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Integration of Simian Virus 40 into Cellular DNA Occurs at or near Topoisomerase II Cleavage Hot Spots Induced by VM-26 (Teniposide)

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Inhibition of DNA topoisomerase II in simian virus 40 (SV40)-infected BSC-1 cells with a topoisomerase II poison, VM-26 (teniposide), resulted in rapid conversion of a population of the SV40 DNA into a high-molecular-weight form. Characterization of this high-molecular-weight form of SV40 DNA suggests that it is linear, double stranded, and a recombinant with SV40 DNA sequences covalently joined to cellular DNA. The majority of the integrants contain fewer than two tandem copies of SV40 DNA. Neither DNA-damaging agents, such as mitomycin and UV, nor the topoisomerase I inhibitor camptothecin induced detectable integration in this system. In addition, the recombination junctions within the SV40 portion of the integrants correlate with VM-26-induced, topoisomerase II cleavage hot spots on SV40 DNA. These results suggest a direct and specific role for topoisomerase II and possibly the enzyme-inhibitor-DNA ternary cleavable complex in integration. The propensity of poisoned topoisomerase II to induce viral integration also suggests a role for topoisomerase II in a pathway of chromosomal DNA rearrangements.

Genomic rearrangements are known to occur in mammalian cells and are often associated with oncogenic transformation. However, very little is known about the molecular mechanism(s) of this process. Attempts to study this process in mammalian cells with transient transfection systems have been carried out. Stable integration of transfected DNA through illegitimate recombination has been demonstrated to be an efficient process. Double-strand breaks stimulate both illegitimate and homologous recombinations (reviewed in references 28 and 34). However, it is not clear whether integration of transfected DNA reflects events of chromosomal DNA rearrangements in cells.

Bacterial DNA topoisomerase II has been implicated in illegitimate recombination through in vitro studies (20). A subunit-exchange model in which the recombination reaction is thought to proceed via the exchange of subunits between two topoisomerase II homodimers with the concomitant exchange of the DNA strands to which the subunits are covalently attached has been proposed. The involvement of DNA replication has also been suggested (21, 22). More recently, bacterial DNA gyrase has also been implicated to be responsible for a *recA*-independent homologous recombination pathway (25). Other type II DNA topoisomerases such as bacteriophage T4 topoisomerase and calf thymus DNA topoisomerase II have also been shown to promote illegitimate recombination in vitro (2, 19). These studies suggest a potential role for topoisomerase II in both illegitimate recombination and homologous recombination.

The breakage-reunion reaction of mammalian DNA topoisomerase II can be interrupted by a large number of intercalative and nonintercalative antitumor compounds (7, 26). These inhibitors stabilize a reversible topoisomerase II-inhibitor-DNA ternary complex, termed the cleavable complex. Denaturation of these cleavable complexes in vitro with strong protein denaturants such as sodium dodecyl sulfate (SDS) or alkali causes their irreversible conversion to topoisomerase-linked DNA strand breaks (reviewed in references 12 and 24). It has been proposed that interaction of these reversible, cleavable complexes with cellular processes such as DNA replication, RNA transcription, or DNA repair in vivo can also produce irreversible topoisomerase II-linked DNA breaks (39). These topoisomerase II-poisoning drugs are known to induce high levels of both homologous and nonhomologous recombination in mammalian cells (reviewed in references 1 and 24). Unlike other DNA-damaging agents, topoisomerase II-poisoning drugs can efficiently induce the formation of quadriradial chromosomes, which presumably is caused by illegitimate recombination between two nonhomologous chromosomes (6). Topoisomerase II has been shown to bind to regions near or at the nuclear matrix attachment sites (10, 14). The potential involvement of topoisomerase II in illegitimate recombination underscores the importance of these sites in genome rearrangements and other processes such as gene amplification (33).

The evidence suggesting a role for topoisomerase II in illegitimate recombination led us to examine its involvement in recombination by using simian virus 40 (SV40)-infected cells. The chromatin structure of the SV40 genome resembles that of the host genome and may serve as a model system for studying illegitimate recombination in mammalian cells. We show that within 1 h of poisoning topoisomerase II in both virus-infected cells and plasmid DNA-transfected COS cells, a population of SV40 DNA is efficiently integrated into host chromosomal DNA. DNA replication appears to be intimately involved in this process. Our results suggest a direct and specific role for topoisomerase II in a pathway of recombination in mammalian cells. The efficiency of this illegitimate recombination sys-

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tem may allow biochemical investigation of topoisomerase II-mediated recombination in mammalian cells.

MATERIALS AND METHODS

DNA, cell lines, enzymes, and chemicals. pA26 DNA is a composite of pBR322 and SV40 DNA and was constructed by cloning the *Eco*RI-digested SV40 DNA into the *Eco*RI site of pBR322 DNA. pLY1 DNA was constructed by cloning the SV40 origin-containing *Hin*dIII fragment of SV40 DNA into the *Hin*dIII site of pBR322 DNA. SV40 (strain 835) was used to infect monkey BSC-1 cells. Bluescript KS-(−) vector was purchased from Stratagene. VM-26 (teniposide) and VP-16 (etoposide) were kind gifts from Bristol-Myers and Squibb Co. Camptothecin lactone (NSC 94600) was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. Aphidicolin and proteinase K were purchased from Boehringer Mannheim Co., and fetal calf serum and tissue culture media were purchased from GIBCO-BRL. Hydroxyurea, 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole (DRB), 5-fluorouracil, 3-aminobenzamide, dinitrophenol, Nα-tosyl-L-lysine chloromethyl ketone (TLCK), calcium ionophore A23187 (A23187), cycloheximide, and mitomycin were purchased from Sigma Chemical Co. ST638, a specific tyrosine kinase inhibitor, was a kind gift from T. Shiraishi (31). Restriction enzymes were purchased from either New England Biolabs or GIBCO-BRL. [α-^{32}P]dCTP was purchased from Dupont/NEN. Incert agarose was purchased from FMC, and Gelase was purchased from Epicentre Technologies.

SV40 infection and treatment with the topoisomerase II inhibitor VM-26. BSC-1 cells, seeded at a density of 2×10^6 cells per 10-cm-diameter plate, were grown for 18 h in an incubator maintained at 37°C with 5% CO_2 in minimal essential media (MEM) supplemented with 10% fetal calf serum. The cells were washed twice with phosphate-buffered saline (PBS; 110 mM NaCl, 2.1 mM KCl, 6.3 mM Na_2HPO_4, 1.2 mM KH_2PO_4) containing 1.0 mM $CaCl_2$ and 0.5 mM $MgCl_2$ and then infected in 1.5 ml of MEM supplemented with 2% fetal calf serum (MEM-2) at a multiplicity of infection of 5 to 10 PFU per cell with an SV40 (strain 835) stock. Following a 2-h incubation at 37°C, with occasional agitation, an additional 8.5 ml of MEM supplemented with 10% fetal calf serum (MEM-10) was added, and the incubation was continued. At 20 h postinfection, VM-26 was added to a final concentration of 100 μM (unless otherwise indicated), and the incubation was continued for 5 hours. The cells were then washed three times with MEM-2, and the incubation was continued for another hour in MEM-10. Cells were washed once with PBS and lysed with 1 ml of lysis buffer (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.5], 50 mM EDTA, 1% SDS). The lysates were digested with 1 mg of proteinase K per ml at 60°C for 18 h.

