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Camptothecin-Stabilized Topoisomerase I–DNA Adducts Cause Premature Termination of Transcription†

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ABSTRACT: The antitumor agent camptothecin stabilizes type I topoisomerase–DNA complexes. One of the primary cellular responses to camptothecin exposure is rapid cessation of RNA synthesis. Results obtained by using an in vitro transcription system supplemented with eukaryotic topoisomerase I show that this inhibition can be attributed to physical blockage of the RNA polymerase by camptothecin-stabilized topoisomerase I–DNA complexes on the DNA template. The site of premature termination is located 10 base pairs upstream of the topoisomerase I linked nucleotide residue on the coding strand, corresponding closely to the border of the protected area obtained in a micrococcus nuclease footprint of topoisomerase I. The RNA polymerase transcribes unimpeded through complexes attached to the noncoding strand. No inhibitory effect of camptothecin on RNA transcription was observed with human topoisomerase I isolated from a camptothecin-resistant cell line. Taken together, the data suggest that part of the cytotoxicity of camptothecin is mediated through adduct formation on transcribed DNA, resulting in interference with transcriptional elongation.

DNA topoisomerases I and II are ubiquitous enzymes influencing essential processes such as replication, chromatid segregation, and transcription through regulation of DNA topology (Wang, 1985; Vosberg, 1985). Recently, DNA topoisomerases have been identified as the intranuclear target for a number of important chemotherapeutic agents (Bodley & Liu, 1988; Liu, 1989). Mechanistically, topoisomerases act by transiently breaking the phosphodiester backbone, and antitumor drugs are thought to interfere with the topoisomerization process by stabilizing a covalent enzyme–DNA reaction intermediate in the normal catalytic cycle (Hsiang et al., 1985). The stabilization of the DNA–topoisomerase complex by antitumor drugs results in inhibition of catalytic activity and DNA breakage in the presence of a protein denaturant.

Camptothecin and its derivatives are topoisomerase I targeting cytotoxic alkaloids with antineoplastic activity (Gallo et al., 1971; Tsuruo et al., 1988; Giovanella et al., 1989).

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Studies have shown that camptothecin strongly inhibits DNA and RNA synthesis and induces extensive fragmentation of chromosomal DNA in eukaryotic cells (Kessel et al., 1972; Covey et al., 1989). The cellular target of the drug has been shown to be DNA topoisomerase I as the drug inhibits the catalytic activity of the purified enzyme (Hsiang et al., 1985). Consistently, some camptothecin-resistant cell lines are insensitive to drug exposure due to the presence of a mutant form of topoisomerase I (Andoh et al., 1987; Gupta et al., 1988); also, yeast mutants totally lacking the enzyme are completely resistant to the drug (Eng et al., 1988). Moreover, expression of human topoisomerase I in yeast lacking a functional endogenous topoisomerase I restores camptothecin sensitivity (Bjornsti et al., 1989).

Actively transcribed genes in the eukaryotic nucleus are highly complexed with topoisomerase I that serves to alleviate the transcription-promoted torsional strain in the DNA template (Fleischman et al., 1984; Stewart & Schütz, 1987; Gilmour & Elgin, 1987; Zhang et al., 1988). Movement of the transcriptional apparatus along the template generates a positively supercoiled domain in front of the transversing RNA polymerase and a negatively supercoiled domain behind it (Wu et al., 1988; Giever & Wang, 1988; Osborne & Guarente, 1988; Brill & Sternglanz, 1988). Perturbation of the relaxing activity of topoisomerase I by treatment with camptothecin might therefore lead to accumulation of torsional strain in the DNA template and eventually stop transcriptional elongation (Garg et al., 1987). Transcriptional arrest may also be caused by inhibition of RNA polymerase movement through the camptothecin-stabilized topoisomerase I–DNA covalent adducts on the DNA template. To test the latter suggestion, we inserted a high-affinity hexadecameric topoisomerase I recognition sequence (Bonven et al., 1985) downstream of a promoter specific for a highly processive viral RNA polymerase (Butler & Michael, 1982; Melton et al., 1984). It is found that topoisomerase I–DNA complexes on a linear template in the presence of camptothecin provoke premature termination of transcription by steric hindrance of elongation.

Experimental Procedures

Purification of Topoisomerase I. Human DNA topoisomerase I and the mutant enzyme CPT-K5 were purified as described by Kjeldsen et al. (1988a,b). *Tetrahymena thermophila* topoisomerase I was purified as described by Thomsen et al. (1987). One unit of activity is defined as the amount of topoisomerase that relaxes 50% of 1 μg of fully supercoiled pBR322 DNA in 30 min at 30 °C.

