The anti-cancer drug camptothecin inhibits elongation but stimulates initiation of RNA polymerase II transcription

Mats Ljungman and Philip C. Hanawalt

Introduction

Camptothecin is a plant alkaloid possessing a broad spectrum of anti-tumor activity (1). Treatment of cells with camptothecin has been shown to result in the inhibition of the synthesis of both DNA (2) and RNA (3–5) as well as the induction of the tumor suppressor protein p53 (6) and the fragmentation of chromosomal DNA (7). Camptothecin also selectively kills tumor suppressor protein p53 (6) and the fragmentation of chromosomal DNA (7). Camptothecin is the inhibition of DNA topoisomerase I (10,11). The principal function of DNA topoisomerase I is to relax DNA torsional stress induced by DNA replication fork movement and by transcriptional elongation (for reviews see 1,12). The mechanism of relaxation has been shown to involve the formation of a transient DNA single-strand break through which the other DNA strand is passed, followed by the rejoicing of the transient break (12–14). Camptothecin specifically interferes with the rejoining step in the topoisomerase reaction which results in the trapping of the topoisomerase I enzyme in a reversible cleavable complex (10,11). It is thought that this trapped topoisomerase complex is a barrier for RNA polymerase elongation (15). It has been shown that the elongation but not initiation of transcription of the ribosomal genes by RNA polymerase I is inhibited by camptothecin in vivo (16).

It has been shown that topoisomerase I is involved in both the initiation (17,18) and elongation (19–21) of RNA polymerase II transcribed genes. The aim of this study was to assess the effect of camptothecin on transcription from the polymerase II transcribed dihydrofolate reductase (DHFR*) gene. Transcription from three different regions of the DHFR gene in Chinese hamster ovary (CHO) B11 cells was assessed both in vivo by [3H]uridine pulse labeling followed by DNA filter excess hybridization, and in vitro by the nuclear run-on technique. Results show that in cells treated with camptothecin, transcription was affected differentially throughout the DHFR gene. While transcription from promoter-distal sequences of the gene was reduced, transcription from the 5'-end of the DHFR gene was stimulated in the presence of camptothecin.

Materials and methods

Cell culture

The methotrexate-resistant Chinese hamster cell line, CHO K1 B11 (0.5), containing an 80-fold amplification of the DHFR gene (22), was plated at a density of 1.5 × 10^6 cells per 60 cm^2 culture dish in minimal essential medium (MEM) supplemented with 10% dialyzed fetal bovine serum, 2 mM L-glutamine, 1% non-essential amino acids, 100 IU penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B and 5000 µg/ml [methyl-3H]thymidine (50 Ci/mmol) (Amersham) (13). The medium was removed 24 h after seeding the cells and the cells were rinsed with PBS. The cells were then supplied with a medium containing 0.1% fetal bovine serum but lacking labeled nucleotides. After 4 days of incubation in the low serum medium (serum starvation), the cells were stimulated by exchanging the medium with medium containing 15% fetal bovine serum. The camptothecin experiments were performed 15 h after the serum addition to allow for maximal transcription of the DHFR gene (23,24).

In vivo transcription

The measurement of in vivo synthesis of nascent RNA was performed as previously described (24) except for the labeling period which was shortened to 15 min. CHO B11 cells were either washed twice in PBS and supplied with camptothecin-free media (chase experiments) or left in 0.8 ml of medium containing 20 µM camptothecin. RNA labeling took place in 1 ml medium with medium containing 15% fetal bovine serum. The camptothecin experiments were performed 15 h after the serum addition to allow for maximal transcription of the DHFR gene (23,24).

In vitro nuclear run-on transcription

The nuclear run-on procedure was performed as previously described (24). Nuclei were isolated from ~10^6 cells and heterogeneous nuclear RNA was labeled in vitro in the absence of camptothecin with 32P-labeled UTP for

Abbreviations: DHFR, dihydrofolate reductase; CHO, Chinese hamster ovary cells.

© Oxford University Press
15 min at 30°C. RNA was then isolated, partially hydrolyzed in cold alkali (25) and hybridized at 65°C for 48 h to 2-4 μg of DNA probes immobilized on Hybond N+ membranes. Membranes were stringency washed at 65°C in 0.1 X SSPE and 0.1% SDS for 60 min. Autoradiographs were scanned using a scanning densitometer (Helena Laboratories), and peak values were corrected for cell input by measuring the tritium count of isolated nuclei prior to the run-on protocol (24).

