULTS

The mechanism of action of both topoisomerases I and II involves the transient formation of DNA strand scissions, with the formation of covalent DNA-protein adducts at the site of cleavage. In the case of topoisomerase I, the protein is attached to a 3' phosphate, while for topoisomerase II the protein is attached to a 5' phosphate (for reviews, see references 21, 41, and 42). Thus, denaturation of the topoisomerase while it is in the covalent DNA-protein intermediate offers the possibility of localizing sites of interaction of the topoisomerases with DNA. Given the short half-lives of these intermediates, only a very low level of cleavage can normally be seen. However, drugs which inhibit the topoisomerases by prolonging the half-lives of the DNA-protein covalent intermediates can be used to increase the frequency of strand scissions (9, 45). This approach was followed here to map the cleavage sites of topoisomerases I and II on Ad5 DNA.

Effects of the topoisomerase II inhibitor VM26 on cleavage of Ad5 DNA. In order to establish that Ad5 DNA cleavage induced by the epipodophylotoxin VM26 occurs through inhibition of the religation step by topoisomerase II, the cofactor requirements and cleavage products were examined. VM26-stimulated DNA cleavage within nuclei prepared from cells 12 h after infection with Ad5 (at which time, under the conditions used here, the viral DNA has been replicated 100- to 200-fold) was assayed for its requirement for ATP and Mg²⁺, cofactors required for topoisomerase II activity. Very little cleavage was observed in vitro in the absence of exogenous ATP, or in the presence of EDTA, relative to the presence of both ATP and Mg²⁺ (Fig. 1). Cleavage of Ad5 DNA induced in vivo by VM26 was reversed when the cells were lysed with Nonidet P-40 and the nuclei were incubated with EDTA before the addition of SDS. Finally, DNA from VM26-treated Ad5-infected HeLa cells was purified by pelleting through a sucrose gradient, cleaved with *Hind*III, and assayed for mobility of the Ad5 DNA fragment from 7.8 to 16.9 m.u. in denaturing agarose gels with and without digestion with proteinase K. Only the fragments with 5' ends generated by the VM26-stimulated cleavage were retarded in mobility in the absence of protease treatment, indicating the presence of a covalently attached protein (data not shown). Thus, the features of the cleavage reaction were all as would be expected if the target of inhibition by VM26 is topoisomerase II—the reaction was reversible, required both ATP and Mg²⁺, and resulted in the covalent attachment of a protein at the 5' end of the cleavage product.

Topoisomerase II cleavage of Ad5 DNA increases in the late phase of the infection. In an attempt to correlate topoisomerase II-induced cleavage of Ad5 DNA with the stage of the infection, the effects of VM26 were examined at various times after infection. At 4 h, when Ad5 DNA replication had not yet begun and transcription was in the early phase, there was a low level of cleavage (Fig. 2). By 8 h, Ad5 DNA replication had begun, the level of viral DNA had increased approximately 10-fold, and the late phase of transcription had begun, and cleavage by topoisomerase II increased. At 12 h, when Ad5 DNA levels had increased 100- to 200-fold

FIG. 1. Cofactor requirements for VM26-stimulated cleavage of Ad5 DNA. HeLa cells, 12 h after infection with *dl309*, were either lysed in hypotonic buffer containing 0.5% Nonidet P-40 to prepare nuclei (in vitro) or treated directly (in vivo). For the in vitro analysis, nuclei were washed, suspended in buffer containing 5 mM $MgCl_2$, divided into aliquots, and treated with various concentrations of VM26 for 15 min at 37°C in the absence of exogenous ATP (lanes marked no ATP) or with 1.5 mM added ATP (lanes marked SDS and EDTA). The nuclei were then lysed by the addition of SDS (lanes marked no ATP and SDS) or incubated for an additional 5 min with 20 mM added EDTA, followed by lysis with SDS. For in vivo analysis, cells were treated with various concentrations of VM26 for 15 min and then lysed by the addition of SDS (lanes marked SDS), or nuclei were prepared, EDTA was added to 20 mM, incubation was continued for 15 min, and SDS was added. DNA was purified, digested with *HindIII*, resolved by agarose gel electrophoresis, transferred to nitrocellulose, and indirectly end labeled with the ^{32}P -labeled Ad5 DNA fragment from 5.7 (*KpnI* site) to 7.8 m.u. (*HindIII* site). DNA size markers (lane M) were prepared by complete *HindIII* digestion and partial *RsaI* digestion. The major bands represent cleavage at 193, 638, 1,358, and 1,781 base pairs from the left end of the viral DNA (the *RsaI* site at 905 base pairs is cleaved less efficiently). A schematic representation of the left end of the Ad5 chromosome is shown. TR, Terminal repeat; ENH, E1A enhancer region. E1A and E1B transcribed regions are indicated.

