Transcription of adenovirus and HeLa cell genes in the presence of drugs that inhibit topoisomerase I and II function

Jerome Schaak*, Paul Schedl[1] and Thomas Shenk
Howard Hughes Medical Institute, Department of Biology and [1]Department of Biology, Princeton University, Princeton, NJ 08544, USA

Received December 1, 1989; Revised and Accepted February 11, 1990

ABSTRACT

The requirements for topoisomerases in transcription of adenovirus and HeLa cell genes were analyzed using drugs that specifically inhibit either topoisomerase I or II. Cleavage of viral DNA by topoisomerases in the presence of either camptothecin or VM26 was used to determine drug concentrations that led to maximal inhibition of ligation in the cleavage and ligation step of topoisomerase I or II respectively. Inhibition of topoisomerase II with VM26 did not cause a direct reduction in transcription of adenoviral genes or HeLa cell heat shock genes. VM26 did, however, interfere with other cellular processes. It reduced nucleoside uptake into HeLa cells from the medium, and it altered the normal nuclear to cytoplasmic ratio of specific RNAs. Treatment of cells with camptothecin to inhibit topoisomerase I reduced but did not abolish transcription of viral and HeLa cell genes. Transcription mediated by both RNA polymerases I and II was reduced. Topoisomerase II did not appear to substitute for topoisomerase I in transcription since treatment of cells with VM26 and camptothecin did not reduce transcript accumulation relative to cells treated with camptothecin alone.

INTRODUCTION

Transcription leads to alterations in the local topology of DNA: positive supercoiling occurs in front of the transcription complex and negative supercoiling occurs behind (1). The torsional stress can be relaxed through the action of topoisomerases. In eukaryotes, the roles of topoisomerases in transcription have been most thoroughly studied in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (e.g., 2, 3). Inactivation of both topoisomerases I and II did not affect transcription by RNA polymerase III, but led to a reduction in total transcription by RNA polymerase II to approximately 30% of the wild-type level (2), and in transcription by RNA polymerase I to approximately 15% (2, 4). Interestingly, transcription of a variety of induced genes by RNA polymerase II was not significantly affected in the absence of topoisomerase activity (2). In general, it appears that transcriptional requirements for either topoisomerase can be assumed by the other in yeasts (2, 4).

The absence of either topoisomerase I or II alone does not lead to a change in the topology of the 2μ circle, demonstrating that both topoisomerases act to relax supercoiling (5). Supercoiling of transcribed regions does occur, but it does so through topological perturbations induced by RNA polymerase (1, 6–8). This supercoiling is a result of transcription, and thus does not appear to represent a control step for transcriptional activation. In yeast cells which lack topoisomerase I, this supercoiling is not relaxed (6, 7), suggesting that topoisomerase I plays the primary transcriptional role.

In higher eukaryotes, the transcriptional functions of the topoisomerases have been studied using inhibitors. A direct role for topoisomerase II has been inferred from studies which demonstrated that RNA polymerase II-directed transcription is inhibited by the topoisomerase II inhibitors novobiocin and VM26 (9, 10). However, suggestive evidence against a transcriptional role for topoisomerase II exists. Topoisomerase II concentrations decrease to levels which are probably insufficient to support a transcriptional role in various terminally differentiated and/or non-dividing cells (11–17). There are exceptions to the relationship between growth rate and topoisomerase II concentration, however. In mature *Drosophila*, a high level of topoisomerase II has been found in non-dividing cells (18). It may be that, in quiescent cells where its concentration is not reduced, topoisomerase II activity is reduced by modification of the protein (19).

There is stronger evidence for a role for topoisomerase I in transcription in higher eukaryotes. Topoisomerase I is recruited to transcriptionally activated genes (20–23). Further, inhibition of topoisomerase I by the anti-tumor drug camptothecin (21, 24, 25) or by injection of antibodies against topoisomerase I (26) led to a reduced rate of transcription by both RNA polymerases I and II.

In this study, we have examined the effects of inhibiting topoisomerases on transcription of both adenovirus and human genes. Transcription by RNA polymerase II was reduced but not

* To whom correspondence should be addressed at Department of Microbiology and Immunology, University of Colorado Health Sciences Center, Campus Box B175, 4200 East 9th Ave., Denver, CO 80262, USA

abolished when topoisomerase I was inhibited by treatment with camptothecin. Transcription was not reduced when topoisomerase II activity was inhibited by treatment with VM26 or novobiocin. VM26 interfered with cellular processes other than transcription. Treatment with this drug altered the normal ratio of nuclear to cytoplasmic RNAs, suggesting that it might inhibit transport of RNAs from nucleus to cytoplasm, and it interfered with the uptake of [^{3}H] uridine by HeLa cells. The inhibition in nucleoside uptake can give the mistaken impression in metabolic labeling experiments that VM26 blocks transcription.

MATERIALS AND METHODS

Cells and viruses

All assays were carried out using HeLa suspended-cell cultures grown in medium containing 10% horse serum. *dl*309, a phenotypically wild-type derivative of adenovirus type 5 (27), was used to infect cells at a multiplicity of 25 pfu/cell.

Topoisomerase inhibitors

Camptothecin, which inhibits topoisomerase I (28), and VM26, which inhibits topoisomerase II (29, 30), were dissolved in DMSO and stored at −20°C. Novobiocin, which inhibits eukaryotic topoisomerase II at much higher concentrations than are required to inhibit eubacterial DNA gyrase (31), was dissolved in H_2O and stored at −20°C.

Assays for cleavage of DNA in the presence of topoisomerase inhibitors

To determine the sites and extent of topoisomerase cleavage, infected cells were treated with either VM26 or camptothecin and then lysed by the addition of 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 using as probe the entire Ad5 chromosome which was [^{32}P] labeled (32). For analysis of VM26-induced double-strand cleavage, the same quantity of Ad5 DNA from each sample was digested with *Hin*dIII, resolved by electrophoresis on a 20 cm long agarose gel, denatured and transfered to nitrocellulose, and indirectly end labeled using a probe that extended from 5.7 (*Kpn*I site) to 7.8 (*Hin*dIII site) mu on the Ad5 chromosome. For analysis of camptothecin-induced single-strand cleavage, the DNA was denatured after cleavage with *Hin*dIII and resolved by electrophoresis on 20 cm long agarose gels containing 40mM NaOH, 5mM EDTA. To probe individual strands, [^{32}P] labeled complementary RNA of high specific activity was synthesized using SP6 or T7 RNA polymerase (33) to transcribe the 5.7 to 7.8 mu Ad5 DNA fragment for use as probe for indirect end labeling.

Analysis of transcription

Cytoplasmic RNA was isolated by lysing cells in hypotonic buffer (10 mM Tris-HCl, pH8.0, 10 mM NaCl, 3 mM $MgCl_2$) containing 0.5% NP40, deproteinizing by digestion with 100 μg/ml proteinase K for 15 min at 37°C, phenol extracting, and ethanol precipitating the aqueous phase two times. Nuclear and total RNA were isolated by acid phenol/chloroform extraction in the presence of guanidinium thiocyanate (34). The relative concentrations of specific RNAs were determined by RNase protection analyses using [^{32}P] labeled probes (33). E1A and E1B mRNAs were analyzed using a transcript which overlaps all of E1A and the first 139 bases of the E1B mRNAs. E2A mRNA was analyzed using a probe which overlaps the 3′-terminal 90 bases. L1 transcription was analyzed using a probe which overlaps the 3′-terminal 700 bases. Heat shock gene transcription was examined with and without heat shock at 43°C using probes which overlap 375 bases from the 5′ end of hsp70 mRNA or 424 bases from the 5′ end of hsx70 mRNA (35).