Results

Camptothecin enhances transcription in the 5'-end of the DHFR gene in vivo

First we estimated the relative rate of transcription in camptothecin-treated CHO B11 cells using an approach in which nascent RNA was labeled in vivo with [5-3H]uridine (Figure 1). Three DNA probes complementary to RNA synthesized from the 5'-end, the middle portion or the 3'-end of the DHFR gene were used to detect any differential effect on transcription throughout the DHFR gene. In addition, a DNA probe complementary to the 3'-half of the ribosomal RNA transcriptional unit was used to assess the effect of camptothecin on polymerase I transcription (Figure 2).

We found that treatment with 20 μM camptothecin for 30 min at 37°C reduced the rate of total RNA synthesis to ~30% of that in untreated control cells as measured by liquid scintillation counting of a small aliquot of isolated total RNA (Figure 3). This inhibitory effect on total RNA synthesis by camptothecin is consistent with results obtained by others (3,5). When studying the effect of camptothecin on the RNA polymerase II transcribing DHFR gene, it was found that transcription was differentially affected throughout the gene. RNA synthesis was enhanced in the 5'-end of the DHFR gene to ~150% of that of untreated control cells, while RNA synthesis was severely inhibited in the middle portion and the 3'-end to 20% and 10% of that of untreated controls, respectively. RNA synthesis from the 3'-half of the rRNA transcriptional unit was virtually abolished in the presence of camptothecin, a result which supports earlier findings (16).

Nuclear run-on technique reveals a dramatic increase in transcription signal from the 5'-end of the DHFR gene

The effect of camptothecin on DHFR transcription was also evaluated using the nuclear run-on technique. In this technique, the in vivo distribution of RNA polymerases on a particular gene is scored by allowing engaged RNA polymerases to elongate in vitro in the presence of [32P]UTP (25,26). The results obtained support the results from the uridine-labeling experiments in that the DHFR RNA synthesis was enhanced in the 5'-end (Figure 4A,B). However, the enhancement of the in vitro nuclear run-on labeling was much greater. In fact, the [32P]UTP-incorporation in the run-on transcripts from the 5'-end was 10--15 times higher in nuclei from camptothecin-treated cells than in untreated control cells. This is to be compared to the 50% increase of transcription in the equivalent experiment using the in vivo uridine-labeling technique (see Figure 3). Another discrepancy between the results obtained using the two different techniques was that the marked inhibi-
Experiments with bars showing the sample standard deviation.

Independent n relative to untreated control cells and represent the mean of from a number of experiments are summarized and the values are expressed relative to untreated control cells (100%) and represent the mean of three independent experiments with bars showing the sample standard deviation.

Recovery of transcription by the removal of camptothecin

In the next set of experiments, we wanted to address the question of whether the effects of camptothecin on transcription were reversible. Cells were treated with 20 μM camptothecin for 30 min at 37°C, and nascent RNA was [3H]uridine-labeled in vivo for 15 min in camptothecin-free media (chase 0–15 min). In addition, some cells were allowed to incubate in drug-free media for 15 min prior to the uridine-labeling of the RNA in drug-free media (chase 15–30 min) (Figure 1).

The results show that a chase of camptothecin-treated cells with drug-free media only partially restored synthesis of total RNA from ~30% to ~50–60% of that synthesized in untreated control cells (Figure 5). The same was true for rRNA synthesis where in vivo [3H]uridine-labeling of nascent RNA rebounded from <1% to ~50% of that of controls. The measurements of transcription from the 5’-end of the DHFR gene revealed that transcription remained elevated at levels of ~50% of that of controls even after 15–30 min of incubation in drug-free media. Transcription from the middle portion of the DHFR gene rebounded from ~20% to ~60% during a 15 min chase and to control levels during a 15–30 min chase. At the 3’-end of the DHFR gene, the transcription showed no signs of recovery during a 0–15 min chase but transcription was fully recovered after a 15–30 min incubation in drug-free media.

Discussion

The effect of the anti-cancer drug camptothecin on RNA polymerase II transcription of the DHFR gene was assessed using both in vivo [3H]uridine-labeling and in vitro nuclear run-ons to study nascent RNA synthesis. Although both assays showed that RNA synthesis was enhanced by camptothecin in promoter-proximal regions while reduced in promoter-distal regions, the two transcription assays gave quantitatively different results. While the in vivo pulse-labeling assay showed a 1.5-fold enhancement of promoter-proximal transcription by camptothecin, the in vitro run-on assay revealed a 10– to 15-fold increase in this region (compare Figures 3 and 4B). Furthermore, transcription from promoter-distal regions appeared more severely inhibited by camptothecin when using the in vivo pulse-labeling technique in comparison to the in vitro nuclear run-on technique (compare Figures 3 and 4B).

