Effects of VM26 (teniposide), a specific inhibitor of type II DNA topoisomerase, on SV40 DNA replication *in vivo*

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ABSTRACT

Simian Virus 40 (SV40) infected cells were pulse labeled with (³H) thymidine and chased either in the absence or in the presence of the cytotoxic drug VM26 (teniposide). We investigated the structure of labeled SV40 DNA and found that VM26 had no significant effect on replicative chain elongation but strongly inhibited the conversion of late replication intermediates to mature DNA daughter molecules. The late replicative SV40 DNA intermediates which accumulate in VM26 treated cells contained essentially full length labeled DNA strands. These newly synthesized strands were not part of two catenated interlocked SV40 monomers suggesting that the block occurred prior to the final ligation reaction. Since VM26 is known to be a specific inhibitor of DNA topoisomerase II we conclude that this enzyme is dispensable for the chain elongation of replicating SV40 DNA, but that it is essential for the termination of SV40 DNA replication cycles.

INTRODUCTION

Type II DNA topoisomerase has been detected in all eukaryotic cells investigated.

The enzyme catalyses *in vitro* the isomerisation of topologically linked DNA molecules introducing an enzyme bridged double strand break staggered by four base pairs. Each subunit of the homodimeric enzyme becomes covalently bound to a 5' end via a tyrosylphosphate linkage. Another DNA segment is then passed through the double strand break which is finally resealed on the expense of the energy trapped in the enzyme-DNA linkage. By this mechanism, DNA topoisomerase II changes the linking number of topologically linked DNA molecules in steps of two and causes the knotting or unknotting of DNA strands (summarized in ref.nr. 1, 2, 3).

The biochemistry of eukaryotic DNA topoisomerase II is reasonably well understood from *in vitro* studies. However, data concerning the *in vivo* functions of the enzyme are relatively scarce. It has been proposed that DNA topoisomerase II may be involved in transcription, chromatin organisation and DNA replication (1, 2, 3).

Studies with yeast strains, mutated in the DNA topoisomerase II gene, suggest a function in the segregation of the intertwined replication products

(4,5). A similar function has been proposed for mammalian DNA topoisomerase II. However, this proposition was based on indirect evidence (6), and an independent confirmation would be important. It has also been proposed that DNA topoisomerase II may be required to remove the torsional stress introduced by positive supercoiling which may occur during the replication of topologically fixed DNA molecules (3).

To investigate this and related questions we choose to study the effects of VM26 (teniposide; 4'-demethylepipodophyllotoxin- 4-(4,6-O-thenylidene- β -D-glucopyranoside)), an inhibitor of DNA topoisomerase II (7, 8), on Simian Virus 40 (SV40) DNA replication in vivo.

Circular, double stranded SV40 DNA, 5243 base pairs long (reviewed in ref.nr.9), is associated with about 25 nucleosomes forming a chromatin structure which resembles cellular chromatin, both, in its resting state (10) and during DNA replication (11). SV40 chromatin is replicated by host cell enzymes, including cellular DNA polymerase α and DNA primase (12, 13, 14), except for the viral "early" gene A product which serves as an initiator protein (15, 16) and as a DNA helicase (17).

The outlines of the SV40 replication cycle are fairly well understood (reviewed in ref.nr.18, 19). Initiation occurs at a defined section of the viral genome, the origin of replication, where two replication forks are established which move bidirectionally and meet in the termination region, located just opposite of the origin on the circular DNA. The experimentally convenient size of SV40 chromatin, its similarity to cellular chromatin and its dependence on host cell functions for DNA replication make the SV40 system a good model to probe for the function of components of the replication apparatus of the cell.

The in vivo action of VM26 on mammalian cells has been studied in some detail. It could be shown, for example, that the drug is rapidly taken up by the cell where it appears to cause extensive breakage of double stranded DNA with DNA topoisomerase II covalently bound to the 5' ends. These results have been interpreted to mean that the action of DNA topoisomerase II is inhibited after the strand scission event (20, 21).

We use the cytotoxic antitumor drug VM26 to specifically inhibit the action of DNA topoisomerase II. The activities of DNA topoisomerase I, DNA primase and DNA polymerase α are not affected by this drug (unpublished).