Labeling of RNA in vivo and analysis of uridine transport

For analysis of transcription, cells were pelleted 4.5 hr after infection with *dl*309 at a multiplicity of 25 pfu/cell. The cells were concentrated 5-fold, unlabeled uridine was added to 14 μM and [^{3}H] uridine added to 0.875 mCi/ml (48 Ci/mmole), and the culture was divided into 4 equal fractions. The fractions received either: no drug, VM26 (100 μg/ml), camptothecin (6.7 μg/ml), or VM26 (100 μg/ml) plus camptothecin (6.7 μg/ml). Aliquots were collected after 10, 20, 30, 45, and 60 min incubation of treated cultures at 37°C, and cytoplasmic and nuclear RNA isolated. Total incorporation of label was determined by acid precipitation followed by liquid scintillation counting. Incorporation of label into specific RNAs was determined by hybridizing the labeled RNA to DNAs immobilized on nitrocellulose followed by liquid scintillation counting.

The transport of uridine into HeLa cells in the presence and absence of topoisomerase-inhibiting drugs was examined by incubating the cells in medium supplemented with 14 μM uridine plus 0.1 mCi/ml (48 Ci/mmole) [^{3}H] uridine at 37°C. Aliquots of cells were taken at 5, 10, and 20 min, diluted 5 fold into ice-cold phosphate buffered saline (PBS) containing 200 μM unlabeled uridine, pelleted by centrifugation, and washed two times in ice-cold PBS. Uptake was quantified by liquid scintillation counting of aliquots of lysed cells spotted on nitrocellulose.

RESULTS

Effects of topoisomerase inhibitors on cleavage of DNA

To ensure that VM26 and camptothecin were inhibiting topoisomerases II and I, respectively, cleavage of adenovirus DNA within the E1A gene induced by the drugs was assayed. Both drugs act by prolonging the half life of the transient covalent DNA-protein intermediate in the cleavage-ligation reaction. Therefore, addition of a protein denaturant such as SDS in the presence of the drug leads to DNA strand scission at the site of topoisomerase cleavage (28−30, 36−38). The effect of drug concentration on cleavage was tested 12 hr after infection.

Little cleavage was induced by VM26 at concentrations of 0.25 μg/ml or less, while nearly maximal levels of cleavage were observed at 10 μg/ml or greater (Fig. 1). There was little or no change in sites of cleavage at the level of resolution achieved in an agarose gel with increasing VM26 concentration. There was also little if any change in the relative amounts of the various cleavage products. At the concentration of VM26 routinely used in these studies (100 μg/ml), the effect on topoisomerase II cleavage was maximal.

Cleavage of DNA was efficiently induced by camptothecin at low drug concentrations, particularly in the non-transcribed stand (Fig. 2). Cleavage of the transcribed strand was nearly maximal at a camptothecin concentration of 1.7 μg/ml, while cleavage of the non-transcribed stand was nearly maximal at a concentration

of 0.07 μg/ml, the lowest concentration tested. Again, increases in drug concentration led to little if any alteration in the sites of cleavage or in the relative intensities of the cleavage products. At the concentration of camptothecin routinely used in these studies (6.7 μg/ml), the effect on topoisomerase I was maximal.

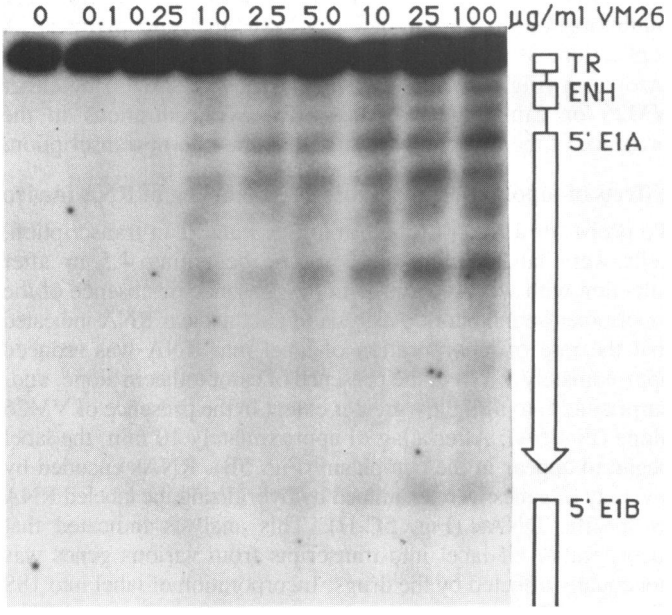

Figure. 1. VM26 Concentration Dependence for Stimulation of Double-Strand DNA Cleavage. HeLa cells 12 hr after infection with *dl*309 were treated for 15 min with VM26 at the concentrations indicated. Cells were then lysed by addition of SDS, DNA purified, digested with *Hin*dIII, resolved by agarose gel electrophoresis, and indirectly end-labeled using a 5.7 to 7.8 mu adenovirus DNA fragment. A schematic representation of the left end of the adenovirus chromosome is shown (TR=terminal repeat; ENH=E1A enhancer region; E1A and E1B transcribed regions are indicated).

Topoisomerase requirement for E1A mRNA accumulation

The effects of inhibiting either or both topoisomerase I and II on E1A gene transcription were examined during the early phase of adenovirus infection (Fig. 3A). Drugs were added either at the time of infection or from 3−4.5 hr after infection. Effects of topoisomerase inhibitors on E1A transcription can be observed at the level of mRNA accumulation after brief periods of inhibition since the half-life of E1A mRNAs is short and the concentration of mRNAs increases rapidly during the early phase of the infection. mRNA accumulation was inhibited by camptothecin, suggesting that topoisomerase I is required for a maximal rate of transcription. Addition of VM26 at the time of infection had little effect (in this case, there was even a slight increase in E1A RNA). This indicates that topoisomerase II is not required in the unpackaging of the adenovirus DNA or in the binding of factors to form a transcription complex. Addition of VM26 during the early phase also had little effect. A slight inhibition in mRNA accumulation was apparent, but this was not routinely observed. Inhibition of both topoisomerases led to no increased inhibition of mRNA accumulation over that observed when topoisomerase I alone was inhibited. Thus, it does not appear that topoisomerase II can substitute for topoisomerase I during E1A gene transcription.

The concentration dependence for inhibition of transcription by camptothecin can be compared to the concentration dependence for induction of single-strand breaks in adenovirus DNA. At concentrations as low as 0.07 μg/ml camptothecin, cleavage was efficient (although a nearly maximal level of cleavage of the transcribed strand was not attained until the camptothecin concentration reached 1.7 μg/ml; Fig. 2), while maximal inhibition of E1A mRNA accumulation required between 0.67 and 3.3 μg/ml (Fig. 3B). Even at 6.7 μg/ml, there was significant appearance of E1A RNA in the cytoplasm. Thus, transcription of the E1A gene may occur relatively efficiently in the presence of low levels of topoisomerase I activity.