Plasmids. pCB641 and pCB651 were constructed by insertion of the *Hin*dIII–*Sal*I fragment spanning from nucleotide positions 29–303 in pNC1 (Thomsen et al., 1987; Busk et al., 1987) into the *Hin*dIII–*Sal*I sites of the polylinker region of pSP64 and pSP65, respectively. pCB645 is a construct similar to pCB641, containing the *Hin*dIII–*Sal*I fragment from pNC5 (Thomsen et al., 1987; Busk et al., 1987).

Topoisomerase I Mediated DNA Cleavage Reactions. 3′ end-labeled restriction fragments (5 fmol) were incubated with 50 units of topoisomerase I in the transcription buffer for 15 min at 30 °C. Sodium dodecyl sulfate (SDS)[1] and EDTA were added to final concentrations of 1% and 10 mM, respectively, and incubation was continued for 5 min at 42 °C. After proteinase K treatment, the cleavage products were analyzed on 6% denaturing polyacrylamide gels. Camptothecin (lactone form, no. 94600) was kindly provided by the National Cancer Institute. Stock solutions, 10 mM in DMSO, were stored at −20 °C.

In Vitro Transcriptions. SP6 RNA polymerase transcriptions were carried out in a 30-μL reaction volume containing 40 mM Tris·HCl, pH 7.6, 5 mM $MgCl_2$, 10 mM DTT, 1 mM spermidine, 0.3 mM ATP, GTP, and CTP, 50 μM UTP, 25 μCi of [α-^{32}P]UTP, 20–30 fmol of DNA template/reaction, 1 unit/μL RNAsin (Promega), and 0.2 unit/μL SP6-polymerase (Promega). pCB641 and pCB645 were linearized at the *Hin*cII site. pCB651 was linearized at the *Hin*dIII site. Transcriptions were carried out at 37 °C for 15 min. Transcription was terminated by addition of EDTA followed by phenol–chloroform extraction and ethanol precipitation. The transcription products were analyzed on 6% or 8% denaturing polyacrylamide sequencing gels.

RNA Transcript Sequencing. RNA sequencing reactions were done as above except for the addition of 3′-deoxyribonucleoside 5′-triphosphate analogues (Pharmacia) of the nucleotide being sequenced. The concentration of 3′-dATP, 3′-dGTP, and 3′-dCTP was 150 μM, and the concentration of 3′-dUTP was 25 μM.

Footprinting Analysis. 3′ end-labeled restriction fragment (20 fmol) was incubated with 100 units of topoisomerase I in a 40-μL reaction volume containing 10 mM Tris·HCl, pH 7.2, 3 mM $CaCl_2$, and 3 mM $MgCl_2$ for 15 min at 30 °C. One aliquot of 10 μL was treated with SDS and EDTA in final concentrations of 1% and 10 mM, respectively, for 5 min at 42 °C. The remaining 30 μL was treated with 0.05 unit micrococcal nuclease for 10 s at 30 °C, and the reaction was terminated by addition of NaCl, EDTA, and salmon sperm DNA to final concentrations of 0.8 M, 10 mM, and 60 μg/mL, respectively. Finally, both aliquots were treated with proteinase K, and the products were analyzed on 6% denaturing polyacrylamide gels.

Quantitation of RNA Transcription Products on Polyacrylamide Gels. A quantitative measure of the relative band intensity was obtained by densitometric scanning of the autoradiograms using a Shimadzu Chromatoscanner, Model CS930.

Results

Camptothecin-Stabilized Topoisomerase I–DNA Adducts Provoke Transcriptional Arrest. To characterize the interference of topoisomerase I–DNA adducts with the transcriptional process, we have taken advantage of a high-affinity hexadecameric binding sequence for topoisomerase I (Bonven et al., 1985; Busk et al., 1987; Kjeldsen et al., 1990). A 274 bp *Hin*dIII–*Sal*I fragment from pNC1 harboring the high-affinity topoisomerase I recognition sequence (Thomsen et al., 1987) was inserted in either orientation downstream of a highly processive viral RNA polymerase promoter. These allowed transcription toward DNA–topoisomerase I adducts formed at the hexadecameric sequence situated either on the coding strand or on the noncoding strand, pCB641 and pCB651, respectively. Since the interaction between the high-affinity recognition sequence and topoisomerase I is confined to a 20 bp region in which the cleavage site is centrally located (Stevnsner et al., 1989), insertion of this 274 bp fragment in either orientation relative to the promoter ensured that complex formation at the hexadecameric sequence occurred at the same frequency when present in the template strand as when present in the nontemplate strand. A schematic representation of these transcription templates is shown in Figure 1. A similar

[1] Abbreviations: SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; DTT, dithiothreitol; DMSO, dimethyl sulfoxide; bp, base pair(s).