Isolation of the HMW DNA. The proteinase K-treated lysate from one 10-cm-diameter dish of virus-infected, VM-26-treated BSC-1 cells was electrophoresed in a 0.6% Incert agarose gel in 1× Tris-acetate-EDTA (TAE) at 1.9 V/cm for 20 h. A fraction of the gel was sliced longitudinally and stained with ethidium bromide to locate the high-molecular-weight (HMW) DNA band which migrated at the limiting mobility of the gel. The band was then excised from the unstained portion of the gel. Immediately prior to use, a portion of the HMW DNA-containing gel piece was melted at 65°C and then cooled to 41°C and digested with Gelase (1 U/100 mg of gel) for 90 min. The digested gel, containing the HMW DNA, was then used directly in subsequent reactions.

Electrophoresis and Southern analysis. Generally, DNA samples from cell lysates were electrophoresed in a 0.7% agarose gel in 0.5× Tris-phosphate-EDTA (TPE) at 1.6 V/cm for 20 h. The gels were then subjected to Southern blot analysis. Nick-translated pA26 DNA was used to probe for SV40 DNA-containing sequences.

UV irradiation of cells. UV irradiation of cells was done at 254 nm in a Stratagene UV Stratalinker model 1800. BSC-1 cells were grown and infected with SV40 as described above. The media were aspirated, and the cells were UV irradiated at the indicated energy level in the uncovered plate. Prewarmed MEM-10 was added to each plate, and the incubation was continued for 6 h.

Cloning and sequence analysis of integrant junction sites. To clone the chromosomal-SV40 junction sites, HMW DNA from VM-26-treated, SV40-infected BSC-1 cells was gel isolated by using 0.6% Incert agarose. Following digestion of the agarose with Gelase, the DNA was divided and each half was digested with two restriction enzymes, *Bgl*II and *Eco*RI (for set 3) or *Spe*I and *Eco*RI (for set 4). The resulting DNA fragments were cloned into the multicloning site of the Bluescript KS(−) vector which had been previously cut with *Bam*HI and *Eco*RI (for set 3) or *Spe*I and *Eco*RI (for set 4) (set 1 and set 2 were digested with *Bgl*II and *Spe*I, respec-

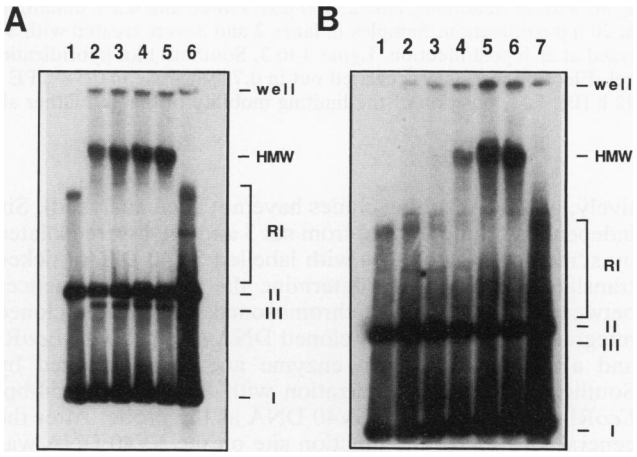

FIG. 1. Poisoning of DNA topoisomerase II with VM-26 stimulates the formation of HMW SV40 DNA. (A) Time course of induction of the HMW DNA. BSC-1 cells were infected with SV40 as described in Materials and Methods. At 20 h postinfection, VM-26 (100 μM in 0.2% dimethyl sulfoxide [DMSO]) or DMSO (0.2%) was added to cells, and incubation continued for indicated times. Cells were washed, incubated for an additional hour in inhibitor-free MEM-10, lysed, and treated with proteinase K. The DNA samples were electrophoresed in a 0.7% agarose gel in 0.5× TPE at 1.6 V/cm for 20 h. The gel was examined by Southern blot analysis with nick-translated pA26 DNA to probe for SV40-containing DNA. Lane 1, lysing at 20 h postinfection; lanes 2 to 5, VM-26 treatment for 1, 2, 3, and 5 h, respectively; lane 6, treatment with 0.2% DMSO for 5 h. (B) Dose-dependent induction of the HMW DNA. The experimental protocol was as described for panel A, except that the VM-26 concentrations were varied and the treatment time was held constant at 5 h. Lane 1, lysing at 20 h postinfection; lanes 2 to 6, VM-26 concentrations were 2.5, 10, 25, 50, and 100 μM, respectively; lane 7, treatment with 0.2% DMSO for 5 h. I, II, III, form I (supercoiled), form II (nicked), and form III (linear), respectively. The minor band just above the RI is at the position for nicked circular dimer.

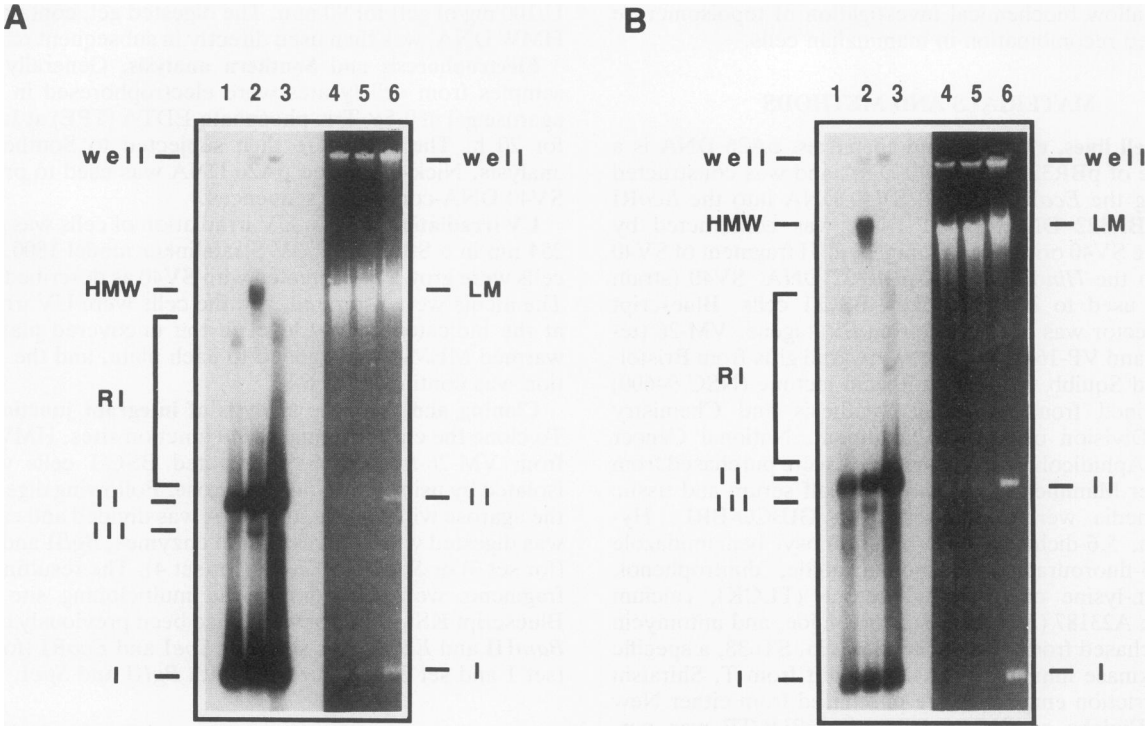

FIG. 2. The HMW DNA is linear and comigrates with cellular DNA. SV40-infected BSC-1 cells were treated with VM-26 as follows: lane 1, no VM-26 treatment; lane 2, 100 μM VM-26 and 0.2% dimethyl sulfoxide (DMSO); lane 3, 0.2% DMSO. The sample in lane 1 was lysed at 20 h postinfection. Samples in lanes 2 and 3 were treated with VM-26 and/or DMSO for 5 h, incubated for 1 h in inhibitor-free media, and lysed at 26 h postinfection. Lanes 1 to 3, Southern blot hybridization with nick-translated pA26 DNA; lanes 4 to 6, ethidium bromide-stained gel. Electrophoresis was carried out in 0.7% agarose in 0.5× TPE buffer at two different conditions: 2.4 V/cm for 17.5 h (A) and 1 V/cm for 42 h (B). LM, position of the limiting mobility of the gel. Other abbreviations are defined in the legend to Fig. 1.