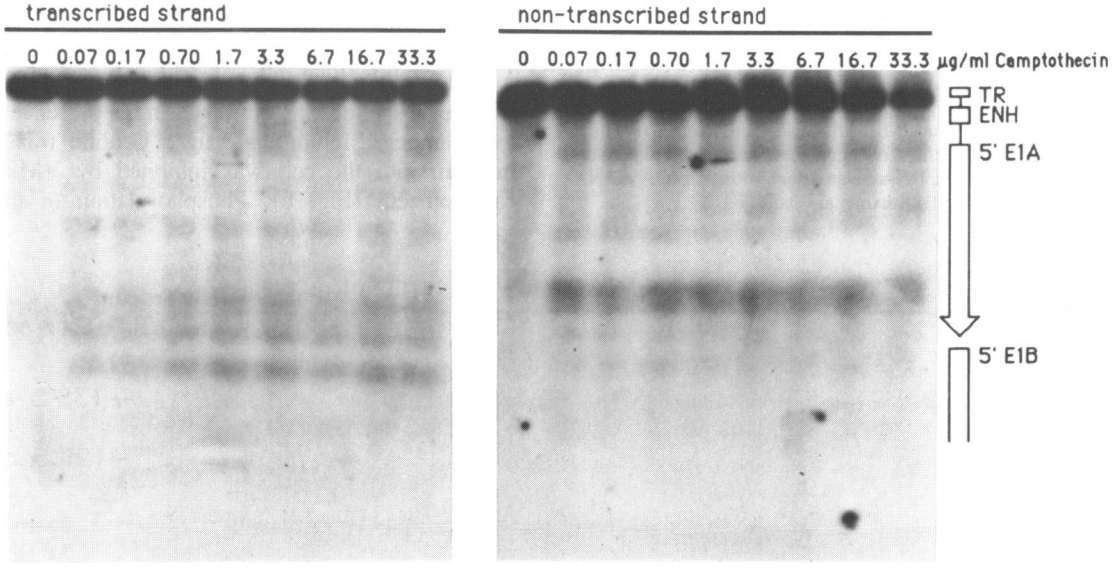

Figure. 2. Camptothecin Concentration Dependence for Stimulation of Single-Strand DNA Cleavage. HeLa cells 12 hr after infection with *dl*309 were treated for 15 min with camptothecin at the concentrations indicated. Cells were then lysed by addition of SDS, DNA purified, digested with *Hin*dIII, resolved by agarose gel electrophoresis under denaturing conditions, and indirectly end-labeled using single-strand RNA probes prepared from each DNA strand of the 5.7 to 7.8 mu fragment. A schematic representation of the left end of the adenovirus chromosome is shown (TR=terminal repeat; ENH=E1A enhancer region; E1A and E1B transcribed regions are indicated).

E1A mRNA half life in the presence of topoisomerase inhibitors

Analysis of E1A mRNA accumulation suggested that transcription is reduced when topoisomerase I, but not when topoisomerase II, is inhibited. mRNA concentrations are reflective of transcription rates if mRNA stability is not affected by treatment with topoisomerase inhibiting drugs. To test if this were so, the effects of the topoisomerase inhibitors on the stability of E1A mRNA was monitored. 4 hr after infection, actinomycin D (4 μg/ml) was added to simultaneously block transcription and the infectious process, thereby controlling for differences in the infectious cycle caused by treatment with the topoisomerase inhibitors (VM26 and camptothecin both block adenovirus DNA replication; 39). E1A RNA was only slightly stabilized in the cytoplasm (Fig. 4) and the nucleus (data not shown) by either VM26 or camptothecin. Thus, RNA concentrations in the presence of the drugs appear to reflect the rate of transcription.

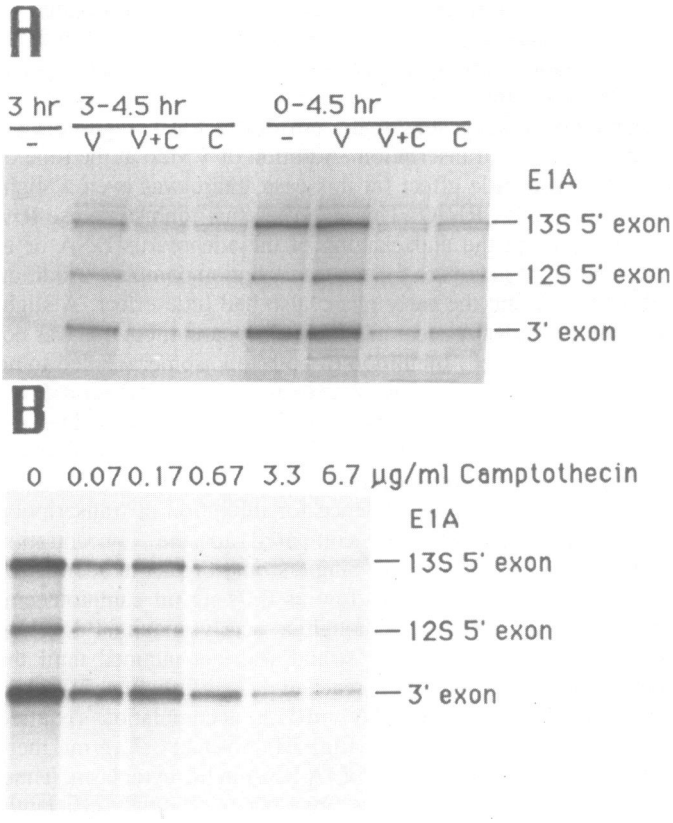

Figure 3. Effects of topoisomerase inhibitors on E1A mRNA accumulation. A) HeLa cells were treated in the absence of drugs (lanes labeled − or 0) or in the presence of 100 μg/ml VM26 (lanes labeled V), 6.7 μg/ml camptothecin (lanes labeled C), or 100 μg/ml VM26+6.7 μg/ml camptothecin (lanes labeled V+C) from 3 to 4.5 hr after infection or from the time of infection until 4.5 hr after infection with *dl*309. Total cellular RNA was probed for E1A mRNAs by RNase protection. The E1A exons are indicated. B) HeLa cells were treated beginning at the time of infection with concentrations of camptothecin as noted. 4.5 hr later, cells were harvested, and cytoplasmic RNA isolated and probed for E1A mRNA by RNase protection analysis. The E1A exons are indicated.

Effects of topoisomerase inhibitors on labeling of RNA in vivo

To probe for a role of topoisomerases I and II in transcription, cells were labeled with [^{3}H] uridine beginning 4.5 hr after infection with wild-type virus in the presence or absence of the topoisomerase inhibitors. Analysis of total nuclear RNA indicated that the rate of incorporation of label into RNA was reduced approximately 5 fold in the presence of camptothecin alone, and, surprisingly, to a slightly greater extent in the presence of VM26 alone (Fig. 5A). After a lag of approximately 10 min, the label began to appear in the cytoplasm (Fig. 5B). RNAs encoded by a variety of genes were examined by hybridizing the labeled RNA to specific DNAs (Fig. 5C-H). This analysis indicated that incorporation of label into transcripts from various genes was not equally affected by the drugs. Incorporation of label into 18S ribosomal RNA was extremely low, and into 28S ribosomal RNA was almost zero, in the presence of camptothecin, in contrast to the moderate inhibition of RNA polymerase II-directed incorporation of label. This strong requirement of HeLa cell RNA polymerase I for topoisomerase I activity has been reported previously (25).

The effect of camptothecin on incorporation of label into E1A RNA is in reasonable agreement with the data generated from total RNA analysis (Fig. 3), but the effects of VM26 in the two assays are in complete disagreement. To determine where the discrepancy might lie, uridine metabolism was examined.