In this communication we present data showing that VM26 inhibits SV40 DNA replication. Pulse-chase experiments demonstrate that the progression of the replication forks is not much affected in drug treated cells. It rather appears that one of the last steps in the replication cycle, including termination and segregation of daughter molecules, is blocked by VM26 suggesting that in mammalian systems a function of DNA topoisomerase II is required for the termination of replication cycles and the segregation of replicating DNA molecules.

MATERIAL AND METHODS

Cells and Virus.

The African Green Monkey Kidney Cell line TC7 was cultivated as described before (22). The cells were infected at half confluency with wild type SV40 at a multiplicity of 20. For long term labeling 10 μ Ci (14 C) thymidine/ml was added to the culture medium at 24 h after infection for about 12 h. The cells were then pulse labeled with 200 μ Ci (3 H) thymidine in a total volume of 2ml culture medium per 14cm (diameter) plate. In pulse-chase experiments, the radioactive medium was removed and the cells were washed twice with prewarmed medium containing 50 μ M non radioactive thymidine. The chase reaction was performed in 5ml prewarmed culture medium containing 50 μ M thymidine and either VM26 (final concentration: 100 μ M) in DMSO (0.2% final concentration) or DMSO (0.2% final concentration) alone. The DNA products formed in the presence of DMSO are indistinguishable from those synthesized in control experiments without DMSO. We have analyzed the effects of VM26 on SV40 DNA replication at concentrations of 50, 100 and 200 μ M and obtained identical results.

Extraction and analysis of DNA.

The infected cells were rinsed several times with prewarmed buffered saline before the cells were lysed with EDTA and sodium dodecyl sulfate (23). Viral DNA was prepared according to the Hirt procedure (23). The Hirt supernatants, containing the labeled SV40 DNA, were first treated with RNase A (10 μ g/ml) for 1 h and Proteinase K (100 μ g/ml) for 2 h at 50°C. The DNA was then purified by phenol/chloroform extraction and ethanol precipitation.

In some experiments, sodium dodecyl sulfate was avoided for viral DNA extraction. In these cases, SV40 chromatin was first eluted from isolated nuclei (6) and used to extract the DNA with 1M NaCl and proteinase K. The DNA was analyzed by zone velocity centrifugation using neutral or alkaline sucrose gradients as described before (22). Electrophoresis was performed in 1-2% horizontal agarose gels in 40mM Tris, 5mM sodium acetate, 1mM EDTA, pH8.0 ("neutral" gel electrophoresis) or, after alkaline denaturation, in 30mM NaOH, 3mM EDTA, 1mM EGTA ("alkaline" gel electrophoresis). The gels were either processed for fluorography (Amplify/ Amersham), or, alternatively, individual lanes of the gel were cut into 1mm slices which were then dissolved in 0.1M HCl and Aquasol. The radioactivity was determined by liquid scintillation counting (22).

Potassium dodecyl sulfate precipitation.

Potassium dodecyl sulfate precipitation was initially described by Trask and Muller (24). Briefly, Hirt supernatants were desalted on PD10 columns (Pharmacia) in the presence of 1% sodium dodecyl sulfate. Potassium chloride was then added to give a final concentration of 500mM. The solution was then

kept at 37°C for 10min. After additional 5min on ice a precipitate formed which was pelleted (2000xg, 10min, 0°C) and washed three times with 500mM potassium chloride in 10mM Tris HCl (pH7.6), 1mM EDTA. The pellet was resolved in 2ml wash puffer and digested with proteinase K (100µg/ml) for 12 h at 37°C. Finally the solution was chilled on ice to precipitate potassium dodecyl sulfate complexes. SV40 DNA remained quantitatively in the supernatant and was phenol/chloroform extracted, precipitated with ethanol and processed as described in the figure legends.

Enzymes and Chemicals.