tively, and the resulting clones have not been analyzed). Six independent clones (each) from set 3 and set 4 were isolated by screening the colonies with labelled SV40 DNA (nicked translated). In order to determine the junction sequences between SV40 DNA and chromosomal DNA in the cloned integrant sequences, each cloned DNA was cut with *Eco*RI and a second restriction enzyme and then analyzed by Southern blot and hybridization with the smaller (154-bp) *Eco*RI-*Acc*I fragment of SV40 DNA as the probe. After the general location of the junction site on the SV40 DNA was determined, each clone was sequenced by using the dideoxy sequencing method and Sequenase 2.0 (U.S. Biochemicals).

Cleavage site determination. The VM-26-induced topoisomerase II cleavage sites on SV40 DNA were determined as described previously (37), using a uniquely 5′-end-labelled SV40 DNA fragment and calf thymus topoisomerase II. The 591-bp DNA fragment from nucleotide 5225 to nucleotide 573 was synthesized by using *Bam*HI-cut SV40 DNA and the polymerase chain reaction (PCR). The fragment was 5′ end labelled with [γ-^{32}P]ATP and T4 kinase as instructed by the manufacturer. After labelling, the DNA was cut with *Bgl*I to generate the uniquely end-labelled fragment. The DNA was precipitated with ethanol, resuspended, and then divided into two portions: one was subjected to G+A chemical sequence analysis, and the other was used to determine VM-26-induced topoisomerase II cleavage sites. The products of both reactions were precipitated, dissolved in formamide sequencing buffer (95% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, 20 mM EDTA), and analyzed with a 6% acrylamide sequencing gel.

RESULTS

Poisoning DNA topoisomerase II induces an HMW DNA. To test whether topoisomerase II poisoning is associated with high-frequency illegitimate recombination, SV40-infected BSC-1 cells were treated with VM-26 (a topoisomerase II-specific poison) at 20 h postinfection. A new, slowly migrating band (labeled HMW) was produced in a time (Fig. 1A)- and dose (Fig. 1B)-dependent manner. Within 1 h of treatment, the HMW band appeared. After 6 h of treatment, approximately 5% of the SV40 DNA was converted into the HMW form, and the intensity of the HMW band appeared to reach a plateau. The disappearance of the replicative intermediates (RI) was reproducibly observed (Fig. 1). The formation of the HMW form of SV40 DNA (HMW DNA) is also dependent on the concentration of VM-26 (Fig. 1B). Approximately 5% of the SV40 DNA was converted into the HMW form at the highest VM-26 concentration (100 μM). Again, the RI progressively disappeared with increasing VM-26 concentrations (Fig. 1B). A minor band at the position expected for nicked (or gapped) circular dimer was also observed in VM-26-treated cells (see Fig. 1B, lanes 2 to 6). HMW DNA formation could also be induced by treatment with VP-16, a related topoisomerase II inhibitor (data not shown).

We have also transfected COS cells with either pSV2.cat DNA or pLY1 DNA and tested the effect of VM-26 treatment (100 μM for 5 h). The HMW DNA was again detected (data not shown). The formation of the HMW DNA was not induced by treatments with other DNA-damaging agents;

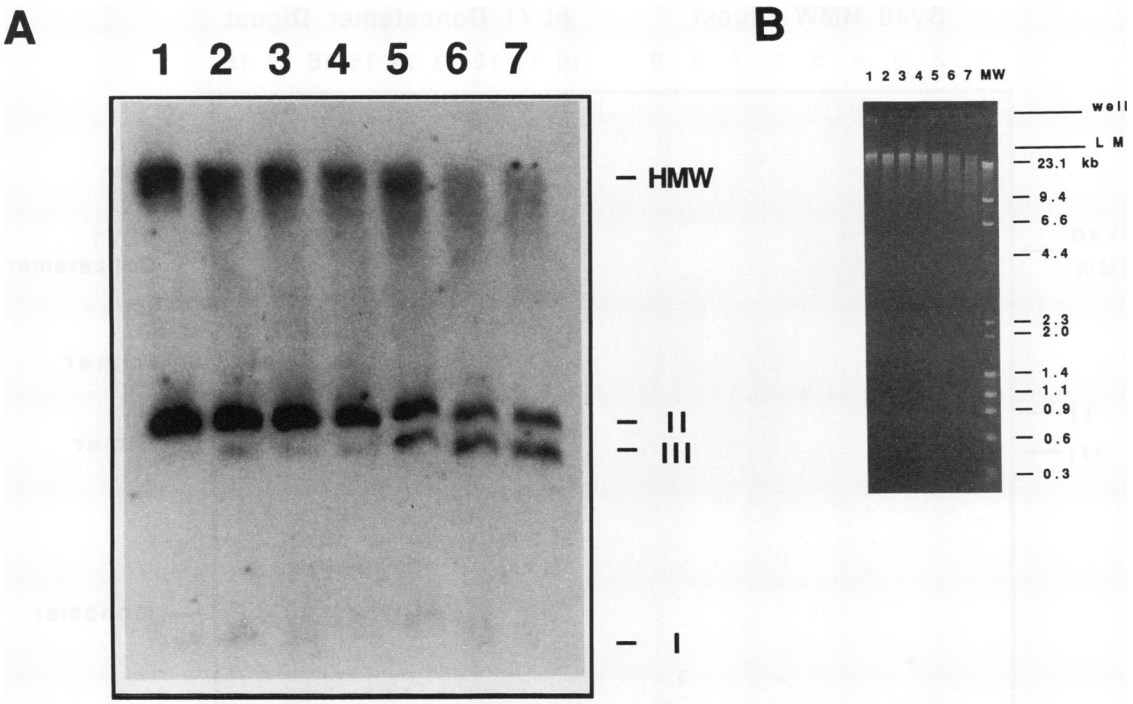

FIG. 3. The HMW DNA is an intact, double-stranded DNA. S1 nuclease digestion of the isolated HMW DNA was performed at 37°C in a reaction mixture containing 30 mM sodium acetate (pH 5.0), 200 mM sodium chloride, 30 mM zinc acetate, 1 mg of the isolated HMW DNA, and S1 nuclease. The S1 nuclease concentrations are as follows: lane 1, 0 U; lanes 2 to 4, 50 U; lanes 5 to 7, 400 U. Incubation times were as follows: 5 min for lanes 2 and 5, 15 min for lanes 3 and 6, and 30 min for lanes 1, 4, and 7. Samples were electrophoresed in a 0.9% agarose gel with 0.5× TPE, and Southern blot analysis was done with nick-translated pA26 DNA. (A) Southern analysis; (B) ethidium bromide-stained gel. Abbreviations are defined in the legends to Fig. 1 and 2.

treatments with mitomycin (10 mg/ml for 5 h), UV (400 or 4,000 mJ/cm^2 followed by a 6-h incubation), or camptothecin (50 μM for 5 h) did not induce the formation of the HMW DNA.