compared with the levels at 4 h, topoisomerase II cleavage had increased greater than 10-fold. By 20 to 24 h, there was little further replication, packaging of viral DNA was under way and transcription continued at a high level, and the amount of topoisomerase II cleavage per Ad5 chromosome decreased dramatically from the 12-h peak (data not shown; Fig. 3). While the yield of cleavage products varied greatly through the course of the infection, the sites utilized and the relative frequency of cleavage at these sites changed little if at all. The efficiency of topoisomerase II cleavage of Ad5 DNA thus appeared to parallel the rate of Ad5 DNA replication much more closely than it paralleled changes in viral transcription.

E1A expression is not required for enhancement of topoisomerase II cleavage in the late phase of infection. To determine whether E1A proteins are required for the increased topoisomerase II cleavage of Ad5 DNA during the late phase of the infection, VM26-induced cleavage of the E1A⁻ virus *dl343* was compared to that of the wild-type virus, *dl309*. The adenovirus E1A proteins are required to

FIG. 2. VM26 stimulation of Ad5 DNA cleavage as a function of time after infection. HeLa cells were treated with various concentrations of VM26 at various times after infection with *dl309*. Cells were lysed by the addition of SDS. DNA was purified, digested with *HindIII*, resolved by agarose gel electrophoresis, and indirectly end labeled by using the 5.7- to 7.8-m.u. Ad5 DNA fragment as a probe. A schematic representation of the left end of the Ad5 chromosome is shown. TR, Terminal repeat; ENH, E1A enhancer region. E1A and E1B transcribed regions are indicated.

FIG. 3. Dependence of VM26-stimulated Ad5 DNA cleavage on E1A expression. HeLa cells were treated with VM26 at 10 µg/ml (lanes V) or with no drug (lanes -) at various times after infection with either *dl309* at a multiplicity of 25 PFU per cell or *dl343* at a multiplicity of 250 PFU/cell. The filter used in this experiment was exposed for a shorter period of time than the filters in Fig. 1 and 2. Thus, minor bands are not as visible in this figure, but the cleavage patterns are virtually identical (e.g., compare the cleavage pattern at 4 h after infection in Fig. 2 with that in Fig. 3). TR, Terminal repeat; ENH, E1A enhancer region.

activate transcription of the early viral genes. Viruses which do not express E1A proteins activate transcription of the other early transcription units poorly and exhibit a prolonged lag before initiation of DNA replication and onset of late gene expression. This lag can be partially bypassed by infection with E1A⁻ viruses at high input multiplicities (31). HeLa cells were infected with either *dl343* (E1A⁻ at a multiplicity of 250 PFU per cell or *dl309* (wild type) at a multiplicity of 25 PFU per cell and treated with VM26 at various times after infection (Fig. 3). Little VM26-induced cleavage of adenovirus DNA was observed 12 h after infection with *dl343*, while cleavage of *dl309* was efficiently induced. Twenty-four hours after infection, a relatively high level of cleavage was observed with *dl343*, while there was little cleavage of *dl309* DNA. Cleavage of *dl343* DNA was efficiently induced at 36 h and induced somewhat less efficiently at 48 h. At all times, the cleavage sites utilized for both E1A⁺ and E1A⁻ viruses were the same. The level of topoisomerase II cleavage per genome was also similar for *dl343* and *dl309* during the phase of rapid replication (compare the 24-h lane for *dl343* with the 12-h lane for *dl309*). Thus, there was not an absolute requirement for E1A proteins in the induction of cleavage by topoisomerase II, but the presence of E1A proteins caused a more rapid induction of the increased level of cleavage.

The efficiency of VM26-induced topoisomerase II cleavage of *dl343* DNA also correlated well with the rate of replication (data not shown). There was no replication of *dl343* DNA 12 h after infection, at which time there was little cleavage induced, while at 24 and 36 h there was a relatively high rate of replication and efficient cleavage, and at 48 h the rate of replication was somewhat reduced, as was the efficiency of cleavage.