All enzymes were purified from calf thymus to homogeneity. The specific activity was 0.5×10^6 units/mg for DNA topoisomerase I, 7.5×10^6 units/mg for DNA topoisomerase II, 15000 units/mg for DNA polymerase α and 800 units/mg for DNA primase. A detailed purification protocol will be described elsewhere. VM26 (98% pure) was a generous gift of Dr.W.Achterrath (Bristol Arzneimittel, Neu-Isenburg).

RESULTS

Effects of VM26 on DNA topoisomerase II in vitro.

It will be useful to briefly consider in this section the mechanism by which VM26 inhibits the action of DNA topoisomerase II in a biochemical assay system. The *in vivo* effects of VM26 will be described later.

Purified calf thymus DNA topoisomerase II was assayed measuring the relaxation of superhelical SV40 DNA in an ATP dependent reaction. Concentrations of 50-100µM VM26 were sufficient to completely block the relaxation of SV40 form I DNA by DNA topoisomerase II (Fig.1A). The structure of the DNA isolated from a VM26 containing reaction mixture depended on the procedure used to terminate the reaction cycle of the enzyme. When DNA topoisomerase II was inactivated by EDTA and digested by proteinase K prior to the sodium dodecyl sulfate treatment we obtained intact DNA circles (Fig.1B; lane 2). However, when sodium dodecyl sulfate was used to inactivate the enzyme we recovered a significant fraction of the DNA in a linearized form (Fig.1B; compare lane 3 to lane 4), which could be precipitated as a potassium dodecyl sulfate complex (Fig.1C; lane 3). These precipitable protein-DNA complexes were not observed when the enzyme was digested with proteinase K prior to the formation of potassium dodecyl sulfate (not shown; see ref.nr. 24).

The observations shown in Figure 1 suggest that VM26 inhibited the strand passing activity of DNA topoisomerase II by stabilizing the covalent enzyme-DNA intermediate of the reaction cycle. Treatment with denaturing agents resulted in the linearisation of DNA and the appearance of covalent DNA topoisomerase II-DNA complexes. These complexes could be specifically precipitated from the reaction mixture as potassium dodecyl sulfate

Fig.1 Inhibition of DNA topoisomerase II.

(A) DNA topoisomerase II (3fmol) was assayed in a 50 μ l volume containing 20mM Tris-HCl(pH7.6),150mM NaCl, 1mM DTT, 0.2mg/ml bovine serum albumin, 10mM MgCl₂, 1mM ATP and 0.6 pmol SV40 form I DNA. After 30min at 37°C the reaction was stopped by the addition of EDTA at a final concentration of 50mM. The drug VM26 was added in the following concentrations: 100 μ M (lane 1), 50 μ M (lane 2), 10 μ M (lane 3), 2 μ M (lane 4), 0.5 μ M (lane 5). The reaction products obtained in the absence of VM26 are shown in lane 6. SV40 DNA, incubated without enzyme under standard conditions in the presence of 100 μ M VM26 is shown in lane 7 and control SV40 DNA in lane 8.(B) 0.6pmol SV40 DNA was incubated with 3pmol DNA topoisomerase II under standard conditions with (lane 2 and 3) or without (lane 4) VM26. Reactions were terminated by addition of EDTA (lane 2) or sodium dodecyl sulfate (final concentration: 1%; lane 3 and 4). The reaction mixture was proteinase K digested prior to electrophoresis. (C) Reactions were performed as in (B) using (³H) labeled SV40 DNA. The reaction products were precipitated with potassium dodecyl sulfate as described in Material and Methods. The fraction of precipitable radioactivity is presented.

complexes. However, when the enzyme was first inactivated by EDTA the VM26 stabilized double strand break was sealed. Consequently, no complexes could be precipitated by potassium dodecyl sulfate. These results will be important for the interpretation of the data obtained after extraction of SV40 DNA from VM26 treated infected cells.

Effects of VM26 on the maturation of replicative intermediates in vivo.