VM26 inhibits the uptake of uridine by cells from the medium

Uptake of uridine was examined in the presence and absence of topoisomerase inhibiting drugs. Uridine transport from the medium into the cell was inhibited by VM26, but not by camptothecin (Fig. 6). Phosphorylation of the uridine was

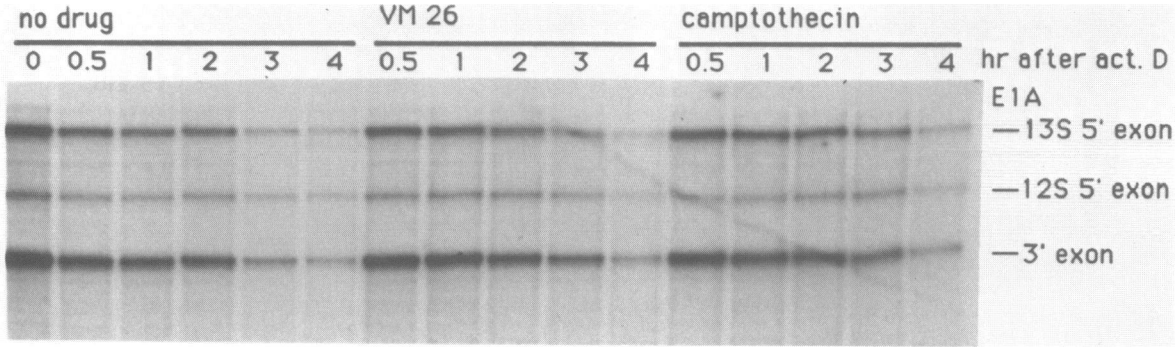

Figure 4. Effects of topoisomerase inhibitors on E1A mRNA half life. Actinomycin D (4 μg/ml) was added to each of three samples of cells along with no additional drugs, 100 μg/ml VM26, or 6.7 μg/ml camptothecin 4 hr after infection with *dl*309. Aliquots were taken at various times after addition of drugs as noted and cytoplasmic RNA assayed for E1A RNA by RNase protection. The E1A exons are indicated.

examined by PEI thin layer chromatography of cell extracts in 0.75 M KPO_4, pH 3.75, and found to be unaltered in the presence of the drugs (data not shown). The block in uridine uptake appears to explain the discrepancy between intracellular concentrations of RNAs and their rate of labeling by [^{3}H] uridine in the presence of VM26.

VM26 increases the nuclear to cytoplasmic ratio of E1A and E1B RNAs

The effects of the topoisomerase inhibitors on E1A RNA accumulation after the onset of adenovirus DNA replication were examined. The drugs were added 9 hr after infection (under the conditions used DNA replication began at 4.5 hr, and by 9 hr the concentration of adenovirus DNA had increased approximately 20-fold; data not shown). Treatment with VM26, but not with camptothecin, caused an increase in the ratio of nuclear/cytoplasmic RNA for both E1A and E1B (Fig. 7). The majority of the E1A nuclear transcripts appeared to be appropriately spliced. There was an increase in the nuclear concentration of RNA species which varied in structure from the mature species, but this may simply result from the general increase in E1A RNA concentration. A similar increase in nuclear concentration and reduction in cytoplasmic concentration of E1A and E1B RNAs was observed during the early phase of the infection (data not shown). This suggests that VM26 interferes with transport of mature transcripts from the nucleus to the cytoplasm.

The nuclear concentration of E1A and E1B RNAs was substantially increased in the presence of VM26 from 9 to 10 hr after infection, while the concentration of cytoplasmic RNAs was only modestly reduced (Fig. 7). Thus, the total amount of E1A and E1B mRNAs that accumulated in the presence of the drug was considerably greater than that which accumulated in its absence. A significant increase in E1A and E1B mRNAs was also observed in infected cells treated with 500 μg/ml novobiocin during the late phase (data not shown). This suggests that, rather than activating transcription of the early viral genes, topoisomerase II directly or indirectly down regulates early gene transcription after the onset of replication.

Requirement for topoisomerases in accumulation of E2A and L1 mRNAs

The E2A transcription unit is located nearer the middle of the viral chromosome (transcription initiates at 75 map units, or 9 kb from the right end of the chromosome) and is much larger than the E1A transcribed region. Thus, if there were a topoisomerase II role in either transcription or activating DNA during unpackaging of the virion, E2A transcription might exhibit a greater dependence on topoisomerase II activity. To test this possibility, total cellular levels of E2A RNA synthesized when the topoisomerases were inhibited were examined (Fig. 8A). Inhibition of topoisomerase II had virtually no effect, while inhibition of topoisomerase I led to virtually complete inhibition of accumulation of E2A mRNA. The effect of camptothecin on E2A mRNA accumulation may be due in part to the reduction in the concentration of E1A protein, since E2A transcription is dependent on activation by E1A proteins (40, 41). Thus, the extent of the inhibition due directly to camptothecin is not immediately clear. The inhibition does appear to be significantly greater than for E1A mRNA, however.

The effects of inhibiting topoisomerases I and II on RNA accumulation from the major late promoter was examined by analyzing L1 RNA (Fig. 8B). Treatment with drugs was limited to 1 hr to minimize the inhibitory effects of the drugs on DNA replication (39). Inhibition of topoisomerase I virtually blocked L1 RNA accumulation. Inhibition of topoisomerase II had little effect on total RNA accumulation. There was relatively little L1 mRNA accumulation in the cytoplasm by 10 hr after infection. Nevertheless, it is apparent that VM26 also interfered with transport of L1 mRNA to the cytoplasm.

Effects of blocking topoisomerase activity on accumulation of heat shock mRNAs

As a host cell control for the effects of topoisomerase inhibition on transcription of the adenovirus genome, heat shock gene transcription was examined. hsx70, which is expressed constitutively and further induced upon heat shock, and hsp70, which is expressed at extremely low levels and greatly induced upon heat shock, were tested. HeLa suspension cells at 37°C

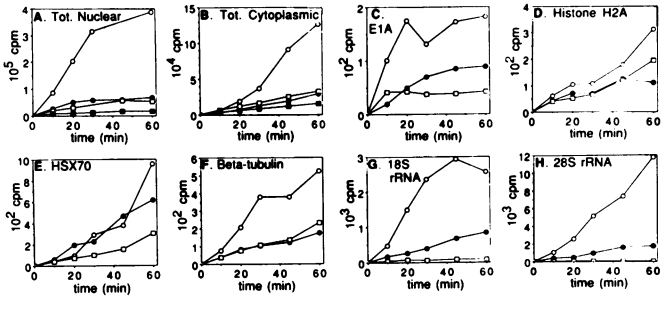

Figure 5. Effects of topoisomerase inhibitors on metabolic labeling of RNA. 4 hr after infection with *dl*309, HeLa cells were continuously labeled with [^{3}H] uridine in the absence of drugs (○) or in the presence of 100 μg/ml VM26 (●), 6.7 μg/ml camptothecin (□), or 100 μg/ml VM26+6.7 μg/ml camptothecin (■). Aliquots were taken at the times indicated after addition of label, and cytoplasmic and nuclear RNA were isolated. Total incorporation and incorporation into specific transcripts were determined. A) total nuclear incorporation. B) total cytoplasmic incorporation. C) nuclear incorporation for adenovirus E1A. D) nuclear incorporation for histone H2A. E) nuclear incorporation for the heat shock gene hsx70. F) nuclear incorporation for beta-tubulin. G) nuclear incorporation for 18S ribosomal RNA. H) nuclear incorporation for 28S ribosomal RNA.