Topoisomerase II cleavage occurs across large segments of the viral chromosome. To determine whether topoisomerase II cleavage occurred in internal DNA fragments as well as near the termini, the major late promoter region and the right-end 25% of the viral DNA were examined at 12 h after infection (Fig. 4). The results demonstrated that strong cleavage sites occurred at fairly regular intervals throughout both of these regions. Thus, during rapid replication of Ad5 DNA, topoisomerase II binds throughout the viral chromosome.

Topoisomerase I cleavage remains nearly constant during Ad5 infection. In order to establish that Ad5 DNA cleavage induced by camptothecin occurs through inhibition of the religation step by topoisomerase I, the cofactor requirements and cleavage products were examined. DNA from camptothecin-treated Ad5-infected HeLa cells was isolated and either treated with proteinase K or not treated, as described above for VM26-treated cells. Only the fragments with 3' ends generated by the camptothecin-stimulated cleavage were retarded in electrophoretic mobility in the absence of protease treatment (indicating the presence of a protein), and treatment of isolated nuclei in the presence of EDTA and in the absence of exogenous Mg²⁺ did not inhibit camptothecin-stimulated cleavage (data not shown). Thus, the characteristics of the camptothecin-stimulated cleavage reaction and products are consistent with inhibition of the topoisomerase I cleavage and religation reaction.

Topoisomerase I cleavage in the presence of camptothecin was assayed within a portion of the viral chromosome including the E1A and E1B genes as a function of time after infection. At 1 and 4 h after infection, the pattern was nearly identical (Fig. 5). At 8 and 12 h, after the onset of the late phase of infection, most of the cleavage products and their

FIG. 4. VM26-stimulated cleavage of the Ad5 chromosome outside of the E1 region. HeLa cells, 12 h after infection with *dl309*, were treated with VM26 at the concentrations noted. DNA was purified, digested with *Hind*III (A) or *Eco*RI (B), resolved by agarose gel electrophoresis, and indirectly end labeled by using the 5.7- to 7.8-m.u. fragment as a probe for *Hind*III-digested DNA and the 75.9- to 77.9-m.u. fragment as a probe for *Eco*RI-digested DNA. Schematic representations of the regions of viral DNA are presented. TR, Terminal repeat. IVa2, the major late promoter (MLP), and E4 transcription regions are indicated.

relative intensities were unchanged. However, new or intensified bands appeared near the 3' end of E1A on the transcribed strand and near the 5' end of the E1B transcription region on the nontranscribed strand.

E1A protein expression is not required for topoisomerase I cleavage of Ad5 DNA. To examine the effect of expression of the E1A proteins on topoisomerase I interaction with adenovirus DNA, camptothecin-induced cleavage was examined 12 h after infection with a wild-type virus (*dl309*) and 36 h after infection with a virus expressing no E1A functions (*dl343*) (Fig. 6). Little or no alteration in either position or relative intensity was observed for either strand of *dl343* relative to *dl309*. Thus, it appears that E1A-induced alterations in either transcription or replication do not affect topoisomerase I function.

Topoisomerase I cleavage was also compared with topoisomerase II cleavage for the wild-type virus (Fig. 6). While sites for the two enzymes occur in close juxtaposition near the left end of the virus, topoisomerase I cleavage occurred with much greater frequency than did topoisomerase II cleavage, even though the time used for this assay was near, or at, the peak of topoisomerase II cleavage of Ad5 DNA.

Topoisomerase inhibitors block Ad5 DNA replication. VM26 and camptothecin were tested for their ability to inhibit Ad5 DNA replication from 8 to 10 h after infection (Fig. 7). Camptothecin at a concentration of 1 µg/ml nearly abolished replication, while high concentrations of VM26 reduced replication to slightly less than 20% of the control level. In the absence of drugs, the Ad5 DNA increased in amount by a factor of 4 to 5 between 8 and 10 h after infection. Thus, in the presence of high concentrations of VM26, the DNA increased in amount by a factor of slightly less than 2. This could represent the completion of one round of replication at a normal rate, followed by complete inhibi-

FIG. 5. Camptothecin stimulation of Ad5 DNA cleavage as a function of time after infection. HeLa cells were treated with camptothecin at 6.7 $\mu\text{g/ml}$ (lanes C) or with no drug (lanes -) at various times after infection with *dl309*. DNA was purified, digested with *HindIII*, resolved by denaturing agarose gel electrophoresis, and indirectly end labeled by using single-stranded RNA transcribed from the 5.7- to 7.8-m.u. fragment. A schematic representation of the left end of the virus is presented. TR, Terminal repeat; ENH, E1A enhancer region. E1A and E1B transcribed regions are indicated.