We have found, in agreement with previous reports on the related drug VP16, that VM26 drastically inhibits the uptake of (³H) thymidine into culture cells (25). For example, a concentration of 100 μ M VM26 in the culture medium reduced the uptake of (³H) thymidine to 80% of a control value obtained in the absence of VM26. Due to this properties of podophylotoxin derivatives all methods using the incorporation of radioactively labeled deoxynucleosides to measure intracellular DNA synthesis are of limited value in these experiments. We therefore pulse-labeled infected cells with (³H) thymidine in absence of VM26. The fate of the replicative intermediates was

then investigated during a chase in the presence of an excess of nonradioactive thymidine and 100 μ M VM26. The results, obtained in a typical pulse-chase experiment are shown in Fig.2. In this experiment, SV40 infected TC7 cells were first uniformly labeled for 12 h with (14 C) thymidine to obtain mature non-replicating DNA as an internal marker. The cells were then pulse labeled for 5min with (3 H) thymidine. SV40 DNA was extracted by the Hirt procedure and investigated by zone velocity gradient centrifugation. As reported previously (26), the majority of replicating (3 H) pulse labeled SV40 DNA sedimented faster (22S-27S) than long term (14 C) labeled mature SV40 DNA (21S) (Fig.2A). After a 20min chase in the absence of VM26, essentially all pulse labeled (3 H) SV40 DNA sedimented like the (14 C) labeled marker DNA suggesting a maturation of replicating DNA (Fig.2B). A different result was obtained when the chase was carried out in the presence of 100 μ M VM26. In this case, a considerable fraction of the (3 H) labeled SV40 DNA remained at the position of the faster sedimenting replicative structures (Fig.2C), showing that VM26 inhibited the maturation of replicating SV40 DNA. Another fraction of (3 H) labeled, chased DNA sedimented like or somewhat slower than the (14 C) marker SV40 DNA. This fraction could include mature replicated DNA and linearized SV40 DNA molecules. To investigate this possibility we performed a centrifugation analysis at pH12.5 which showed that, in the absence of VM26, most replicating intermediates had matured to give

Fig.2 Inhibition of SV40 DNA replication *in vivo*.

Replicating SV40 DNA was pulse labeled with (3 H) thymidine (—●—) for 5min (A, D) and chased for 20min in absence (B,E) or presence (C,F) of 100 μ M VM26. Total SV40 DNA was isolated from Hirt supernatants as described and analyzed by sucrose gradient centrifugation under neutral (A-C) or alkaline (D-F) conditions. Mature SV40 DNA was long term labeled with (14 C) thymidine (—○—).

superhelical form I SV40 DNA which sedimented as a random coil at about 53 S in an alkaline sucrose gradient (Fig.2E). In contrast, only a small fraction of pulse labeled replicative intermediate DNA was converted to superhelical DNA when VM26 was present during the chase (Fig.2F). The nature of the fast sedimenting (^3H) labeled SV40 DNA, after a 20 min pulse in the presence of VM26 (see Fig.2C) was further investigated by agarose gel electrophoresis (Fig.3). Again, (^3H) labeled SV40 DNA was prepared from Hirt supernatants after a 5 min pulse and after 20 min chase both, in the absence and in the presence of $100\mu\text{M}$ VM26. The DNA was separated on agarose gels and visualized by fluorography. We found, as expected from previous experiments (22, 26), a broad spectrum of pulse labeled replicative intermediate DNA (Fig.3, lane 1) which was converted to mature form I and form II DNA during a normal chase (Fig.3, lane 2). After a chase in the presence of VM26 the heterogeneous fraction of intermediate sized replicative SV40 DNA had disappeared. Instead slow migrating structures, most likely late Cairns' forms accumulated together with linearized unit length SV40 DNA and some mature form I DNA (Fig.3, lane3).

We have performed several experiments of the type shown in Figure 2 with pulses from 1 to 4 min and chase times up to 40 min. A summary of the results is shown in Figure 4. In agreement with previous reports (22), we observed that pulse labeled replicative intermediates were normally converted to mature DNA within 20 min. Clearly, VM26 reduced the efficiency of this process. Even after chase periods of 40 min only about 25 percent of (^3H) pulse labeled DNA was found in the form of mature superhelical DNA.

The experiments, shown in Figure 2 and 3, were repeated using a DNA

Fig.3 Neutral agarose gel electrophoresis of SV40 replication products.