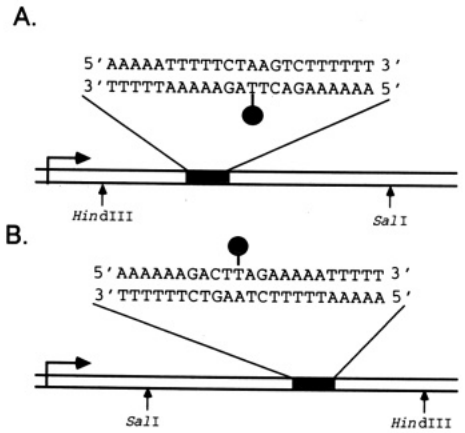

FIGURE 1: Schematic representation of the in vitro transcription templates pCB641 (A) and pCB651 (B). The arrows indicate the transcription initiation sites for SP6 polymerase. The outlined sequence demonstrates the position of the topoisomerase I–DNA covalent adduct with respect to the transcribed strand. The template pCB645 is identical with pCB641 except for a single base mutation at the site of covalent attachment which abolishes topoisomerase I binding.

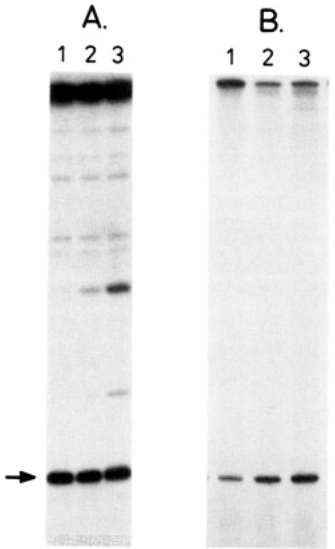

FIGURE 2: Topoisomerase I mediated DNA cleavages on the transcription template. Five femtomoles of *Hin*dIII–*Hin*cII fragment of pCB641 3′ end-labeled at the *Hin*dIII site was used as substrate. (A) lanes 1–3, 50 units of human topoisomerase I and 0, 10, and 100 μM camptothecin, respectively. (B) lanes 1–3, 50 units of *Tetrahymena* topoisomerase I and 0, 50, and 100 μM camptothecin, respectively. Arrow indicates cleavage at the hexadecameric sequence motif.

construct, pCB645, was made with a single base mutation within the hexadecameric sequence that abolishes binding of topoisomerase I (Thomsen et al., 1987; Stevnsner et al., 1989).

Sites at which topoisomerase I interacts with DNA can be identified by treatment of these complexes with the strong protein denaturant sodium dodecyl sulfate (SDS), resulting in formation of single-stranded DNA breaks with topoisomerase I covalently linked to the 3′ end. In order to map the topoisomerase I mediated cleavages on the transcribed DNA, a *Hin*dIII–*Hin*cII fragment from pCB641 encompassing the template was 3′ end-labeled at the *Hin*dIII site which is located 7 bp downstream of the transcription initiation site. Subsequently, a fixed amount (5 fmol) of this fragment was incubated with 50 units of topoisomerase I from higher (human) or lower eukaryotic organisms (*Tetrahymena*) (panels A and B, respectively, of Figure 2) in the presence of varying amounts of camptothecin. Following SDS treatment, the cleavage products were analyzed on a denaturing polyacrylamide gel. The experiment shows that DNA cleavage