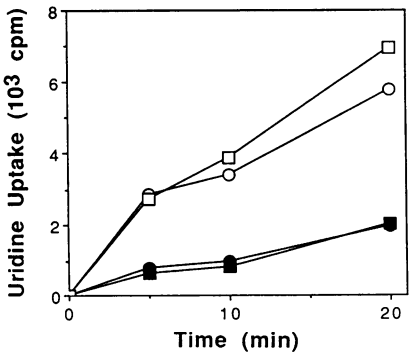

Figure 6. VM26 inhibits cellular uptake of uridine. HeLa cells were incubated at 37°C in medium containing [^{3}H]-uridine in the absence of drugs (○) or in the presence of 100 μg VM26/ml (●), 6.7 μg camptothecin/ml (□), or 100 μg VM26/ml+6.7 μg camptothecin/ml (■). Aliquots were taken at the times indicated and total uptake of [^{3}H] uridine determined and plotted as a function of time of incubation.

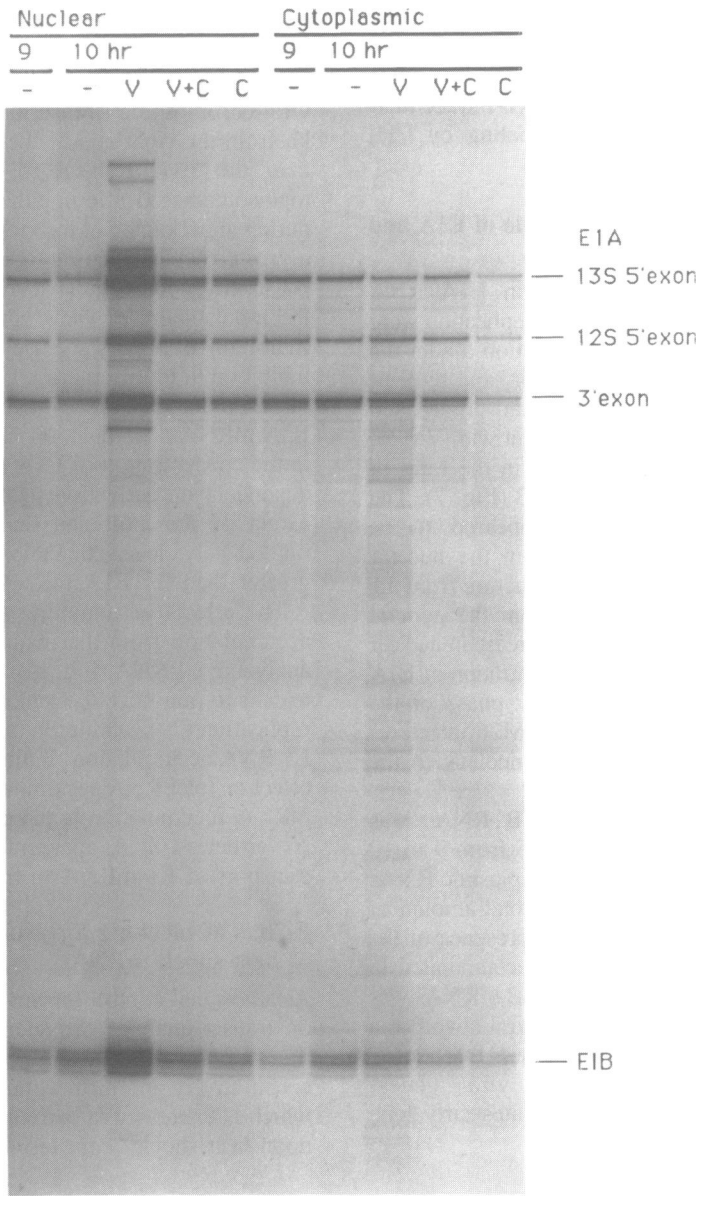

Figure 7. The effects of topoisomerase inhibitors on E1A and E1B mRNA accumulation after the onset of replication. HeLa cells infected with *dl*309 were either treated in the absence of drugs (lanes labeled −) or in the presence of 100 μg/ml VM26 (lanes labeled V), 6.7 μg/ml camptothecin (lanes labeled C), or 100 μg/ml VM26+6.7 μg/ml camptothecin (lanes labeled V+C) from 9−10 hr after infection. Cytoplasmic and nuclear RNA were prepared, and probed for E1A and E1B RNAs by RNase protection analysis. The protected fragments are indicated.

were treated for 15 min with topoisomerase inhibitors and then placed at 43°C (at which temperature both hsx70 and hsp70 mRNAs are strongly induced, ref. 29). Aliquots were collected at various times after heat shock, and total (Fig. 9A and D), cytoplasmic (Fig. 9B and E), and nuclear (Fig. 9C and F) RNA prepared. The samples were probed for hsp70 (Fig. 9A−C) and hsx70 (Fig. 9D−F) RNAs. Two protected bands were observed in the RNase protection analysis of hsx70 mRNAs, presumably reflecting transcription from two HSX70 alleles (35). The drugs had a greater effect on hsp70 RNA, but both genes responded in a similar manner. At 15 min of heat shock, VM26 had no discernable effect, and at 30 min very little effect, on total heat shock RNA accumulation. By 60 min, however, there was a modest reduction (approximately 2 fold). Examination of nuclear and cytoplasmic RNA indicated that, at early times, there was an excess accumulation of heat shock RNA in the nucleus of VM26-treated cells. At the same time, there was a reduction in cytoplasmic accumulation. The inhibition of RNA accumulation was delayed relative to transcriptional activation, which indicates that the inhibition was not due to a direct effect on transcription. Further, the transport of mRNA from the nucleus to the cytoplasm appeared to be inhibited, as was observed for adenovirus RNAs.

Inhibition of RNA accumulation by camptothecin was observed at all times, and the inhibition was linear with respect to time. Camptothecin did not affect the relative proportion of nuclear and cytoplasmic RNAs. The combination of VM26 and camptothecin led to a summation of the effects observed for each of the drugs alone. RNA accumulation at early times after heat shock was inhibited to the same degree as in cells treated with

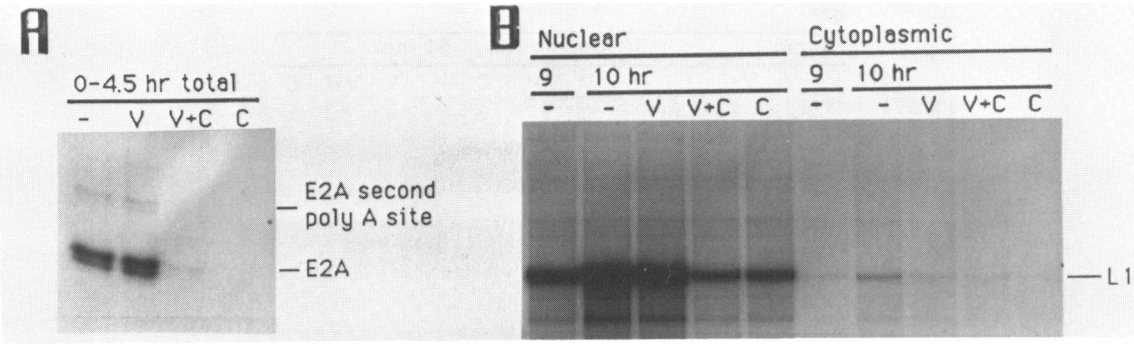

Figure 8. Effects of topoisomerase inhibitors on E2A and L1 mRNA accumulation. A) HeLa cells infected with *dl*309 were either treated in the absence of drugs (lane labeled −) or in the presence of 100 μg/ml VM26 (lane labeled V), 6.7 μg/ml camptothecin (lane labeled C), or 100 μg/ml VM26+6.7 μg/ml camptothecin (lane labeled V+C) from the time of infection. 4.5 hr later, cells were harvested and total cellular RNA isolated and probed for E2A mRNA by RNase protection analysis. The E2A protected species are indicated. B) HeLa cells infected with *dl*309 were treated in the absence of drugs (lanes labeled −) or in the presence of 100 μg/ml VM26 (lanes labeled V), 6.7 μg/ml camptothecin (lanes labeled C), or 100 μg/ml VM26+6.7 μg/ml camptothecin (lanes labeled V+C) from 9 to 10 hr after infection with *dl*309. The cells were harvested 10 hr after infection and nuclear and cytoplasmic RNA prepared and probed for L1 mRNA by RNase protection analysis. Protected species are indicated.

camptothecin alone. Inhibition was greater at later times, and transport to the cytoplasm was inhibited at all times. This suggests that the inhibition of both topoisomerases had no more direct effect on RNA polymerase II directed transcription than did inhibition of topoisomerase I alone.