The HMW DNA is linear and double stranded and comigrates with host chromosomal DNA. The HMW DNA comigrated with cellular chromosomal DNA during gel electrophoresis under all electrophoresis conditions tested. Figure 2 shows the electrophoresis results from two different voltage gradients. In both cases, the HMW DNA comigrated with cellular chromosomal DNA, which migrated at the limiting mobility of the gel (compare lanes 2 and 5 in both panels of Fig. 2), suggesting that the HMW DNA is linear and doublestranded. The size of the HMW DNA cannot be determined by this electrophoresis system. Since it always comigrates with the cellular DNA at the limiting mobility of the gel, it should have a size greater than 23 kb (see Fig. 5, compare the HMW band with the DNA markers). Comigration of the HMW DNA with the bulk chromosomal DNA was also observed under alkaline gel electrophoresis conditions (data not shown). Brief heating of the DNA samples to 65°C for 10 min did not affect the mobility of the HMW DNA in the gel (data not shown). Furthermore, the HMW DNA was not preferentially sensitive to S1 nuclease digestion relative to the bulk cellular DNA (compare Fig. 3A and B). These results suggest that the HMW DNA does not contain a significant number of nicks or gaps and is not held together by short, cohesive ends.

The majority of HMW DNA is not head-to-tail linear concatemeric SV40 DNA. The HMW DNA could be either linear head-to-tail concatemeric SV40 DNA or linear cellular DNA covalently joined to SV40 DNA, or a combination of both. If the HMW DNA represented head-to-tail concatemeric SV40 DNA, digestion of the HMW DNA with a restriction enzyme that cuts SV40 DNA only once would generate primarily unit-length linear SV40 DNA. As shown in Fig. 4 (compare lanes 1 and 9), exhaustive *Eco*RI digestion converted only about 10% of the HMW DNA into unit-length linear SV40 DNA. The majority of the HMW DNA was converted to a smear, most of which migrated more slowly than linear SV40 DNA (Fig. 4, lane 9). These results strongly suggest that the majority of the HMW DNA is not head-to-tail concatemeric SV40 DNA. The fact that 10% of the HMW DNA was digested to full-length linear SV40 DNA suggests that a small fraction of the HMW DNA does contain head-to-tail tandem repeats of unit-length SV40 DNA. Similar results were also observed when *Bgl*I, *Kpn*I, or *Bam*HI (Fig. 5, lanes 6, 7, and 8, respectively) was used. The structure of the HMW DNA was also assessed by a partial digestion with *Eco*RI. If the HMW DNA represented head-to-tail concatemers of SV40 DNA, partial *Eco*RI digestion is expected to generate an array of linear SV40 DNAs representative of partial digestion products (monomers, dimers, trimers, etc.). Results of such an experiment are shown in Fig. 4. At different concentrations of *Eco*RI, the HMW DNA was gradually converted to a smear and unit-length linear SV40 DNA. The digestion pattern remained unchanged above 25 U of *Eco*RI (Fig. 4, lanes 7 to 9). No dimers or multimers of linear SV40 DNA were detectable at any concentrations of *Eco*RI. As a control, a plasmid DNA was made into linear concatemers (pLY1 DNA was digested with *Eco*RI and then ligated with T4 DNA ligase) and then

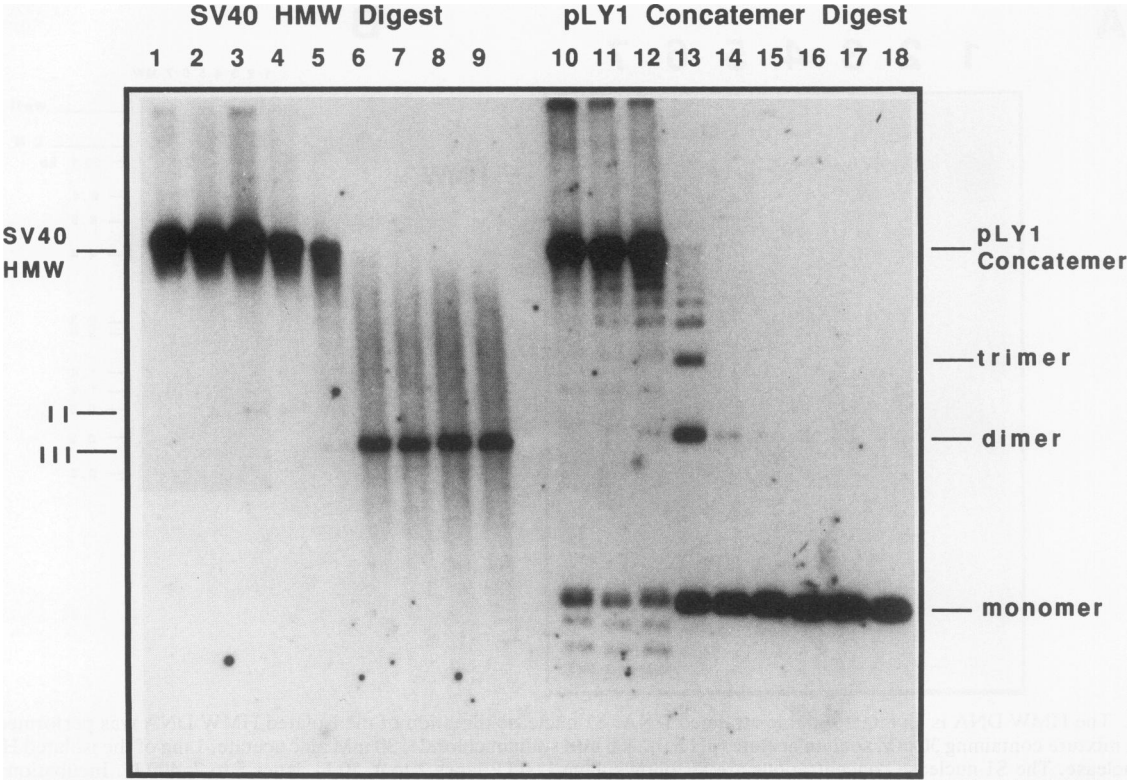

FIG. 4. The HMW DNA does not involve extensive head-to-tail concatemerization. Approximately 250 ng of gel-isolated HMW DNA was digested with different concentrations of *Eco*RI for 60 min at 37°C. Reactions were stopped with SDS (final concentration, 1%), reduced in volume by butanol extraction, and mixed with loading buffer. As a positive control, pLY1 DNA was linearized with *Eco*RI and then ligated with T4 DNA ligase into long linear concatemers. The linear concatemers of pLY1 DNA (3 ng each lane) were then redigested with *Eco*RI under digestion conditions identical to those described above. Lanes 1 to 9, *Eco*RI digests of HMW DNA; lanes 10 to 18, *Eco*RI digests of pLY1 DNA. *Eco*RI concentrations in each sample are as follows: zero for lanes 1 and 10, 0.008 U for lanes 2 and 11, 0.04 U for lanes 3 and 12, 0.2 U for lanes 4 and 13, 1.0 U for lanes 5 and 14, 5.0 U for lanes 6 and 15, 25 U for lanes 7 and 16, 50 U for lanes 8 and 17, and 75 U for lanes 9 and 18. Abbreviations are defined in the legend to Fig. 1.

digested with increasing concentrations of *Eco*RI. In this case, intermediates corresponding to linear DNA with multiple copies of the plasmid DNA were clearly demonstrable (Fig. 4, see lane 13 for the ladder). The partial digest results also indicate that the number of SV40 DNA repeats in the HMW DNA that is concatemeric is less than two (i.e., between one and two).