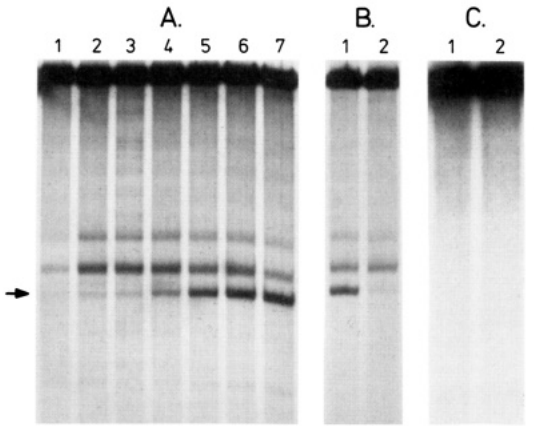

FIGURE 3: (A) Accumulation of premature transcription products in the presence of increasing amounts of camptothecin. Lanes 1–7, transcription of 20 fmol of pCB641 in the presence of 100 units of *Tetrahymena* topoisomerase I and increasing amounts of camptothecin, 0, 1, 5, 10, 50, 80, and 100 μM, respectively. The arrow marks the camptothecin-induced termination product. (B) Abortive transcriptional elongation depends on the presence of a high-affinity topoisomerase I binding sequence. Transcription in the presence of 100 units of *Tetrahymena* topoisomerase I, 100 μM camptothecin, and as template 20 fmol of pCB641 containing the high-affinity topoisomerase binding site (lane 1) or 20 fmol of pCB645 containing the mutated topoisomerase I binding sequence (lane 2). (C) RNA polymerase transcribes unimpeded through adducts on the noncoding strand. Transcription of 20 fmol of pCB651 using 100 units of *Tetrahymena* topoisomerase I in the absence (lane 1) and in the presence (lane 2) of 100 μM camptothecin.

in the absence of camptothecin is largely confined to the high-affinity topoisomerase I binding sequence for both the human enzyme (Figure 2A, lane 1) and the *Tetrahymena* enzyme (Figure 2B, lane 1). In the presence of increasing amounts of camptothecin, the cleavage frequency of the human enzyme at the different sites is altered dramatically (Figure 2A, lanes 2 and 3), whereas the cleavage pattern of the *Tetrahymena* enzyme is relatively unaffected (Figure 2B, lanes 2 and 3). Camptothecin has, however, a marked influence on the *Tetrahymena* enzyme at the hexadecameric sequence motif where the cleavage frequency is increased from 25–30% to approximately 70%.

Studies on the effect of camptothecin on transcription were done by using the SP6 RNA transcription system. The SP6 RNA polymerase is a highly processive and strictly promoter-specific enzyme that has proven to be suitable for high-yield RNA synthesis (Melton et al., 1984) as well as molecular studies of various aspects of transcription (Lorch et al., 1987; Losa & Brown, 1987). To study the effect of a single, well-defined camptothecin-stabilized topoisomerase I–DNA adduct on RNA synthesis, the initial experiments were performed using *Tetrahymena* enzyme. Figure 3A shows the results of an experiment where 20 fmol of linearized pCB641 was incubated 15 min at 37 °C with 6 units of RNA polymerase and 100 units of *Tetrahymena* topoisomerase I in the presence of increasing amounts of camptothecin. The transcription reactions were terminated with EDTA, and the RNA was purified followed by analysis by denaturing polyacrylamide gel electrophoresis. The experiment reveals the formation of a specific drug-induced termination product (marked with an arrow) accumulating in a drug concentration dependent manner (lanes 2–7). In the absence of drug, there is no effect on transcription (lane 1). The various RNA transcripts present in all lanes are sequence-dependent termination products, and their relative intensities are subject to variations in transcription efficiency. No correlation to the presence of topoisomerase I or camptothecin has been observed. Such sequence-de-

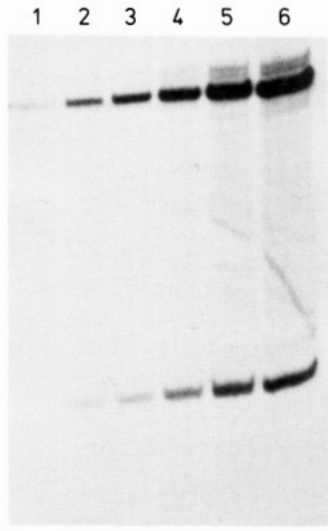

FIGURE 4: Time course study on transcriptional termination. Aliquots (30 μL) were withdrawn from a 200-μL reaction containing 600 units of *Tetrahymana* topoisomerase and I and 100 μM camptothecin. Lanes 1–6, the transcriptions were stopped by addition of EDTA at 30 s and 1, 3, 5, 8, and 10 min, respectively.