Replicating SV40 DNA was pulse labeled for 5min with (^3H) thymidine (lane 1). A chase reaction was performed for 20min in the absence (lane 2) or in the presence (lane 3) of VM26. The DNA was recovered from Hirt supernatants. No (^{14}C) thymidine label was used in this experiment. (ICS= intermediate Cairns' structures; LCS= late Cairns' structures; I= superhelical SV40 monomer DNA; II= relaxed SV40 monomer DNA; III= linearized SV40 DNA).

Fig.4 Maturation of replicating SV40 DNA. Replicating SV40 DNA was pulse labeled for 5 min. The labeled DNA was chased for 2, 5, 10, 20 and 40 min in the absence (-o-) or in the presence (-●-) of VM26. Total SV40 DNA was isolated from Hirt supernatants as described and analyzed by alkaline sucrose gradient centrifugation (see Fig.2, C and F). The amount of (^3H) labeled DNA, sedimenting with 5.3 S, is presented as the percentage of total (^3H) labeled DNA.

extraction procedure in which the sodium dodecyl sulfate treatment of the SV40 chromatin was avoided (see: Material and Methods). (^3H) labeled SV40 DNA, isolated without sodium dodecyl sulfate from VM26 treated cells, had the same sedimentation and electrophoretic properties as the SV40 DNA prepared by the Hirt procedure (data not shown) except for the fact, that linearized DNA molecules could not be observed and that no complexes, precipitable by potassium dodecyl sulfate, were obtained (see Fig.1 and the following section).

It has been reported, that SV40 catenanes, multiply interlocked superhelical DNA circles accumulate in infected cells, kept at hypertonic growth conditions. This result had been interpreted to mean, that a DNA topoisomerase II function was inhibited under these conditions causing a block in the segregation of replicated DNA molecules but not in the termination of DNA synthesis cycles. We obtained catenated SV40 DNA (C catenanes) under hypertonic growth conditions exactly as previously described (6). However, no such structures were found in VM26 treated cells (data not shown).

VM26 inhibits the termination of SV40 DNA replication.

Detergent lysed cells offer the opportunity to investigate DNA molecules carrying covalently linked DNA topoisomerase II. We found indeed that DNA-protein complexes became precipitable by potassium dodecyl sulfate soon after VM26 treatment of the cells. With increasing length of the chase period, the fraction of precipitable pulse labeled DNA increased suggesting an accumulation of structures carrying covalently bound DNA topoisomerase II (Fig.5). To learn more about DNA chain growth in the presence of VM26 we investigated the precipitable DNA using alkaline gel electrophoresis. Pulse labeled DNA extracted from the Hirt supernatant before the chase was purified by proteinase K treatment and phenol/chloroform extraction. The DNA samples taken at times after the chase in the presence of VM26 were first precipitated with potassium dodecyl sulfate and then purified as

Fig.5 Potassium dodecyl sulfate precipitation of replicating SV40 DNA.

Replicating SV40 DNA was pulse labeled for 5 min with (³H) thymidine and chased for 2, 5, 10, and 20 min with 100μM VM26. Hirt supernatants were prepared and potassium dodecyl sulfate precipitation was performed as described under Material and Methods. The percentage of precipitable radioactivity was determined for (³H) pulse labeled DNA (—●—) and for (¹⁴C) long term labeled DNA (—○—) as well as for (³H) pulse labeled DNA from control experiments without VM26 (—Δ—). No precipitable DNA was recovered when the samples had been treated with proteinase K before addition of potassium chloride to the Hirt supernatants.

described in Material and Methods. The DNA was denatured and analyzed by gel electrophoresis at pH12.5. To facilitate a quantitative evaluation of the results individual tracks of the gel were cut into 1 mm slices to determine the radioactivity by liquid scintillation counting (Fig.6). The pulse labeled DNA migrated in a broad spectrum ranging from a few hundred to four thousand nucleotides (Fig.6A). With increasing chase times in the presence of VM26 the (³H) DNA strands became more uniform and finally reached within 20 min the size of denatured unit length SV40 DNA strands (Fig.6B-E).