The HMW DNA is a recombinant with SV40 sequences covalently joined to cellular DNA. The possibility that SV40 DNA is integrated into cellular DNA was tested by digesting the HMW DNA with restriction enzymes (e.g., *Bgl*II, *Bst*BI, and *Sma*I) that do not cut SV40 DNA. Digestion of isolated HMW DNA with *Bgl*II (Fig. 5, lane 2) or *Bst*BI (Fig. 5, lane 3) or a mixture of three (Fig. 5, lane 4) or six (Fig. 5, lane 5) restriction enzymes that do not cut SV40 DNA converted the HMW DNA into a smear of faster-migrating DNA. The fact that the mobility of the HMW DNA can be increased by restriction enzymes that do not cut SV40 DNA suggests that the HMW DNA is a recombinant with SV40 DNA sequences covalently joined to cellular DNA.

Integration of SV40 DNA into cellular DNA involves DNA replication. The disappearance of the RI in VM-26-treated cells suggested that SV40 DNA replication might be involved in integration of SV40 DNA into cellular DNA (Fig. 1). The involvement of DNA replication and other metabolic processes in VM-26-induced viral integration was analyzed with a number of metabolic inhibitors. Table 1 shows the effects of various inhibitors on the formation of the HMW DNA. Figure 6A shows a representative Southern analysis of the effects of these inhibitors. DNA replication inhibitors such as aphidicolin, hydroxyurea, and 5-fluorouracil strongly inhibited the formation of the HMW DNA, suggesting that DNA replication is involved in the formation of the HMW DNA. Aphidicolin, being the most specific and potent inhibitor of DNA replication, did not completely abolish the formation of the HMW DNA (70% reduction) (Fig. 6A, lane 5) even though DNA synthesis was inhibited more than 95%. To determine whether this residual HMW signal was caused by slower transport of aphidicolin into the cells relative to that of VM-26, aphidicolin was added either 30 min prior to VM-26 addition (Fig. 6B, lane 4) or at the same time as VM-26 addition (Fig. 6B, lane 5). In both cases, the level of HMW DNA was reduced to 30% of the level observed with VM-26 treatment alone. These results suggest that cellular processes other than DNA replication also induce integration of SV40 DNA following VM-26 treatment.

Transcription inhibitors such as DRB (Fig. 6A, lane 7), actinomycin D, and camptothecin (Fig. 6C, lane 4) inhibited the formation of HMW DNA to variable extents. However, none of these inhibitors is specific. The mode of action of DRB is not completely clear (38). Camptothecin is also a replication inhibitor (37). Actinomycin D, an intercalator,

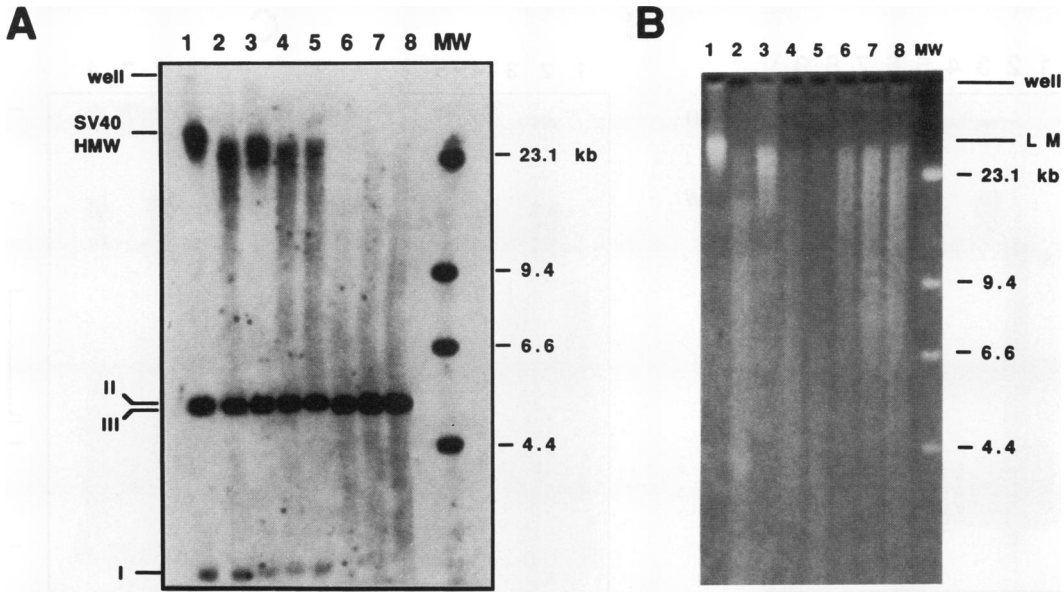

FIG. 5. The HMW DNA is linked to chromosomal DNA. The gel-isolated HMW DNA (250 ng each lane) was digested overnight with the following restriction enzymes: lane 1, no enzyme; lane 2, 40 U of *Bgl*II; lane 3, 100 U of *Bst*BI; lane 4, 16 U of *Bgl*II, 16 U of *Nru*I, and 80 U of *Sma*I; lane 5, 8 U of *Bgl*II, 8 U of *Nru*I, 40 U of *Sma*I, 2 U of *Aat*II, 5 U of *Sst*I, and 5 U of *Nhe*I; lane 6, 50 U of *Bgl*I; lane 7, 25 U of *Kpn*I; lane 8, 100 U of *Bam*HI. (A) Southern blot analysis of the gel, probed with nick-translated pA26 DNA; (B) the corresponding ethidium bromide-stained gel. Abbreviations are defined in the legends to Fig. 1 and 2.

may affect many DNA processes, including DNA replication. It is interesting that *m*-AMSA, which is also a topoisomerase II poison, did not induce the HMW DNA. However, *m*-AMSA, *o*-AMSA, and ethidium bromide inhibited the formation of the HMW DNA induced by VM-26. These results suggest that integration of SV40 DNA can be readily abolished by DNA intercalators.

The protein synthesis inhibitor cycloheximide also reduced the HMW DNA formation by about 50%. However, cycloheximide is known to inhibit host cell DNA replication (30). This result indicates either host cell DNA replication or protein synthesis is important for SV40 integration induced by VM-26.

Integration of SV40 DNA into cellular DNA occurs at or near topoisomerase II cleavage hot spots. To investigate the mechanism of integration, the junction sites of several integrants were cloned and sequenced. The locations of the junction sites with respect to the SV40 DNA were first determined by restriction enzyme mapping and then by sequence analysis as described in Materials and Methods. All the clones (3P, 4F, 4GH4, 4R, and 4T) contain one SV40 DNA-chromosomal DNA junction site (Fig. 7). Clone 3P contained, in addition, an SV40 DNA-SV40 DNA junction. The sequences of all these junctions are shown in Fig. 7. The arrows in Fig. 7 indicate the end of SV40 DNA sequences. The sequences downstream from the arrows are presumably host chromosomal DNA sequences (except for the first junction in clone 3P, which is an SV40 DNA-SV40 DNA junction). The positions of the arrows mark the exact junction sites. However, since the chromosomal DNA sequences prior to integration are not known, the actual junction sites may be several base pairs upstream of the arrows if there are several base pairs of homology between the SV40 DNA and chromosomal DNA at the sites. In the case of clone 3P, the SV40 DNA-SV40 DNA junction site apparently does not involve sequence homology at the junction sites.

