Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 1060–1064, February 1988
Biochemistry

Involvement of DNA topoisomerase I in transcription of human ribosomal RNA genes

(transcriptional swivel/template supercoiling/camptothecin/RNA polymerase I)

HUI ZHANG*, JAMES C. WANG†, AND LEROY F. LIU*

*Department of Biological Chemistry, Johns Hopkins University, 725 North Wolfe Street, Baltimore, MD 21205; and †Department of Biochemistry and Molecular Biology, Harvard University, 7 Divinity Avenue, Cambridge, MA 02138

Contributed by James C. Wang, November 9, 1987

ABSTRACT **Treatment of HeLa cells with a DNA topoisomerase I-specific inhibitor, camptothecin, results in rapid cessation of the synthesis of the 45S rRNA precursor. The inhibition of rRNA synthesis is reversible following drug removal and correlates with the presence of camptothecin-trapped topoisomerase I–DNA abortive complexes, which can be detected as topoisomerase I-linked DNA breaks upon lysis with sodium dodecyl sulfate. These breaks were found to be concentrated within the transcribed region of human rRNA genes. No such sites can be detected in the inactive human rRNA genes in mouse–human hybrid cells, suggesting a preferential association of topoisomerase I with actively transcribed genes. The distribution of RNA polymerase molecules along the transcription unit of human rRNA genes in camptothecin-treated HeLa cells, as assayed by nuclear run-on transcription, shows a graded decrease of the RNA polymerase density toward the 3′ end of the transcription unit; the density is minimally affected near the 5′ start of the transcription unit. These results suggest that DNA topoisomerase I is normally involved in the elongation step of transcription, especially when the transcripts are long, and that camptothecin interferes with this role.**

Transcription of DNA requires the rotation of RNA polymerase and its associated components around the DNA or the rotation of DNA around its helical axis. It has been suggested that the process might be facilitated by the introduction of a "swivel" into the DNA, which would allow the rotation of segments of DNA being transcribed without turning the other parts of DNA or the transcription ensembles (1–4). The evidence now indicates that eukaryotic DNA topoisomerase I is preferentially associated with actively transcribed regions on chromatin (5). Immunological and UV-crosslinking studies have shown that following heat shock, there is an enrichment of topoisomerase I within the transcribed regions of the heat shock loci of *Drosophila* chromosomes (6, 7). Immunofluorescence studies have shown that nucleoli, in which rRNA genes are being transcribed at a high rate, are highly enriched in topoisomerase I (6, 8). Consistent with these data, isolated nucleoli have also been shown to contain a high level of topoisomerase I activity (8, 9). Further, it was found that topoisomerase I-mediated nicking of DNA occurred upstream of the promoter of rRNA genes in *Tetrahymena* macronuclei, in the same region as the DNase I-hypersensitive sites (10). Using mutants defective in one or both topoisomerases, it was demonstrated that the synthesis of rRNA in the distantly related yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* was most severely inhibited when both topoisomerase activities were absent; there was little effect on rRNA synthesis in either *top1* or *top2* single mutants. These results suggest that a topoisomerase activity is required for rRNA transcription and that either topoisomerase I or topoisomerase II is sufficient for such a requirement (11, 12).

Recently, several specific inhibitors for mammalian DNA topoisomerases have been identified and characterized (13, 14). For example, camptothecin and VM-26 (teniposide) specifically inhibit mammalian DNA topoisomerases I and II, respectively, by trapping an abortive enzyme–DNA complex termed the cleavable complex. Treatment of these complexes with protein denaturant leads to DNA strand breaks and the concomitant linking of a topoisomerase polypeptide to the 3′ end (in the case of topoisomerase I) or the 5′ end (in the case of topoisomerase II) of the broken DNA strands. These inhibitors may thus be used to study both the functional involvement of topoisomerase activities and the chromosomal locations of topoisomerases.

In this communication, we report studies on the involvement of topoisomerase I in the transcription of human rRNA genes by RNA polymerase I, using the topoisomerase I-specific inhibitor camptothecin. Our results indicate that DNA topoisomerase I is enriched within the transcribed region of human rRNA genes and its presence correlates with the transcriptional state of the genes. A similar conclusion was drawn recently by Gilmour and Elgin (15) and by Stewart and Schutz (16) for several *Drosophila* heat shock genes and for rat tyrosine aminotransferase and phospho*enol*pyruvate carboxykinase genes, which are transcribed by RNA polymerase II. We show further that the inhibitory effect of camptothecin is more pronounced toward the 3′ end of the long rRNA transcription unit; transcripts near the start of the transcription unit are affected minimally.