FIG. 6. Dependence of camptothecin-stimulated Ad5 DNA cleavage on E1A expression. HeLa cells were treated with camptothecin at 6.7 $\mu\text{g/ml}$ (lanes C) or with no drug (lanes -) 12 h after infection with *dl309* and 36 h after infection with *dl343*. DNA was purified, digested with *HindIII*, resolved by electrophoresis on denaturing agarose gels, and indirectly end labeled by using single-stranded RNA from the 5.7- to 7.8-m.u. fragment. For comparison of topoisomerase I and II cleavage, DNA from *dl309*-infected HeLa cells treated at 12 h after infection with VM26 at 100 $\mu\text{g/ml}$ (lanes V) is shown. A schematic representation of the left end of the virus is presented. TR, Terminal repeat; ENH, E1A enhancer region. E1A and E1B transcribed regions are indicated.

tion, or a general reduction in the rate of replication. To distinguish between these possibilities, the kinetics of replication in the presence and absence of VM26 at 100 $\mu\text{g/ml}$ were examined (Fig. 8). Replication occurred at a normal rate for the first 30 min after the addition of the drug or until the concentration of Ad5 DNA had increased slightly less than twofold. After 30 min, there was little further replication in the absence of topoisomerase II activity.

DISCUSSION

Topoisomerase II interaction with Ad5 DNA. Sites of interaction of topoisomerase II with the SV40 chromosome *in vivo* have been mapped (45). There is little topoisomerase II cleavage until late in the infection, when a single prominent site near the origin of replication is evident. In contrast, there are numerous sites within the Ad5 E1 region alone (Fig. 1 to 3).

FIG. 7. Effect of topoisomerase inhibitors on Ad5 DNA replication. HeLa cells, 8 h after infection with *dl309*, were treated for 2 h with various concentrations of VM26 or camptothecin. DNA was purified, and serial dilutions of the DNA were immobilized on nitrocellulose and probed with ^{32}P -labeled Ad5 DNA. Hybridization was quantified by liquid scintillation counting, and the extent of Ad5 DNA replication which occurred between 8 and 10 h after infection was plotted as the percentage of replication in the absence of drugs versus the concentration of VM26 (A) or camptothecin (B).

FIG. 8. Kinetics of Ad5 DNA replication in the presence of VM26. HeLa cells, 8 h after infection with *d*/309, were treated with no drug or with VM26 (100 µg/ml). Samples of cells were taken at the times noted, DNA was purified, and dilutions were immobilized on nitrocellulose and probed with ³²P-labeled Ad5 DNA. Hybridization was quantified by liquid scintillation counting and plotted as fold replication of Ad5 DNA in the absence (○) or presence (●) of VM26.

The sequence specificity of cleavage by topoisomerase II is likely to be the same in vivo and in vitro. However, fewer sites are utilized in vivo than in vitro (e.g., there is a dramatic decrease in the number of topoisomerase II cleavage sites in SV40 chromatin in vivo relative to naked SV40 DNA [19, 45]), presumably because of exclusion of topoisomerase II from certain potential sites by chromatin proteins (37). Topoisomerase II cleavage sites tend to be found near nuclease-hypersensitive sites (28, 29, 45), which may be relatively protein-free. In the case of topoisomerase II cleavage of Ad5 DNA, there was little difference in either the choice of sites or their relative frequencies of utilization when the cleavage was induced in isolated nuclei relative to in vivo cleavage (Fig. 1). Thus, any loss of DNA-binding proteins which occurred during preparation of nuclei did not significantly alter the cleavage of Ad5 DNA. This raises the possibility that the Ad5 DNA is recognized as virtually naked throughout the infection, although it is bound, at least in the early part of the infection, by adenovirus-encoded core proteins (2). This may explain why so many topoisomerase II sites are utilized on Ad5 chromatin in vivo.

It has been suggested that specific attachment of DNA to the nuclear matrix is mediated by topoisomerase II (4, 8). However, this does not appear to be the case for Ad5 DNA. Ad5 DNA binds tightly to the nuclear matrix through its covalently attached terminal protein, with no apparent specific binding of internal fragments (J. Schaack, W. Ho, P. Freimuth, P. Schedl, and T. Shenk, submitted for publication). Binding to the nuclear matrix occurs very early after infection, when there is little interaction of topoisomerase II with viral DNA. Topoisomerase II interaction with viral DNA increased dramatically during replication (Fig. 2), but there was no concomitant increase in nuclear matrix association. Furthermore, there was no relative enrichment of topoisomerase II cleavage sites at the termini, and strong sites occurred in internal fragments (Fig. 1 to 4), even though the terminal fragments contain the sites of strongest association with the nuclear matrix (1; Schaack et al., submitted).