Discrepancies in the results obtained from in vivo [3H]uridine pulse-labeling and in vitro nuclear run-on transcription have been reported before (27). It is believed that the two assays measure different aspects of transcription. While the in vivo uridine pulse-labeling technique is thought to reflect transcription elongation in intact cells, the in vitro nuclear run-on technique merely reflects the abundance of RNA polymerases.

Fig. 4. Relative rate of in vitro nuclear run-on transcription. Nuclei were prepared from cells treated with 20 μM camptothecin at 37°C for 30 min and from untreated control cells, and nascent RNA was labeled with [33P]UTP at 30°C for 15 min. RNA was then isolated and hybridized to DNA probes described in Figure 2. (A) Autoradiograph showing that camptothecin enhanced the run-on signal in the 5’-region of the DHFR gene and suppressed the signal in the 3’-half of the RNA gene. (B) The results from a number of experiments are summarized and the values are expressed relative to untreated control cells and represent the mean of n independent experiments with bars showing the sample standard deviation.

Fig. 5. Reversal of the effect of camptothecin on RNA synthesis. Cells were treated with 20 μM camptothecin for 30 min at 37°C for 30 min followed by the labeling of nascent RNA in vivo with [3H]uridine according to the experimental design described in Figure 1. RNA was isolated and hybridized to DNA probes described in Figure 2. The [3H] counts were measured in a scintillation counter and the c.p.m. values were in the range of ~50–400 c.p.m. above background for the DHFR sequences and ~600–8000 for the rRNA sequence. The values are expressed relative to untreated control cells (100%) and represent the mean of n independent experiments with bars showing the sample standard deviation.
These results suggest that camptothecin enhances transcription in vitro the run-on signal (10 to 15-fold) was not matched by results. Furthermore, the majority of these polymerases were gene is reduced following camptothecin treatment (unpublished results). This leads to the accumulation of RNA polymerases at the 5′-region of the gene. When the effect of camptothecin is chased with a drug-free medium, some of the RNA polymerases that accumulated during the drug treatment are permitted to elongate. (D) Following a longer chase, the polymerases are once again spaced throughout the gene.

Fig. 6. Cartoon illustrating the effect of camptothecin on the synthesis of RNA from the DHFR gene. (A) Before the treatment with camptothecin, RNA polymerases are spaced throughout the DHFR gene. (B) Following a 30 min treatment with camptothecin, the translocation of RNA polymerases is blocked while free RNA polymerases are able to initiate transcription. (C) When the effect of camptothecin is chased with a drug-free medium, some of the RNA polymerases that accumulated during the drug treatment are permitted to elongate. (D) Following a longer chase, the polymerases are once again spaced throughout the gene.

Within a specific gene. Thus, the nuclear run-on assay is not necessarily a good measure of rates of transcription in cells (27). However, this technique is still informative on the abundance and location of polymerases within a gene whether they are elongating or not. The release of blocked polymerases in the run-on assay may result from the preparation of nuclei which introduces DNA nicks and modifies the interactions of proteins with DNA.

Assuming that the in vitro run-on technique reflects the abundance of RNA polymerases in a particular region and that the in vivo pulse-labeling technique reflects the rate of RNA synthesis in vivo (27), we conclude that a large number of RNA polymerases accumulated in the promoter-proximal region of the DHFR gene during the camptothecin treatment (Figure 6B). In support of this, we have recently obtained results using a psoralen photocross-linking approach suggesting that the accessibility of the DNA in the promoter region of the DHFR gene is reduced following camptothecin treatment (unpublished results). Furthermore, the majority of these polymerases were not capable of elongating in vivo since the huge increase in the in vivo run-on signal (10 to 15-fold) was not matched by a comparable increase in the in vivo pulse-labeling (1.5-fold). These results suggest that camptothecin enhances transcriptional initiation but that the elongation of the polymerases is blocked shortly after initiation, most likely by a topoisomerase I complex trapped by camptothecin on the transcribed strand.