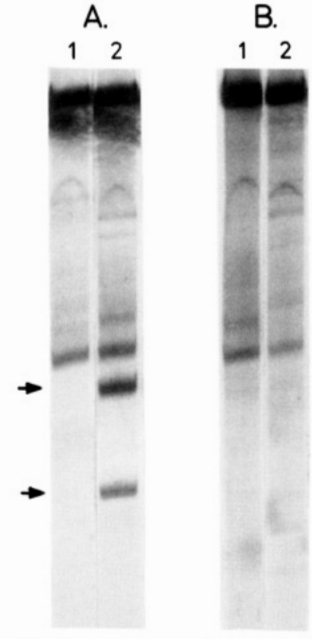

FIGURE 5: Camptothecin-resistant topoisomerase I does not interfere with transcription. (A) Transcriptions in the presence of 50 units of wild-type human topoisomerase I without camptothecin (lane 1) or in the presence of 50 μM camptothecin (lane 2). (B) Transcription using 50 units of camptothecin-resistant human topoisomerase I, CPT-K5, without camptothecin (lane 1) or in the presence of 50 μM camptothecin (lane 2). Arrows mark the most prominent camptothecin-induced termination products.

pendent termination of the SP6 RNA polymerase has previously been described (Mead et al., 1985).

To assess whether transcriptional arrest depends on the presence of the high-affinity topoisomerase I binding sequence, we examined the effect of a single base pair mutation within the hexadecameric sequence motif that abolishes topoisomerase I binding. Figure 3B shows that employment of this template with reduced ability to bind topoisomerase I efficiently restores full-length RNA synthesis. These results demonstrate that the RNA polymerase is blocked at the topoisomerase I binding site in the presence of camptothecin, thus causing termination or pausing of transcription.

SP6 Polymerase Transcribes Unimpeded through Topoisomerase I–DNA Complexes Located on the Noncoding Strand. Using linearized pCB651 as template, we tested the effect of DNA–topoisomerase I adducts attached to the noncoding strand. The result of this analysis is shown in Figure 3C (lanes 1 and 2) where no transcriptional inhibition is observed either in the presence or in the absence of camptothecin. Thus, SP6 polymerase only interacts with the camptothecin-stabilized topoisomerase I–DNA complexes when the cleaved strand is transcribed. The lack of interference on the noncleaved strand may, however, be related to the small size and the high processivity of the SP6 polymerase, and it cannot be excluded that the larger eukaryotic RNA polymerases are sterically inhibited by adducts on the noncoding strand.

Blocked RNA Transcripts Are Generated by Termination Rather than Pausing. To distinguish whether the blocked RNA transcripts were formed as a result of pausing or termination, we performed time course studies where aliquots were taken over a 10-min reaction period. In the case of pausing, the blocked transcripts would be released from the polymerases upon stopping the reaction with EDTA, and a constant amount of blocked transcripts would be expected. However, Figure 4 (lanes 1–6) shows an accumulation of the blocked transcripts proportional to the formation of full-length transcripts, suggesting that they are generated by termination rather than pausing.

The amounts of full-length transcripts and camptothecin-induced blocked transcripts were determined by densitometry and corrected to accommodate that the two RNA species do not contain the same amount of radioactive label. We estimate that the percentage of drug-induced terminated transcripts at the high-affinity topoisomerase I binding sequence amounts to approximately 20%.

Complexes Formed with a Camptothecin-Resistant Topoisomerase I Have No Inhibitory Effect on RNA Synthesis. Topoisomerases from higher eukaryotes are more sensitive to camptothecin than enzymes from lower eukaryotes as assayed by the yield of drug-induced topoisomerase-linked DNA breaks (Figure 2; Kjeldsen et al., 1988b). The above experiment was therefore repeated using human topoisomerase I. As expected, the transcriptional interference analysis now revealed the formation of several blocked RNA transcripts, the most prominent ones marked with arrows in Figure 5A. The upper arrow corresponds to cleavage at the high-affinity sequence and the lower to an additional but weaker cleavage site (data not shown).

A human lymphoblastoid cell line containing a camptothecin-resistant form of topoisomerase I has previously been shown to escape the effects of camptothecin on RNA synthesis (Andoh et al., 1987), and it was therefore of interest to investigate the drug-resistant enzyme in our transcription assay. Figure 5B shows that in contrast to the results obtained with the wild-type enzyme, the topoisomerase I–DNA adducts formed with the camptothecin-resistant enzyme have no inhibitory effect on RNA polymerase movement along the template.