The effect of the topoisomerase inhibitors on expression of hsp70 and hsx70 RNAs was also examined in the absence of heat shock (Fig. 9G). Treatment with camptothecin did not lead to induction of either RNA. Treatment with 500 μg/ml novobiocin efficiently induced hsx70 mRNA. Treatment with 100 μg/ml VM26 weakly induced hsx70 mRNA (middle panel), while treatment with 10 μg/ml had no effect (right panel). Treatment with 10 μg/ml VM26 also had no effect on hsx70 mRNA accumulation during heat shock (data not shown). Neither topoisomerase II inhibitor induced hsp70 RNA.

DISCUSSION

We have presented evidence suggesting that topoisomerase II is not required for a maximal rate of transcription in HeLa cells. In contrast, a correlation was observed between the rate of transcription and inhibition of topoisomerase I. These studies employed drugs which induce breaks in the DNA with the introduction of covalent DNA-protein adducts. Therefore, it is possible that any direct effect on transcription reflects, at least in part, damage to the DNA rather than simply the inhibition of the topoisomerases. Camptothecin altered the site utilization of purified human topoisomerase I on *Tetrahymena* DNA in vitro (42), suggesting that this drug may alter the action of topoisomerase I. These effects could lead to overestimation of the role of topoisomerase I in transcription. However, camptothecin did not alter site utilization by purified mouse topoisomerase I on SV40 DNA in vitro (43). Finally, it is possible that, although camptothecin efficiently prolongs the half-life of the covalent protein-DNA intermediate (28), it does not severely inhibit the catalytic activity of topoisomerase I. Thus, the role of topoisomerase I in transcription may be underestimated from these studies.

For proper conclusions regarding the role of the topoisomerases in transcription to be drawn from studies utilizing loss of activity through inhibition by drugs, genetic manipulation, or depletion by antibodies, it is necessary to demonstrate that any affects on transcription are directly due to the reduction in topoisomerase activity. A careful attempt was made to identify potential effects of the drugs not directly related to the inhibition of the topoisomerases. While such effects were observed in the presence of VM26, none were found to occur in the presence of camptothecin. Nevertheless, they cannot be ruled out. With these potential problems for interpretation of the results in mind, the roles of the topoisomerases in transcription by RNA polymerase II in HeLa cells are examined.

Topoisomerase I and transcription

There appears to be a requirement for topoisomerase activity to permit a maximal rate of transcription (1−3, 8). Topoisomerase I is capable of fulfilling that role in several systems (2, 21, 24, 26). Our evidence suggests that topoisomerase I also serves a transcriptional role for RNA polymerase II in HeLa cells. The presence of the topoisomerase I inhibitor camptothecin led to a reduction in accumulation of both adenovirus mRNAs (Figs. 3, 5, 7, and 8) and HeLa mRNAs (Figs. 5 and 9). It is possible that the inhibition of RNA accumulation in the presence of camptothecin is due, at least in part, to the introduction of covalent DNA-protein adducts (28). However, the evidence that a topoisomerase activity is required for efficient transcription, coupled with the apparent inability of topoisomerase II to fulfill such a role (see below) suggests that topoisomerase I is required for a maximal rate of transcription.

The evidence that the inhibition in RNA accumulation caused by camptothecin occurs at the level of transcription is strong: there was little alteration in the half life of RNAs examined (Fig. 4) or in their transport to the cytoplasm (Figs. 7−9); the inhibition of RNA accumulation appeared to be linear with respect to time (Fig. 9); and, most importantly, the incorporation of label into specific RNAs in vitro (data not shown) and in vivo (Fig. 5) indicated that the rate of transcription was reduced.

Transcription of the E1A and E1B genes was not completely inhibited by high concentrations of camptothecin. The level of inhibition was no greater when topoisomerase II was also inhibited. Thus, there may be a substantial level of transcription which occurs independent of topoisomerase activity. The efficiency of transcription of specific genes in the presence of camptothecin depended on the length of the transcribed region. L1 RNA formation was virtually completely inhibited by