To test whether topoisomerase II-cleavable complexes are directly involved in VM-26-induced integration, in vitro topoisomerase II cleavage sites in the presence of VM-26 were mapped at the sequence level by using uniquely 5′-end-labelled, PCR-amplified fragments of SV40 DNA. A hot spot for topoisomerase II-mediated cleavage was determined to occur between nucleotides 367 and 368 on the sense strand (early RNA) of SV40 DNA (Fig. 8). Topoisomerase II polypeptide is presumably covalently linked to the 5′ phosphate of nucleotide 367. The corresponding cleavage site on the complementary strand for this staggered double-strand break is between nucleotides 363 and 364. This major cleavage site maps to the junction sites of clones 4R and 4T. In both clones 4R and clone 4T, the ends of the SV40 DNA

TABLE 1. Effects of enzyme inhibitors on VM-26-induced formation of HMW DNA

Inhibitor[a]	Concn	% Inhibition
Aph	30 μM	70
5FU	10 μM	60
HU	5 mM	50
CHX	100 μM	50
DRB	100 μM	0
DNP	1 mM	70
3AB	3 mM	0
ST638	100 μM	100
TLCK	270 μM	100
Camp	50 μM	70–80

[a] Inhibitors were added to SV40-infected BSC-1 cells (20 h postinfection) either 15 min (Aph, CHX, and Camp) or 30 min (5FU, HU, DRB, DNP, 3AB, ST638, and TLCK) prior to addition of 100 μM VM-26. Incubation was continued for 5 h at 37°C. The cells were washed, and the incubation was continued for an additional hour in MEM-10. The samples were worked up as described in Materials and Methods. Abbreviations: Aph, aphidicolin; 5FU, 5-fluoruracil; HU, hydroxyurea; CHX, cycloheximide; DNP, dinitrophenol; 3AB, 3-aminobenzamide; Camp, camptothecin lactone.

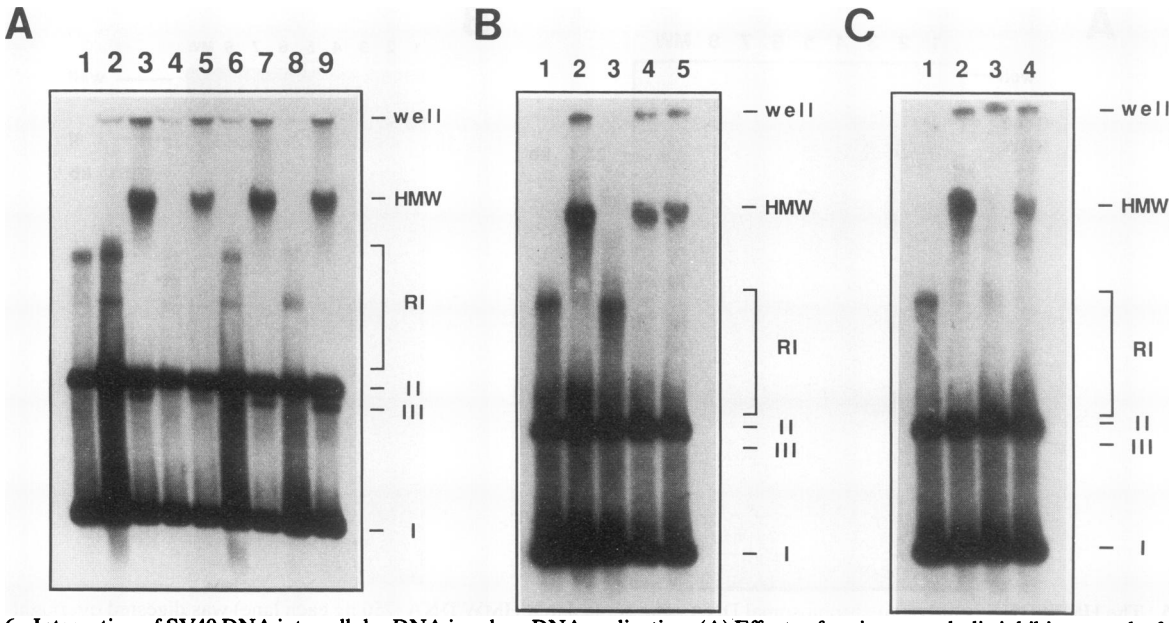

FIG. 6. Integration of SV40 DNA into cellular DNA involves DNA replication. (A) Effects of various metabolic inhibitors on the formation of VM-26-induced HMW DNA were analyzed. BSC-1 cells were infected with SV40 and treated with VM-26 and/or various metabolic inhibitors at 20 h postinfection. All metabolic inhibitors were added 30 min prior to the addition of VM-26 (100 μM in 0.2% dimethyl sulfoxide [DMSO]). Treatment continued for 5 h and then for 1 h in inhibitor-free MEM-10. Cells were lysed with SDS and treated with proteinase K. The samples were electrophoresed in a 0.7% agarose gel with 0.5× TPE buffer at 1.6 V/cm for 20 h. Southern analysis with pA26 DNA was done to probe for SV40-containing sequences. Lane 1, zero time control, no inhibitor treatment, and cells lysed 20 h postinfection; lanes 2, 4, 6, and 8, treatment with 0.2% DMSO and the indicated metabolic inhibitor, lanes 3, 5, 7, and 9, treatment with 100 μM VM-26 and the indicated metabolic inhibitor. Lanes 2 and 3, no metabolic inhibitor; lanes 4 and 5, 30 μM aphidicolin; lanes 5 and 7, 100 μM DRB; lanes 8 and 9, 100 μM cycloheximide. (B) Effects of aphidicolin treatment on the formation of VM-26-induced HMW DNA. VM-26 (100 μM) was added to infected cells at 20 h postinfection. Aphidicolin (30 μM) was added either 30 min prior to VM-26 (lane 4) or at the same time as VM-26 (lane 5). Treatment continued for 5 h. Cell lysis and DNA isolation were done as described in for panel A. Lane 1, untreated control, lysed at 20 h postinfection; lane 2, VM-26 treatment only; lane 3, aphidicolin treatment only. (C) Effects of camptothecin treatment on the formation of VM-26-induced HMW DNA. Experimental protocol was as described for panel B, except that camptothecin (50 μM) was used in place of aphidicolin as the metabolic inhibitor. Lane 1, no treatment, cells lysed at 20 h postinfection; lane 2, VM-26 treatment only; lane 3, camptothecin treatment only; lane 4, camptothecin and VM-26 cotreatment; camptothecin was added 30 min prior to VM-26. Abbreviations are defined in the legend to Fig. 1.

sequences in the integrant sequences are identical and correspond to the topoisomerase II cleavage site. The divergent chromosomal DNA sequences in these two clones suggest that this region of the SV40 DNA is a hot spot for integration. Additionally, a hot spot for VM-26-induced topoisomerase II cleavage was mapped between nucleotides 1771 and 1772 on the strand corresponding to the sense strand for the early RNA. This cleavage site corresponds exactly to one of the internal SV40-SV40 junction sites for clone 3P. The correlation between the junction sites and VM-26-induced, topoisomerase II-mediated cleavage suggests a direct involvement of topoisomerase II in the process of illegitimate recombination induced by VM-26.