Transcriptional Termination Is Located 10 bp Upstream of the Site of Topoisomerase I Mediated DNA Cleavage. The size of the *Tetrahymena* topoisomerase I binding region was determined in a micrococcus nuclease footprinting assay (Figure 6A). 3′ end-labeled transcription template was incubated in the absence or presence of 100 units of *Tetrahymena* topoisomerase I. One aliquot was withdrawn from both reaction mixtures and treated with SDS (lanes 1 and 2). The remainder of both reactions was treated with 0.05 units of micrococcus nuclease for 10 s (lanes 3 and 4). Densito-

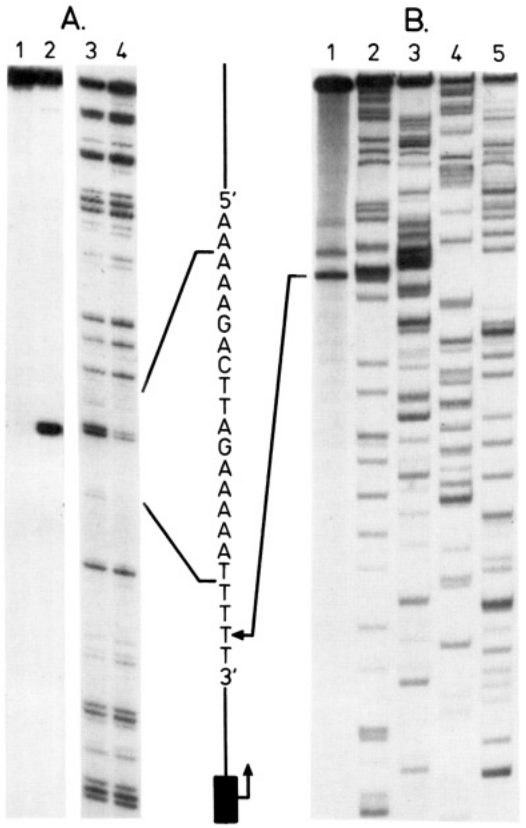

FIGURE 6: (A) Footprinting analysis of *Tetrahymena* topoisomerase I. 3′ end-labeled *Hin*dIII–*Hin*cII fragment of pCB641 was used as substrate. Lanes 1 and 2, SDS-treated samples. Lanes 3 and 4, micrococcus nuclease digested samples. The amounts of topoisomerase I employed are 0 units (lanes 1 and 3) and 100 units (lanes 2 and 4). (B) RNA polymerase terminates 10 base pairs in front of the topoisomerase I linked nucleotide residue. Lane 1, transcription of 20 fmol of pCB641 in the presence of 100 units of *Tetrahymena* topoisomerase I and 100 μM camptothecin. Lanes 2–5, RNA transcript sequencing using 150 μM 3′-dATP, 25 μM 3′-dUTP, 150 μM 3′-dGTP, and 150 μM 3′-dCTP, respectively. The outlined sequence denotes the coding strand with the SP6 promoter shown as a filled box. Brackets mark the topoisomerase I binding region, and the arrow indicates the position of premature termination.

metric scannings of the micrococcal nuclease cleavages and the topoisomerase I mediated cleavage product revealed approximately 80% protection and 30% enzyme-mediated cleavage at the hexadecameric sequence. In agreement with a previous report (Stevnsner et al., 1989) in which the protection boundaries were deliniated more precisely, the footprinting analysis demarcates the topoisomerase I binding area on the cleaved strand to a 16 base pair region symmetrically around the cleavage site.

To accurately define the site of transcriptional termination relative to the protected area, we sequenced the RNA transcripts, using 3′-deoxynucleotide analogues for chain termination. Electrophoresis of the camptothecin-blocked transcript (Figure 6B, lane 1) in parallel to the RNA transcript sequence analysis (Figure 6B, lanes 2–5) locates the site of transcriptional termination to position −10 relative to the site of topoisomerase cleavage. This position corresponds closely to the border of protection in the micrococcus nuclease footprint of the topoisomerase I–DNA complex, advocating that the RNA polymerase stops prematurely due to physical blockage by the topoisomerase I–DNA adduct covalently linked to the DNA template.

Discussion

Due to the central role of topoisomerase I in macromolecular DNA metabolism, this enzyme constitutes an important target for chemotherapy. Recently, a camptothecin derivative with low overall toxicity, 20(*RS*)-9-aminocamptothecin, has been shown to be a potent antitumor agent against human colon cancer in xenografts (Giovanella et al., 1989). The underlying molecular mechanisms responsible for the antitumor activity of camptothecin and its derivatives still remain to be fully elucidated.