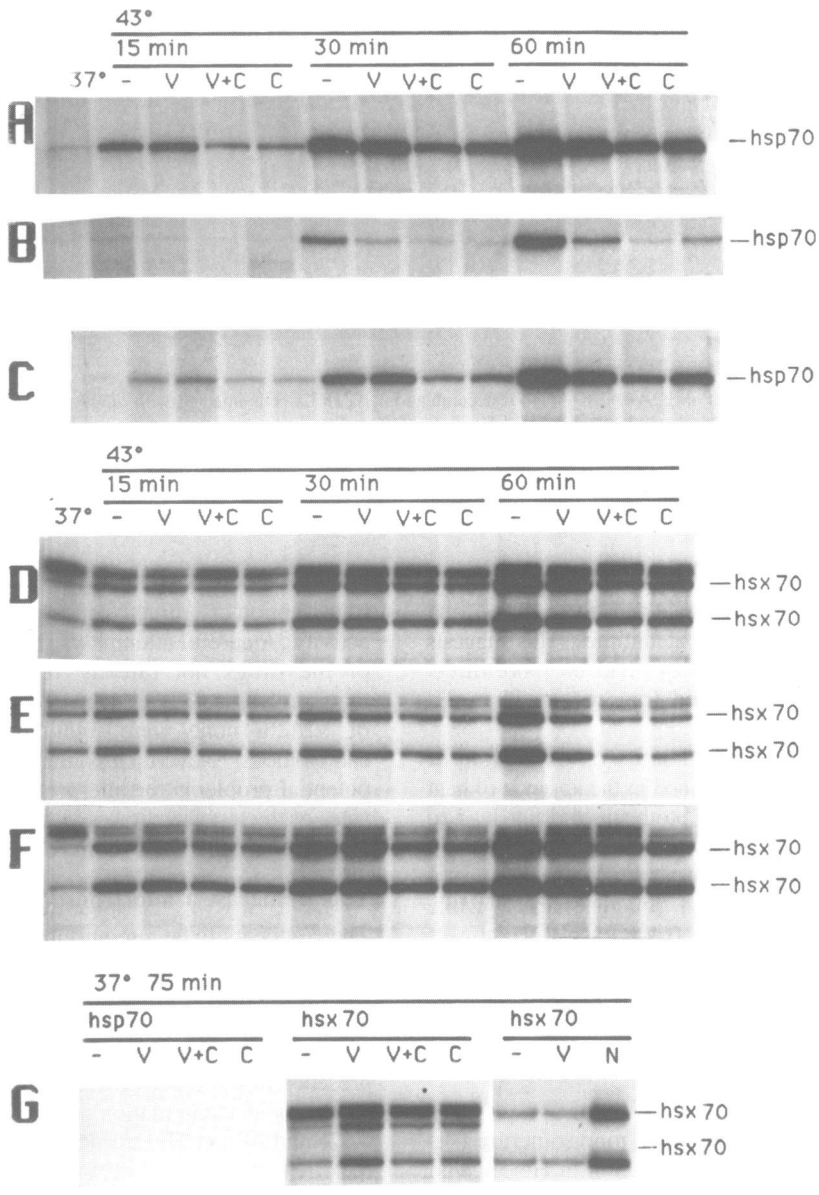

Figure 9. Effects of topoisomerase inhibitors on hsp70 and hsx70 mRNA accumulation. HeLa cells were treated at 37°C in the absence of drugs (lanes labeled −) or in the presence of 100 μg/ml VM26 (lanes labeled V), 6.7 μg/ml camptothecin (lanes labeled C), or 100 μg/ml VM26+6.7 μg/ml camptothecinh (lanes labeled V+C) beginning 15 min before the cells were shifted to 43°C. Aliquots were taken at the times indicated after the temperature shift, cells harvested, and total (panel A), cytoplasmic (panel B), and nuclear (panel C) RNA isolated and probed for hsp70 mRNA by RNase protection analysis. Total (panel D), cytoplasmic (panel E), and nuclear (panel F) RNA were also probed for hsx70 mRNA by RNase protection analysis. Two RNA species, presumably representing transcripts from two hsx70 alleles, protect this probe (35) and are indicated. Additionally, a slower migrating background band of variable intensity resulting from the probe itself was observed. Cells were also treated with 100 μg/ml VM26, 6.7 μg/ml camptothecin, or 100 μg/ml VM26+6.7 μg/ml camptothecin (left and center panels of part G), or 500 μg/ml novobiocin or 10 μg/ml VM26 (right panel of part G) as noted at 37°C for 75 min, total RNA isolated, and probed for either hsp70 or hsx70 RNA as noted. Protected species are indicated. In the experiment in which activation of the HSX70 gene by novobiocin was examined the probe background band was not observed.

camptothecin (Fig. 8). The formation of the L1 transcript requires that transcription proceed at least 7,000 bases, while E1A transcript formation requires polymerization of fewer than 1,200 bases. This apparent size dependence is supported by studies of the transcription of genes of altered length in *S. cerevisiae*, where the degree of supercoiling induced was found to be proportional to the length of the transcribed region (7).

The effect of camptothecin on transcription by RNA polymerase II also varies between host genes. While transcription of all genes was reduced in the presence of camptothecin, the effect was greatest for beta-tubulin, and less for the histone H2A and HSX70 genes (Figs. 5 and 9). Aside from a likely dependence on the size of the transcription unit, it may be that inhibition is reduced in certain genetic structures (e.g., if two nearby genes are found in a head-to-tail arrangement, the build-up of torsional strain may partially cancel).

Non-Transcriptional effects of VM26

A modest inhibition of heat shock gene RNA accumulation was observed when cells were treated with 100 μg/ml VM26, but this inhibition was not apparent until at least 30 min after the initiation of the 43°C heat shock (Fig. 9). In contrast, treatment

of adenovirus-infected cells at 37°C with VM26 did not lead to inhibition of RNA accumulation even after 4.5 hr (Fig. 3). This difference might be due to an increased effect at elevated temperatures of high concentrations of VM26 on uridine uptake. Cellular uptake of uridine from the medium was inhbited by 100 μg/ml VM26 (Fig. 6), but other pathways for UTP biosynthesis were presumably utilized. When the cells were subjected to heat shock, proteins involved in alternative pathways may have been inactivated, leading to a reduction in cellular UTP (and possibly other NTP) concentration which did not occur at 37°C.

Treatment of cells with VM26 inhibited accumulation of both heat shock and viral mRNAs in the cytoplasm, while elevated levels of these RNAs accumulated in the nucleus. The majority of the E1A transcripts appeared to be appropriately spliced. This raises the possibility that the inhibition occurs directly at the level of transport, although it does not demonstrate that topoisomerase II is involved. Release of ovalbumin mRNA from hen oviduct nuclear matrices in vitro was reduced in the presence of topoisomerase II inhibitors, including both ATP analogs and intercalating agents (44). However, the inhibition of mRNA release is not likely to be related directly to inhibition of topoisomerase II, since the concentration of novobiocin required to reduce mRNA release to approximately 50% (44) was far below the K_i for *Drosophila* topoisomerase II (45). Given the effects of VM26 on nucleoside metabolism, it appears more likely that its effect on mRNA transport reflects inhibition of a nucleotide-dependent step in mRNA transport rather than a direct role of topoisomerase II in this process.

Topoisomerase II and transcription

Inhibition of topoisomerase II did not lead to a reduction in transcription of the genes which we have studied. Inhibition of both topoisomerases led to no increased inhibition of transcription over that observed when topoisomerase I alone was blocked (Figs. 3, 7–9), suggesting that topoisomerase II does not serve in place of topoisomerase I. However, it is possible that transcription of other genes does respond to topoisomerase II, so we cannot rule out a transcriptional role. Induced transcription in *Saccharomyces cerevisiae* appears to be independent of topoisomerase activity (2), and all of the genes for which we can draw conclusions were transcriptionally induced. In higher eukaryotes, a potential role for topoisomerase II exists in the decondensation of non-transcribed chromatin into potentially 'active' chromatin (e.g., ref. 46). It is possible that genes in inactive domains would require topoisomerase II for activation, while inactive genes in already activated domains would not. If all of the genes which we studied are located in domains which are already active, a requirement for topoisomerase II would not have been apparent.

While our results suggest that topoisomerase II plays little or no role in transcription by RNA polymerase II in human cells (Figs. 3, 7–9), there is evidence for a role in transcription in other systems. The rate of synthesis of total poly A^+ RNA was reduced approximately 3-fold in yeast expressing no topoisomerase function relative to those expressing either topoisomerase I or II (2). Treatment of *Drosophila* cells with either VM26 or novobiocin before heat shock blocked transcription of the HSP70 gene (9, 10), although the secondary effects associated with the use of novobiocin (47) and VM26 (Fig. 6) indicate that this effect may not be due to inhibition of topoisomerase II. It is possible that the apparent contradictions between our data and results from other organisms can be explained, at least in part, by differences in the transcriptional requirement for topoisomerase II activity between yeasts, *Drosophila*, and human cells. However, our conclusion that topoisomerase II does not play a general transcriptional role in HeLa cells is in agreement with findings that topoisomerase II concentration is reduced at least two orders of magnitude in terminally differentiated and quiescent cells relative to rapidly dividing cells (e.g., ref. 12).

It is possible that topoisomerase II plays an inhibitory role in the control of adenovirus transcription. Shortly after the onset of adenovirus DNA replication, accumulation of nuclear RNAs encoded by E1A and other early region genes was enhanced when topoisomerase II was inhibited by addition of either VM26 or novobiocin (Fig. 7 and data not shown). These drugs both cause changes in cellular metabolism that are not related to topoisomerase II, but no overlap in the potential artifacts caused by the two drugs is apparent. Thus, it is likely that the increased transcription is due directly to inhibition of topoisomerase II. There is an increase in the amount of topoisomerase II interaction with the adenovirus chromosome after the onset of replication (39), and this may lead to down-regulation of E1A transcription. Other early regions may also be transcriptionally down-regulated by topoisomerase II binding, or may simply be transcribed at lower efficiency due to the resultant reduction in the concentration of E1A transcriptional activator proteins.