Topoisomerase I interaction with Ad5 DNA. In the region of the viral chromosome tested, the strongest topoisomerase I cleavage sites occurred primarily near the borders of the E1A and E1B genes (Fig. 5 and 6). Topoisomerase I sites did not change much in either position or intensity with the onset of DNA replication and occurred at much greater frequency

than did topoisomerase II sites. These data are consistent with a role in transcription, in addition to the previously suggested role in replication. Interestingly, the frequency of topoisomerase I cleavage was affected little by E1A proteins (Fig. 6), even though E1A encodes a transcriptional activator. Furthermore, the frequency of topoisomerase I cleavage of the E1 region increased little from 1 to 12 h after infection (Fig. 5), even though the rate of transcription increased dramatically. This suggests that transcriptional activation of the Ad5 genome does not lead to recruitment of topoisomerase I, as in *Drosophila* genes (9, 10), but that topoisomerase I interacts with Ad5 chromatin independently of transcription.

Topoisomerase requirement for Ad5 DNA replication. The roles of the topoisomerases in replication have been studied in a number of organisms. In *S. cerevisiae* and *S. pombe*, in which the genetics of the topoisomerases have been well studied, replication proceeds to completion in the absence of topoisomerase I. In the absence of topoisomerase II, replication is blocked at mitosis, with sister chromatids failing to segregate (6, 14, 38, 39). In SV40, replication has been studied in the presence of topoisomerase-inhibiting drugs and by immunodepletion of topoisomerases. These studies indicated that the presence of topoisomerase I activity alone permits replication only until the terminal stages, while the presence of both topoisomerase activities or topoisomerase II activity alone permits complete replication (26, 32, 33, 43, 46). Thus, it appears that either topoisomerase can relax torsional stress induced ahead of the SV40 replication fork, while topoisomerase II is absolutely required for resolution of daughter molecules. The fact that replication-elongation steps can proceed in the presence of the steric block of the covalent DNA-protein cleavage intermediates induced by either camptothecin or VM26 indicates that there is not an artifactual block to replication in the presence of the drugs.

We cannot be absolutely certain that either drug exerts its effect on adenovirus DNA replication (Fig. 7) as a direct result of its inhibition of topoisomerase I or II. However, both drugs inhibited viral DNA replication, raising the possibility that both topoisomerases play a role in the replication process.

Adenovirus DNA replication proceeds from the chromosomal termini (the replication origins) by a mechanism which involves displacement of a single strand of DNA (for a review, see reference 18). In vitro, full-length replication of viral DNA occurs in the presence of topoisomerase I without topoisomerase II (24). In vivo, inhibition of topoisomerase I with camptothecin very efficiently blocked replication (Fig. 7). This suggests that, unlike replication of SV40 DNA (32, 33, 46), topoisomerase II cannot assume the role of topoisomerase I during elongation steps in Ad5 DNA replication. The apparent absolute requirement for topoisomerase I may result from the fact that adenovirus DNA replication proceeds one strand at a time, with displacement of a single strand.

Topoisomerase II binding to the Ad5 chromosome increased with increasing rate of replication and then decreased as replication slowed (Fig. 2 and 3). This provides a correlation between topoisomerase II activity and replication but does not indicate at which steps in replication topoisomerase II might function. At concentrations of VM26 which cause maximal inhibition, replication was reduced to slightly less than 20% of the uninhibited value when assayed between 8 and 10 h after infection (Fig. 7). During this time, the concentration of Ad5 DNA in cells not treated with drugs increased between four- and fivefold. Thus, the inhibition is

consistent with the completion of approximately one round of replication after addition of the drug. Analysis of the kinetics of replication when topoisomerase II was inhibited demonstrated that replication occurred normally for approximately 30 min, or slightly less than the time required for a doubling in the concentration of Ad5 DNA, but then virtually ceased (Fig. 8). The simplest explanation for these data is that, in the presence of the drug, one round of replication was completed. This suggests that topoisomerase II may be required at the terminal stage of replication, possibly to separate daughter molecules. Freely soluble, linear DNA molecules presumably would not require topoisomerase II activity in order to allow this separation, as is the case for *in vitro* replication (26). This supports the suggestion that the Ad5 chromosome is topologically constrained *in vivo*, consistent with the presence of tight nuclear matrix binding sites at the termini (1; Schaack et al., submitted).

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