In the present study, we have investigated the mechanism of camptothecin-induced cessation of RNA synthesis. The data demonstrate that topoisomerase I–DNA complexes stabilized by camptothecin provoke transcriptional arrest by direct physical impediment of the advancing polymerase. In spite of 80% protection in a nuclease footprinting assay (in the absence of camptothecin), topoisomerase I per se does not block transcription. This demonstrates that the enzyme–DNA complexes in the absence of drug are too transient to impede RNA polymerase movement, which was to be anticipated considering that topoisomerase I is preferentially associated with regions of chromatin undergoing active transcription (Fleischmann et al., 1984; Stewart & Schütz, 1987). Furthermore, since camptothecin is believed to stabilize the cleavable complexes by slowing the rate of religation, the finding that transcription inhibition is totally dependent upon camptothecin indicates that the blockage is caused by covalent intermediates. The difference in the level of topoisomerase I mediated cleavage and the amount of aborted transcription products in the presence of camptothecin (70% versus 20%) probably arises from the fact that even in the presence of camptothecin the dissociation of the covalent enzyme–DNA complexes is not permanently impaired. Supportive of this interpretation is the observation that the rate of religation at the hexadecameric sequence is only marginally affected (reduced approximately 2-fold) by camptothecin (Kjeldsen et al., 1988b). Thus, even in the presence of camptothecin, readthrough accompanying dissociation of topoisomerase I is to be expected. Incomplete inhibition of transcriptional elongation presumably due to the reversible nature of the camptothecin-stabilized topoisomerase I–DNA complexes has also recently been observed in vivo (Stewart et al., 1990).

In the experiments, advantage was taken of the well-characterized interaction between a high-affinity topoisomerase I binding sequence and *Tetrahymena* topoisomerase I (Busk et al., 1987; Stevnsner et al., 1989). This enabled us to establish the close correlation between the topoisomerase I binding region and the site of camptothecin-induced blockage of transcription, demonstrating that camptothecin inhibition of transcription in vitro can be explained by physical blockage of RNA polymerase movement. It is, however, noteworthy that blockage of transcription in the presence of the drug is not restricted to the hexadecameric sequence as employment of the human topoisomerase I which exhibits less sequence specificity as compared to *Tetrahymena* topoisomerase I (Figure 2) in the transcription assay provoked the formation of several blocked transcripts (Figure 5). Thus, although the low-affinity sites are expected to obstruct elongation at a lower rate as compared to the hexadecameric sequence, these results indicate that physical blockage of RNA polymerase elongation is a general phenomenon.

Our results are consistent with the findings of Zhang et al. (1988) showing that camptothecin gradually reduces nuclear run-on transcripts along the rRNA transcription unit, as the probability of encountering an adduct is proportional to the length of the transcription unit. Apparently, only topoisomerase I–drug adducts located on the coding strand inhibit

elongation whereas adducts linked to the noncoding strand have no effect. This observation is particularly intriguing as experiments have suggested that topoisomerase I is asymmetrically distributed with a preference for the coding strand on actively transcribed genes (Stewart & Schütz, 1987). However, on the basis of the present in vitro SP6 polymerase transcription studies, it cannot be excluded that also drug-stabilized topoisomerase I–DNA adducts linked to the noncoding strand may cause blockage of the bulky eukaryotic transcription apparatus in vivo.

A camptothecin-resistant lymphoblastic leukemia cell line with a mutated topoisomerase I gene has previously been shown to be refractory to the effects of camptothecin on RNA synthesis (Andoh et al., 1987). In agreement with this result, the in vitro RNA synthesis is unaffected by camptothecin when employing topoisomerase I purified from the drug-resistant cell line.

A previous study has indicated that the inhibitory effect of camptothecin on RNA synthesis reflects impeded movement of the polymerase due to lack of relaxation of the topological stress associated with transcription rather than blockage of the polymerase by bound topoisomerase I (Garg et al., 1987). Our results show that the RNA polymerase terminates on encountering a camptothecin-stabilized topoisomerase I–DNA complex on a linear template. Sequence fine mapping showed that the polymerase stops 10 base pairs 3′ to the nucleotide residue covalently linked to topoisomerase I. This distance closely matches the border of the region protected by topoisomerase I in the micrococcus nuclease footprinting assay, demonstrating that the inhibition of transcription in the presence of camptothecin is due to physical blockage of RNA polymerase movement. Thus, it appears that two mechanisms are responsible for the cessation of RNA synthesis in the presence of camptothecin. However, considering the fact that the amount of prematurely terminated transcripts at a single adduct approaches 20%, our results show that the toxic effect of camptothecin on RNA transcription is particularly attributable to steric hindrance of elongation caused by adduct formation on actively transcribed DNA.

Our observations lend support to the idea that camptothecin primarily exerts its physiological effect through formation of deleterious topoisomerase I–DNA adducts rather than simple inhibition of catalytic activity. Other lines of evidence in accordance with this mechanism come from studies on cell hybridization between camptothecin-resistant and camptothecin-sensitive cell lines, showing that the drug-resistant phenotype behaves recessively (Gupta et al., 1988). Consistent with the model are also recent results of Hsiang et al. (1989), showing arrest of SV40 replication intermediates in the presence of camptothecin due to interaction between the advancing replication forks and drug-stabilized topoisomerase I–DNA adducts.