Following the removal of camptothecin we found that synthesis of DHFR RNA recovered in a wave that progressed in a 5′ to 3′ direction (Figure 6C). The rate of RNA synthesis from the middle portion of the gene was approaching control levels within the first 15 min of the chase, while little recovery was detected in the 3′-end until after an additional 15 min of chase (Figure 6D). Interestingly, the polymerases that had accumulated at the 5′-end during drug treatment did not appear to translocate down the gene all at once following drug removal. Regarding the kinetics of RNA synthesis recovery throughout the gene, it has been estimated that the rate of elongation in vivo for mammalian RNA polymerases is ~30 nucleotides per second (28). That means that it would take an RNA polymerase ~17 min to traverse the 30 kb long DHFR gene from start to finish. We found in our study that the synthesis of RNA from the 5′-end of the DHFR gene resumed control levels following a 15–30 min chase in camptothecin-free media. This result is in excellent accord with the estimated elongation rate of the RNA polymerase (28) and suggests that RNA synthesis following drug removal is either carried out by newly initiated polymerases or by polymerases that accumulated at the 5′-end of the DHFR gene during the camptothecin treatment.

What are the mechanisms for the enhanced initiation of the DHFR gene by camptothecin? The role of DNA topoisomerase I during the transcription process is to relax the torsional strain induced by the elongating RNA polymerase (16,21,29). Inhibition of topoisomerase I by camptothecin may result in the escape of transcription-induced torsional tension. Although transcriptional elongation is limited in the presence of camptothecin (15,16, this study), some transcription-induced supercoiling may still be generated both in the wake of and ahead of the polymerase (29). Since the DHFR promoter region is located between two divergently transcribing genes (30), the inhibition of topoisomerase I may lead to an accumulation of transcription-generated negative torsional tension in this region. Negative torsional tension has been found to enhance the early stages of transcription both in vitro (31–33) and in vivo (34–37). We have previously found evidence for the presence of negative torsional tension in the promoter region of the DHFR gene in both human (38) and hamster cells (24).

In accordance with results from a previous study (16), camptothecin was found to severely reduce the rate of RNA synthesis from promoter-distal regions of the RNA polymerase I transcribed ribosomal RNA genes (Figures 3 and 4). However, the results obtained for the RNA polymerase II transcribed DHFR gene following camptothecin treatment differ somewhat from the results obtained on the transcription of the ribosomal genes. Our nuclear run-on data from camptothecin-treated cells suggest a much greater relative accumulation of RNA polymerases at the 5′-end of the DHFR gene (10 to 15-fold) than reported for the 5′-end of the ribosomal genes (1.4-fold) (16). Perhaps the high transcriptional activity of the ribosomal genes in dividing cells does not allow for any additional polymerases to accumulate onto the DNA template even in the presence of camptothecin. In fact, a higher relative accumulation of RNA polymerases at the 5′-end of the ribosomal genes by camptothecin has been observed when confluent cells were used (16).

In conclusion, camptothecin was found to enhance the rate of transcriptional initiation while inhibiting the rate of elongation by RNA polymerase II in the DHFR gene. This resulted in the accumulation of RNA polymerases in the 5′-region of the DHFR gene. Following drug removal, the RNA
synthesis returned in a 5' to 3' direction. A possible application of a protocol of camptothecin treatment and reversal is that it could be used in studies where a synchronized wave of transcription from the 5'-end to the 3-end is desired. Of interest would be to study the phenomena of transcription-coupled DNA repair in different parts of a gene in which transcription proceeds in such a synchronized manner. In addition, further studies will determine how important the effects on RNA synthesis are for the anti-tumor activity of camptothecin.

Acknowledgements

We would like to thank the members of our research group at Stanford University for stimulating discussions and ideas that have been very valuable for this study. We thank Joyce Hunt and Maria Turner for technical assistance, Robert Schimke for his generous gift of the CHO Bl 1 cell line and Graciela Spivak. Isabel Mellon, Linus Ho and Lawrence Chasin for their generous gifts of DNA probes. We also thank Sara Ljungman and Al Rehemtulla for critically reading this manuscript. This work was supported by an outstanding gift of DNA probes. We would like to thank the members of our research group at Stanford University for stimulating discussions and ideas that have been very valuable for this study. We thank Joyce Hunt and Maria Turner for technical assistance, Robert Schimke for his generous gift of the CHO Bl 1 cell line and Graciela Spivak. Isabel Mellon, Linus Ho and Lawrence Chasin for their generous gifts of DNA probes. We also thank Sara Ljungman and Al Rehemtulla for critically reading this manuscript. This work was supported by an outstanding investigator award from the National Cancer Institute and a postdoctoral fellowship from the National Institute of Health.

References


Received on July 21, 1995; revised on 13 September, 1995; accepted on September 21, 1995.