The fact that novobiocin blocks transcription of many genes, but not transcription of the adenovirus chromosome, is likely due to differences in chromatin protein composition. Novobiocin precipitates histones (47). Transcription of the early genes of SV40 DNA, which is bound by histones, is inhibited by novobiocin, but not by VM26 (data not shown). In contrast, adenovirus transcription is not inhibited by novobiocin (data not shown), consistent with the report that adenovirus DNA is not bound by histones (48).

ACKNOWLEDGEMENTS

We thank Evangelia Vakalopoulou for critical reading of the manuscript.

This work was supported by a Public Health Service grant from the National Cancer Institute (CA 41086) to T. Shenk and by grants from The March of Dimes and the National Institutes of Health to P. Schedl. J. Schaack was a postdoctoral fellow of the Jane Coffin Childs Memorial Fund for Medical Research, and T. Shenk was an American Cancer Society Research Professor during the initial phase of this work.

ADDENDUM

Wong and Hsu recently reported that transcription of adenovirus genes is reduced in the presence of camptothecin, with apparent premature termination of transcription from the major late promoter (49), in agreement with our results. However, they found that ellipticine, an intercalating inhibitor of topoisomerase II, inhibited transcription of both HeLa and adenovirus genes (49).

REFERENCES

1. Liu, L.F. and Wang, J.C. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 7024–7027
2. Brill, S.J., DiNardo, S., Voelkel-Meiman, K., and Sternglanz,R. (1987) *Nature* **326**, 414–416
3. Uemura, T., Morino, K., Uzawa, S., Shiozaki, K., and Yanagida, M. (1987) *Nucleic Acids Res.* **15**, 9727–9739

4. Yamagishi, M. and Nomura, M. (1988) *Curr. Genet.* **13**, 305–314
5. Saavedra, R.A. and Huberman, J.A. (1986) *Cell* **45**, 65–70
6. Brill, S.J. and Sternglanz, R. (1988) *Cell* **54**, 403–411
7. Osborne, B.I. and Guarente, L. (1988) *Genes Dev.* **2**, 766–772
8. Tsao, Y.-P., Wu, H.-Y., and Liu, L.F. (1989) *Cell* **56**, 111–118
9. Han, S., Udvardy, A., and Schedl, P. (1985) *J. Mol. Biol.* **183**, 13–29
10. Rowe, T.C., Wang, J.C., and Liu, L.F. (1986) *Mol. Cell. Biol.* **6**, 985–992
11. Miskimins, R., Miskimins, W.K., Bernstein, H., and Shimizu, N. (1983) *Exp. Cell Res.* **146**, 53–62
12. Heck, M.M. and Earnshaw, W.C. (1986) *J. Cell Biol.* **103**, 2569–2581
13. Nelson, W.G., Cho, K.R., Hsiang, Y.H., Liu, L.F., and Coffey, D.S. (1987) *Cancer Res.* **47**, 3246–3250
14. Sullivan, D.M., Latham, M.D., and Ross, W.E. (1987) *Cancer Res.* **47**, 3973–3979
15. Fairman, R. and Brutlag, D.L. (1988) *Biochemistry* **27**, 560–565
16. Heck, M.M., Hittelman, W.N., and Earnshaw, W.C. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 1086–1090
17. Hsiang, Y.H., Wu, H.Y., and Liu, L.F. (1988) *Cancer Res.* **48**, 3230–3235
18. Hadlaczky, G., Praznovszky, T., Sofi, J., and Udvardy, A. (1988) *Nucleic Acids Res.* **16**, 10013–10023
19. Ackerman, P., Glover, C.V., and Osheroff,N. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 3164–3168
20. Fleischmann, G., Pflugfelder, G., Steiner, E.K., Javaherian, K., Howard, G.C., Wang, J.C., and Elgin, S.C. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 6958–6962
21. Gilmour, D.S. and Elgin, S.C. (1987) *Mol. Cell. Biol.* **7**, 141–148
22. Gilmour, D.S. and Lis, J.T. (1987) *Mol. Cell. Biol.* **7**, 3341–3344
23. Gilmour, D.S., Pflugfelder, G., Wang, J.C., and Lis, J.T. (1986) *Cell* **44**, 401–407
24. Rowe, T.C., Couto, E., and Kroll, D.J. (1987) *NCI Monogr.* **4**, 49–53
25. Zhang, H., Wang, J.C., and Liu, L.F. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 1060–1064
26. Egyhazi, E. and Durban, E. (1987) *Mol. Cell. Biol.* **7**, 4308–4316
27. Jones, N. and Shenk, T. (1979) *Cell* **17**, 683–689
28. Hsiang, Y.H., Hertzberg, R., Hecht, S., and Liu, L.F. (1985) *J. Biol. Chem.* **260**, 14873–14878
29. Chen, G.L., Yang, L., Rowe, T.C., Halligan, B.D., Tewey, K.M., and. Liu, L.F. (1984) *J. Biol. Chem.* **259**, 13560–13566
30. Minocha, A. and Long, B.H. (1984) *Biochem. Biophys. Res. Commun.* **122**, 165–170
31. Osheroff, N., Shelton, E.R., and Brutlag, D.L. (1983) *J. Biol. Chem.* **258**, 9536–9543
32. Feinberg, A.P. and Vogelstein, B. (1983) *Analyt. Biochem.* **132**, 6–13.
33. Melton, D.A., Krieg, P.A., Rebagliate, M.R., Maniatis, T., Zinn, K., and Green, M.R. (1984) *Nucleic Acids Res.* **12**, 7035–7056
34. Chomczynski, P. and N. Sacchi. (1987) *Analt. Biochem.* **162**, 156–159
35. Moore, M., Schaack, J., Baim, S.B., Morimoto, R.I., and Shenk, T. (1987) *Mol. Cell. Biol.* **7**, 4505–4512
36. Yang, L., Rowe, T.C., and Liu, L.F.. (1985) *Cancer Res.* **45**, 5872–5876
37. Yang, L., Rowe, T.C., Nelson, E.M., and Liu, L.F. (1985) *Cell* **41**, 127–132
38. Long, B.H., Musial, S.T., and Brattain, M.G. (1985) *Cancer Res.* **46**, 3809–3816
39. Schaack, J., Schedl, P., and Shenk T. (1990) *J. Virol.* **64**, 78–85
40. Berk, A.J., Lee, F., Harrison, T.F., Williams, J.F, and Sharp, P.A. (1979) *Cell* **17**, 935–944
41. Jones, N. and Shenk, T. (1979) *Proc. Nat. Acad. Sci. USA* **76**, 3665–3669
42. Kjeldsen, E., Mollerup, S., Thomsen, B., Bonven, B.J., Bolund, L., and Westergaard, O. (1988) *J. Mol. Biol.* **202**, 333–342
43. Jaxel, C., Kohn, K.W., and Pommier, Y. (1988)) *Nucleic Acids Res.* **16**, 11157–11170
44. Schröder, H.C., Trölltsch, D., Friese, U., Bachmann, M., and Müller, W.E.G. (1987) *J. Biol. Chem.* **262**, 8917–8925
45. Osheroff, N., Shelton, E.R., and Brutlag, D.L. (1983) *J. Biol. Chem.* **258**, 9536–9543
46. Udvardy, A., Schedl, P., Sander, M., and Hsieh, T.-S. (1986) *J. Mol. Biol.* **191**, 231–246
47. Cotten, M., Bresnahan, D., Thompson, S., Sealy, L., and Chalkley, R. (1986) *Nucleic Acids Res.* **12**, 3671–3686
48. Chatterjee, P.K., Vayda, M.E., and Flint, S.J. (1986.) *EMBO J.* 5, 1633–1644
49. Wong, M.-L. and Hsu, M.-T. (1990) *J. Virol.* **64**, 691–699