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Structure Calculations for Single-Stranded DNA Complexed with the Single-Stranded DNA Binding Protein GP32 of Bacteriophage T4: A Remarkable DNA Structure†

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ABSTRACT: In this study it is established by calculation which regular conformations single-stranded DNA and RNA can adopt in the complex with the single-stranded DNA binding protein GP32 of bacteriophage T4. In order to do so, information from previous experiments about base orientations and the length and diameter of the complexes is used together with knowledge about bond lengths and valence angles between chemical bonds. It turns out that there is only a limited set of similar conformations which are in agreement with experimental data. The arrangement of neighboring bases is such that there is ample space for aromatic residues of the protein to partly intercalate between the bases, which is in agreement with a previously proposed model for the binding domain of the protein [Prigodich, R. V., Shamoo, Y., Williams, K. R., Chase, J. W., Konigsberg, W. H., & Coleman, J. E. (1986) *Biochemistry 25*, 3666–3671]. Both C2′endo and C3′endo sugar conformations lead to calculated DNA conformations that are consistent with experimental data. The orientation of the O2′ atoms of the sugars in RNA can explain why the binding affinity of GP32 for polyribonucleotides is lower than for polydeoxyribonucleotides.

The gene 32 protein (GP32) of bacteriophage T4 is a much studied example of the single-stranded DNA binding proteins (Chase & Williams, 1986), and it binds much more strongly to single-stranded DNA (ssDNA) than to double-stranded DNA (dsDNA) in a more or less nonspecific way (Jensen et al., 1976). The binding to polydeoxyribonucleotides is stronger than to polyribonucleotides (Newport et al., 1981). The binding is characterized by a high cooperativity, leading to a continuous covering of the DNA by the protein (Delius et al., 1972); estimates for the cooperativity factor range from 2×10^2 to 10^4 (Kowalczykowski et al., 1981; Lohman, 1984; Watanabe, 1989; Kuil et al., 1989). There has been much discussion about the size of the binding site (n) and values obtained range from 5 to 11 nucleotides per protein, but it has been argued that inactivation of a fraction of the protein has caused the lower values (Bobst et al., 1982; Scheerhagen et al., 1986b). Minimizing the contribution of inactive protein during the determination of the site size led to values for n close to 10 (Bobst et al., 1982; Scheerhagen et al., 1986b; Kuil et al., 1988). In addition, Watanabe (1989) reported that in the analysis of a titration experiment the cooperativity can easily be overestimated, which could explain the lower values of n. Reanalysis of titration curves yielded a value of $n = 9$ for the binding of GP32 to poly(rA), in agreement with our recent results (Kuil et al., 1989).

As was reviewed by Chase and Williams (1986), the protein is involved in replication, repair, recombination, and translation processes, and it protects ssDNA against nuclease attack. The protein is also involved in late transcription (Gauss et al., 1987).

The crystal structure of GP32 is not known and neither is that of the complex with ssDNA. Much of what is known about this complex structure stems from spectroscopic measurements. In Scheerhagen (1986) and Scheerhagen et al. (1989) a model for the complex is presented that is largely based on hydrodynamic and spectroscopic measurements (Scheerhagen et al., 1985a–c, 1986a,b; Scheerhagen, 1986). The DNA is held in a more or less rigid, regular conformation, at least over a region of several bases. The DNA strand is rather extended in the complex, and the base–base distance projected along the overall complex axis is between 4.3 and 5.4 Å. From hydrodynamic measurements using complexes with small DNA fragments (about 100 bases), it was concluded (Scheerhagen et al., 1985b; Scheerhagen, 1986) that the local complex axis (e.g., at the level of one protein) is not parallel to the overall complex axis, which implies that the true base–base distance is larger than the distance projected on the overall axis. This can reflect either flexibility of the complex or a tertiary structure. Since at that time the interpretation of the hydrodynamic measurements in terms of flexibility seemed difficult, Scheerhagen chose to model the complex as a regular superhelix, where the local complex axis at every position in the complex has the same orientation with respect to the overall axis (superhelix axis). Recently, hydrodynamic studies (Kuil et al., 1988, 1990) were undertaken with larger fragments of single-stranded DNA with the aim of obtaining more specific data about the flexibility of the GP32–single-

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