DISCUSSION

Topoisomerases have been shown to affect recombination in both prokaryotic and eukaryotic cells. In *Escherichia coli*, a *topA* null mutant has been shown to reduce plasmid recombination 1,000-fold (13). *E. coli* DNA topoisomerase II has been suggested to be involved in both illegitimate and homologous recombination (20, 25). Poisoning of *E. coli* topoisomerase II with oxolinic acid stimulates both recombination processes (20, 25). Poisoning of mammalian topoisomerase II with VM-26 stimulates deletions in the plasmid DNAs that have been transfected into COS cells (1). Studies with yeast cells have also suggested a complex role for topoisomerases in recombination. Topoisomerases can suppress mitotic recombination between genes coding for rRNA (rDNA) repeats. Mutations in either the *TOP1* or the *TOP2* gene increase mitotic recombination between rDNA repeats (9). A related study has revealed a different role for topoisomerase activities in affecting the directionality of excision and integration of rDNA repeats (23). The yeast *TOP3* gene was originally isolated as a hyperrecombination yeast mutant (35). These studies indicate that topoisomerases may participate in recombinational processes in a complex manner. The catalytic activities of topoisomerases may affect recombination (e.g., suppression of rDNA recombination) quite differently from their aborted cleavage reaction intermediates (e.g., stimulation of homologous and nonhomologous recombination).

Our results show that poisoning topoisomerase II leads to rapid and efficient induction of the HMW DNA in SV40-infected BSC-1 cells. We conclude that the HMW DNA, induced by topoisomerase II poisoning, is integrated SV40 DNA. Our conclusion is based on three major pieces of evidence. First, digestion of the HMW DNA with restriction enzymes that do not cut SV40 DNA reduced the size of the HMW DNA. This result strongly suggests that the HMW DNA represents SV40 DNA physically joined to host DNA. Second, digestion of the HMW form of SV40 DNA with

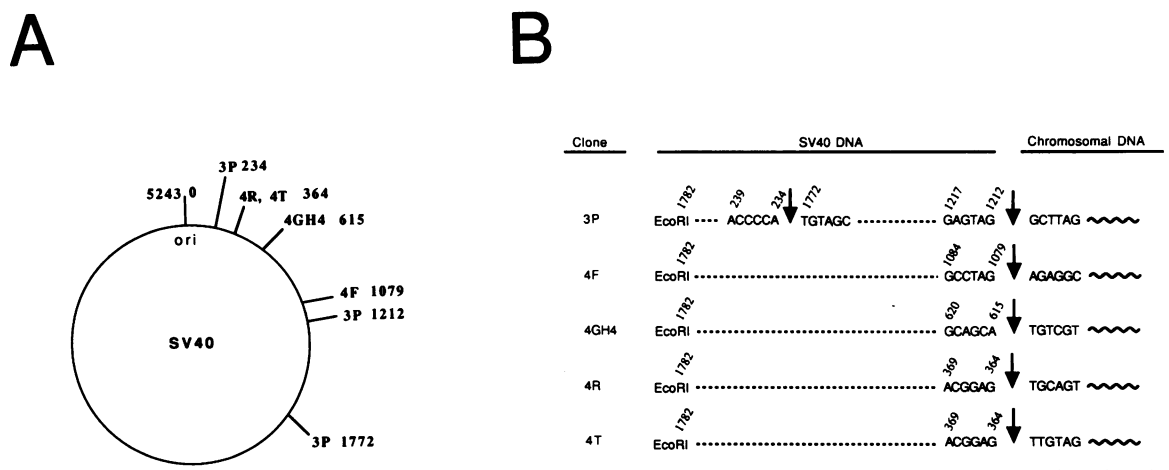

FIG. 7. Sequence analysis of the junction sites on cloned integrants. (A) Diagram showing the locations of the seven junction sites on the SV40 DNA map. Clone 3P is listed three times, as it is a molecule in which SV40 recombined with a second SV40 molecule in addition to integrating into the host chromosome. (B) Nucleotide sequences at the junctions sites of five cloned integrants. The SV40 DNA and chromosomal DNA sequences at the junction sites are shown for each of the five clones. Clone 3P contains an internal junction site which represents the recombination between two SV40 molecules in addition to the SV40-host chromosome junction site. The nucleotide numbering refers to the nucleotide position on SV40 DNA.

restriction enzymes that have a single cutting site on SV40 DNA converted only 10% of the HMW DNA into unit-length linear DNA. The majority of the HMW DNA was converted into heterogeneously sized fragments which comigrated with digested host DNA. The 10% recovery of the HMW DNA as unit-length linear SV40 DNA at limiting digestion suggests that the HMW DNA contains either 10% head-to-tail linear concatemeric SV40 DNA or 10 to 20% integrated SV40 DNA with fewer than two tandem repeats. Third, partial digestion of the HMW DNA with a singly cutting restriction enzyme did not produce a ladder of multimeric linear SV40 DNAs. This result is inconsistent with the HMW DNA being head-to-tail linear concatemeric SV40 DNA. One explanation is that all the HMW DNA represents integrated SV40 DNA.

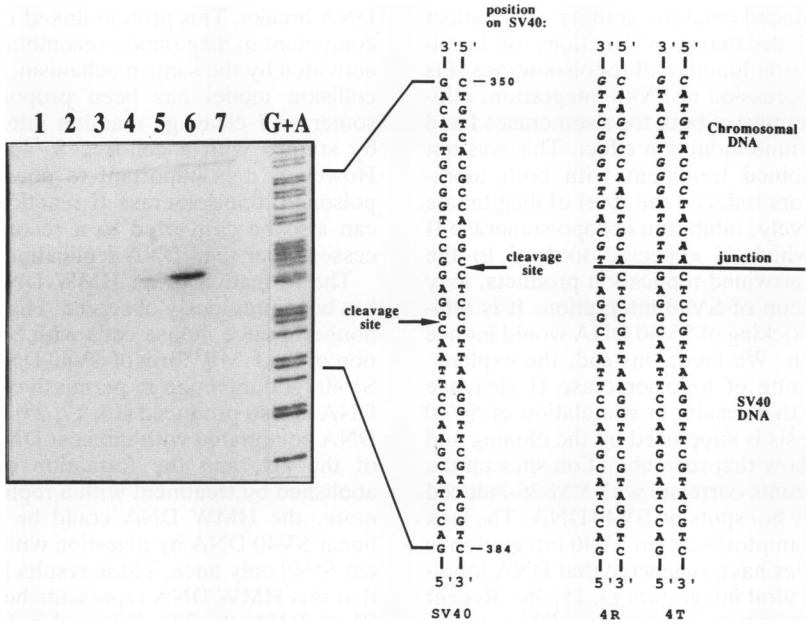

FIG. 8. Recombination occurs at or near topoisomerase II-mediated cleavage hot spots on SV40 DNA. VM-26-induced, topoisomerase II-mediated DNA cleavage was mapped at the sequence level on a fragment of SV40 DNA known to contain the site of recombination for two clones, 4R and 4T. A 591-bp fragment of SV40 DNA, between nucleotides 573 and 5225, was amplified by PCR and then 5′ end labelled with T4 kinase and [γ-^{32}P]ATP. Subsequent digestion with *BglI* produced uniquely end-labelled DNA, with the label on nucleotide 573. Part of the DNA was subjected to G+A chemical sequencing, while the remainder was treated with calf thymus topoisomerase II and various concentrations of VM-26 for 30 min. Lane 1, labelled DNA, 1% dimethyl sulfoxide (DMSO), no enzyme, no drug; lanes 2 to 7, labelled DNA, 1% DMSO, plus 20 ng of calf thymus topoisomerase II; lanes 3 to 7, labelled DNA, 20 ng of calf thymus topoisomerase II, plus 0.03, 0.3, 3.0, 30, and 300 μM VM-26, respectively; lane G+A, products of G+A chemical sequencing of the DNA fragment.