Fig.6 Size distribution of potassium dodecyl sulfate precipitable SV40 DNA. Replicating SV40 DNA was pulse labeled and chased as described under Figure 5. Protein-DNA complexes were precipitated from Hirt supernatants using potassium chloride. SV40 DNA was purified from the precipitates and separated on 1.8% alkaline agarose gels. Pulse: total pulse labeled (³H) SV40 DNA; VM26. 2 min - 20 min: precipitable (³H) labeled DNA after various chase times in the presence of VM26. Marker: (A) EcoR I restricted SV40 DNA, 5.2 kb; (B) SV40 Hind III A-fragment, 1.7 kb; (C) SV40 Hind III B,C-fragment, 1.1 kb; (D) SV40 Hind III D,E-fragment, 0.5 kb; (E) SV40 Hind III F-fragment, 0.2 kb. The resolution of the gel is approximately 260 bp between individual points.

Fig.7 Restriction enzyme digest of potassium dodecyl sulfate precipitable SV40 DNA.

Pulse labeled DNA was chased in the presence of VM26 for 20min. Potassium dodecyl sulfate precipitable DNA was isolated from Hirt supernatants and purified as described. The sample was divided into three portions. 2 μ g of unlabeled SV40 form I DNA was added to serve as internal marker and the DNA was digested with Bgl I (B) or Bam HI (C). Unrestricted DNA solely incubated in restriction enzyme buffer served as control (A). The digested DNA was separated on an alkaline agarose gel. Marker: (1) Eco RI restricted SV40 DNA; (2) SV40 Hind III A-fragment; (3) SV40 Hind III B,C-fragment.

To find out whether the (^3H) labeled strands were linearized at random or not the 20 min chase DNA structures, extracted from potassium dodecyl sulfate precipitates, were treated with the restriction endonuclease Bgl I and, in a separate experiment, with Bam HI. The former enzyme cuts the origin region of the SV40 genome whereas Bam HI cuts close to the replication terminus (8). After restriction the DNA was denatured and analyzed again by alkaline gel electrophoresis. We found that the (^3H) labeled strands from Bgl I treated DNA (Fig.7B) had approximately half the size of unit length DNA whereas the (^3H) labeled strands from Bam HI restricted DNA (Fig.7C) were only slightly smaller than the undigested control (Fig.7A). Thus, the newly synthesized DNA strands were not randomly cut. It rather appeared that their ends were located around the Bam HI site, i.e. close to the replication terminus.

We conclude that VM26 caused an accumulation of late replicative intermediate DNA with almost full length newly synthesized DNA strands. Since these structures precipitated in the presence of potassium dodecyl sulfate they most likely carried an attached protein, probably DNA topoisomerase II, trapped in an abortive attempt to separate the replicating DNA molecules.

DISCUSSION

Biochemical tests show that VM26 inhibits type II DNA topoisomerase but not type I DNA topoisomerase and none of the established replication functions such as DNA polymerase α and DNA primase (unpublished data). This

specificity makes VM26 much more useful than novobiocin, a widely used DNA topoisomerase II inhibitor, which also inhibits DNA polymerase α (27) and which causes an aggregation of chromatin (28).

When VM26 was added to the culture medium of uninfected or SV40 infected cells we observed a virtually immediate reduction of the incorporation of (^3H) thymidine into acid precipitable cellular and viral DNA. The reduction was dose dependent and reached 20 percent of a control value at $100\mu\text{M}$ VM26. However, we found that the reduction of (^3H) thymidine incorporation was largely due to an inhibition of the cellular nucleoside transport system (data not shown). This property of VM26 may be due to its podophylotoxin component which had previously been shown to be a potent inhibitor of the transport of nucleosides through the cytoplasmic membrane (25).

Thus, all results obtained by (^3H) thymidine incorporation into the DNA of VM26 treated cells are difficult to interpret. We noted, however, that the reduced radioactivity which did appear in viral DNA after (^3H) thymidine pulses of VM26 treated cells was associated with structures which resembled normal replicative intermediates by all criteria tested (not shown) indicating that replicative chain elongation proceeded undisturbed in VM26 treated cells.

Since it is difficult to reliably interpret the incorporation of (^3H) thymidine into the DNA of VM26 treated cells we performed pulse-chase experiments. Untreated SV40 infected cells were labeled for a few minutes with (^3H) thymidine of high specific radioactivity followed by a chase with an excess of unlabeled thymidine in the presence of VM26. These experiments allowed us to decide whether VM26 affected the progression of the replication forks and/or the segregation of replicated daughter molecules. However, it was not possible to find out whether the drug inhibits the initiation of new rounds of replication.

A major conclusion which we like to draw from the pulse-chase experiments described above is that high doses of VM26 fail to inhibit DNA chain elongation and the progression of replication forks. This conclusion is based on the observation that small (^3H) pulse labeled DNA fragments invariably grow to full length SV40 DNA strands during a chase in the presence of VM26. We cannot entirely exclude the possibility that the rate of replicative chain elongation may be slightly reduced in VM26 treated cells, but drastic effects could not be observed.

On the other hand, VM26 had drastic effects on the rate by which late replicative intermediates were converted to mature daughter SV40 DNA molecules. Since the structures which accumulated in VM26 treated cells consist of virtually unit length DNA strands we suggest that VM26 reduced (but does not completely inhibit, see Fig.4) the segregation of the replicated products.

Given the specificity of VM26 as an inhibitor of DNA topoisomerase II we suggest that DNA topoisomerase II is not essential to remove the torsional stress which might accumulate in topologically fixed replicating SV40 DNA molecules. This function must be performed by DNA topoisomerase I or by an as yet undetected enzyme. It cannot be excluded though that DNA topoisomerase II normally functions at replication forks, but this function is dispensable and can be replaced by other enzymes. However, DNA topoisomerase II certainly performs an important function at the end of the replication cycle. It seems that this function includes the maturation of late replicative intermediates as an essential step preceding the segregation of daughter DNA molecules. We conclude this because pulse labeled DNA strands grow in the presence of VM26 until they reach unit length size. A small fraction of pulse labeled material matures to give intact form I and form II DNA, but we have no evidence for the conversion of (^3H) labeled DNA to interlocked SV40 daughter molecules. One would expect to obtain interlocked superhelical circles (C catenanes) if DNA chain growth continued without segregation.

The termination of SV40 DNA replication has been investigated in some detail. Two or three pathways have been discussed.

In hypertonic culture medium replication continues beyond the termination region producing multiply interlocked catenated dimers. The catenanes are quickly released to give complete monomeric DNA circles after a shift to isotonic medium (6, 29). A role of DNA topoisomerase II for the maturation of catenanes is immediately apparent since the ability to resolve a catenated network of interlocked double stranded DNA molecules is an established property of the enzyme. However, under normal growth conditions in isotonic culture medium late replicative intermediates usually do not mature via double stranded interlocked catenanes (30).

Another termination pathway has been recently described (11). This pathway includes hemicatenanes as intermediate structures. In SV40 hemicatenanes, two replicated SV40 DNA molecules are interlocked in the termination region via a single strand DNA bridge. These structures have been detected in relatively large numbers in SV40 infected cells at normal growth conditions. The existence of SV40 hemicatenanes, connected via single strand bridges in the termination region, may indicate a role for DNA topoisomerase I in daughter strand separation since this enzyme functions by cutting and resealing of DNA single strands.

The major termination pathway most probably proceeds via late replicative intermediates to gapped circles. It had been shown that SV40 DNA replication rapidly proceeds until 90 to 95 percent completion. Late replicating intermediates accumulate compared to molecules at earlier stages in replication. One of the last steps in replication is the unwinding of the remaining section of the parental DNA and the release of monomeric DNA

circles with gaps of up to 50 bases. DNA synthesis continues rapidly until the gap is filled (summarized in ref.nr.18). The data presented in this communication demonstrate that DNA topoisomerase II may have a function in this termination pathway. Inhibition of the enzyme by VM26 results in the accumulation of late replicating intermediates and a drastic inhibition of the maturation of SV40 DNA molecules. Thus DNA topoisomerase II could be a component of an elaborate termination complex which unwinds the last unreplicated section of parental DNA and, at the same time disentangles the SV40 daughter molecules.

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