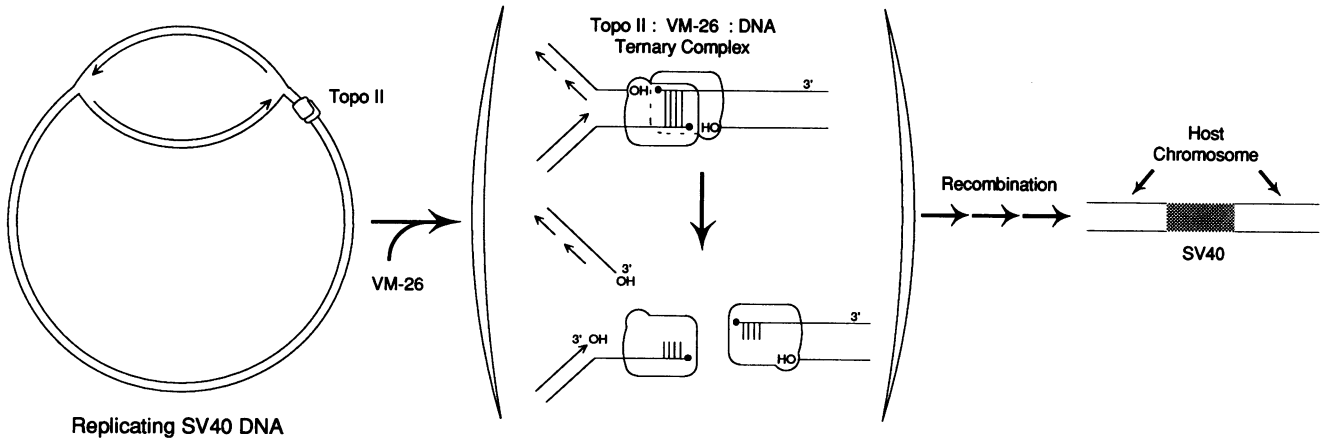

FIG. 9. Model for a possible interaction between a replication fork and topoisomerase II-cleavable complexes.

Some of the SV40 DNA can integrate into the host DNA as tandem repeats, but the number of repeats for those integrants is very small (e.g., two). Homologous recombination between two SV40 DNA molecules prior to integration can result in two tandem repeats in the integrants. The small fraction of nicked circular dimer we have observed in VM-26-treated cells may be due to such a recombinational event. Alternatively, specific breakage of the replication forks (e.g., breakage only at the leading-strand side of the fork) due to topoisomerase poisoning can also generate partial duplication of SV40 sequences.

The stimulatory effect of VM-26 on SV40 integration could be caused by reduced topoisomerase II catalytic activity, poisoned topoisomerase II-cleavable complexes, or a combination of both. The reduced catalytic activity could affect both the relaxation and decatenation functions of topoisomerase II. If the relaxation function of topoisomerase II is normally involved in suppression of SV40 integration, inhibition of the relaxation activity of both topoisomerases I and II may have a more profound induction effect. This was not observed. In fact, combined treatment with both topoisomerase I and II inhibitors reduced the level of illegitimate recombination. Alternatively, inhibition of topoisomerase II decatenation activity, which is expected to lead to the formation of multiply intertwined replication products, may be responsible for induction of SV40 integration. It is difficult to imagine how interlocking of SV40 DNA would induce illegitimate recombination. We favor, instead, the explanation that it is the poisoning of topoisomerase II cleavage intermediates by VM-26 that results in stimulation of SV40 integration. This hypothesis is supported by the cloning and sequencing data which show that recombination sites on the SV40 portion of the integrants correlate with VM-26-induced topoisomerase II cleavage hot spots on SV40 DNA. The lack of a significant effect of camptothecin on SV40 integration is surprising. Previous studies have suggested that DNA topoisomerase I is involved in viral integration (3, 15, 36). Recent studies have also shown that vaccinia virus DNA topoisomerase (a type I DNA topoisomerase with a M_r of 32,000) can promote illegitimate recombination in *E. coli* (32). It is possible that the extremely high frequency of illegitimate recombination produced by poisoning topoisomerase II overshadows the very low frequency of illegitimate recombination mediated by topoisomerase I in our system.

DNA replication appears to be involved in the integration of SV40 DNA following topoisomerase II poisoning. However, the fact that residual integration still occurs in the absence of DNA synthesis suggests the presence of a parallel pathway(s). Previous studies of the cytotoxic mechanisms of topoisomerase II poisons have suggested that there are at least two parallel pathways that can trigger cell death; one involves DNA replication (39), and the other involves transcription (11). It seems possible that both processes also trigger illegitimate recombination. A hypothetical interaction between replication forks and poisoned topoisomerase II-DNA complexes is shown in Fig. 9. The interaction between an advancing replication fork and a poisoned topoisomerase II complex results in fork breakage and the conversion of the reversible cleavable complex into topoisomerase II-linked DNA breaks. This protein-linked DNA break may be highly competent in illegitimate recombination. Host DNA may be activated by the same mechanism. A similar replication fork collision model has been proposed for poisoned topoisomerase I cleavage reaction intermediates and confirmed by studies with a cell-free SV40 replication system (18). However, it is important to point out that the reversibly poisoned topoisomerase II reaction intermediates probably can also be converted to a recombinogenic form by processes other than DNA replication (39).

The formation of an HMW DNA in SV40-infected cells has been previously observed. High-multiplicity infection of nonpermissive mouse cells with SV40 results in the formation of the HMW form of SV40 DNA (8). At the late stage of SV40 lytic infection in permissive monkey cells, the HMW DNA is also produced (16, 17, 27). In both cases, the HMW DNA comigrated with the host DNA at the limiting mobility of the gel, and the formation of the HMW DNA was abolished by treatment with a replication inhibitor. Furthermore, the HMW DNA could be converted to unit-length linear SV40 DNA by digestion with restriction enzymes that cut SV40 only once. These results have led to the suggestion that this HMW DNA represents head-to-tail concatemers of SV40 DNA (8, 27). While this HMW DNA may be the precursor for viral integration, it is not by itself an integrant. The striking similarity in the properties of the HMW DNA produced in these two systems versus our own suggests a common pathway leading to the formation of the HMW DNA.

Topoisomerase II is a sensitive target for poisoning by many compounds of extremely diverse structures (29).

These topoisomerase II targeting drugs are known to induce increased levels of illegitimate recombination. Our results show that topoisomerase II appears to be intimately involved in the molecular mechanism of this process. Cloning of the SV40-host chromosomal junctions of these integrants shows that the junction sites precisely align with the VM-26-induced topoisomerase II cleavage sites on SV40 DNA. These results indicate that recombination occurs at or near topoisomerase II cleavage hot spots induced by VM-26. Although VM-26 was used in our studies, topoisomerase II may participate in certain cellular illegitimate recombination events, albeit at a much lower frequency, in the absence of this drug. The hot spots for these illegitimate recombination events would be expected to correlate with the natural cleavage hot spots of topoisomerase II. Additionally, a mutant topoisomerase II might produce higher levels of topoisomerase II-linked DNA breaks which could result in a higher basal level of DNA rearrangements.

These studies have important implications for understanding the mechanisms of some types of chemically induced carcinogenesis and certain types of drug resistance caused by DNA rearrangements. Further studies are necessary to firmly establish the mechanism of drug-induced, topoisomerase II-mediated, illegitimate recombination and to confirm the relevance of this process to many drug-mediated cellular phenomena.

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