MATERIALS AND METHODS

Cells, Plasmids, and Chemicals. Mouse–human hybrid cell lines 56-05 F5 (MH1) and 56-05 F1 (MH2) were generously provided by C. Croce (Wistar Institute, Philadelphia). Plasmid RSB was obtained from N. Arnheim (University of Southern California, Los Angeles). All other human rRNA gene subfragments were generously provided by J. E. Sylvester and R. D. Schmickle (University of Pennsylvania, Philadelphia) or subcloned from the clones provided. Radioisotopes and GeneScreen membrane filters were obtained from New England Nuclear. The lactone form of camptothecin (NSC 94600) was obtained from the National Cancer Institute. The drug was dissolved in dimethyl sulfoxide (Me_2SO) and stored at −20°C before use.

DNA Isolation and Indirect End-Labeling. Cells (3×10^6) were lysed with 1% $NaDodSO_4$/2 mM EDTA, pH 8.0. Proteinase K and NaCl were added to lysates to final concentrations of 400 μg/ml and 0.1 M, respectively, and the mixtures were incubated at 37°C overnight. DNA was iso-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: Me_2SO, dimethyl sulfoxide.

lated from the lysates by phenol extraction and ethanol precipitation. Isolated DNA was digested with restriction enzymes and analyzed by electrophoresis in either nondenaturing or formamide-containing denaturing agarose gel (17). DNA was blotted onto GeneScreen membrane filters and hybridized with various human rRNA gene probes that had been ^{32}P-labeled by the hexanucleotide random-priming method (18).

Isolation of Protein-Linked DNA. HeLa cells were treated with either 60 μM camptothecin from a 20 mM stock solution (in Me_2SO) or the same amount of Me_2SO (final concentration, 1%) for 15 min and then lysed with the $NaDodSO_4$/EDTA buffer. The lysates were passed through a 20-gauge needle to shear DNA into small pieces and then extracted three times with an equal volume of phenol. After centrifugation to separate the proteinaceous interface from the aqueous supernatant, the supernatant was carefully pipeted into a clean tube. The proteinaceous material in the interface was also collected. DNA in the interface and supernatant was then precipitated with ethanol, digested with proteinase K, and processed as described above.

Nuclear Run-On Transcription. Nuclear run-on assays were done according to Greenberg and Ziff (19) with the following modifications. Cells that had been treated with Me_2SO or camptothecin were chilled on ice and washed with ice-cold phosphate-buffered saline (PBS: 136.8 mM NaCl/2.6 mM KCl/8 mM Na_2HPO_4/1.4 mM KH_2PO_4). Cells were then lysed with an ice-cold buffer containing the nonionic detergent Nonidet P-40 (10 mM Tris·HCl, pH 7.8/10 mM NaCl/3 mM $MgCl_2$/0.3% Nonidet P-40/0.1 mM dithiothreitol), and nuclei were isolated by centrifugation. The isolated nuclei were resuspended in 90 μl of a nuclear run-on transcription buffer [90 mM KCl/5 mM $MgCl_2$/25% (vol/vol) glycerol/10 mM Tris·HCl, pH 7.8/2.5 mM dithiothreitol/250 μM ATP/250 μM CTP/250 μM GTP]. [α-^{32}P]UTP was added to 1 μCi/ml (1 μCi = 37 kBq) and the run-on transcription was assayed at 26°C for 15 min.

Blot Hybridization. Plasmid DNAs containing inserts from different regions of rRNA genes were nicked by adding DNase I to 10 μg/ml and ethidium bromide to 250 μg/ml at 25°C for 90 min. DNA samples (10 μg per slot), which had been phenol-extracted and ethanol-precipitated, were blotted onto GeneScreen membrane filters using a "slot blotter" (Minifold II; Schleicher & Schuell).

RESULTS

Localization of Topoisomerase I on Human rRNA Genes with Camptothecin. There are about 200–300 copies of human rRNA genes per genome. They are organized as

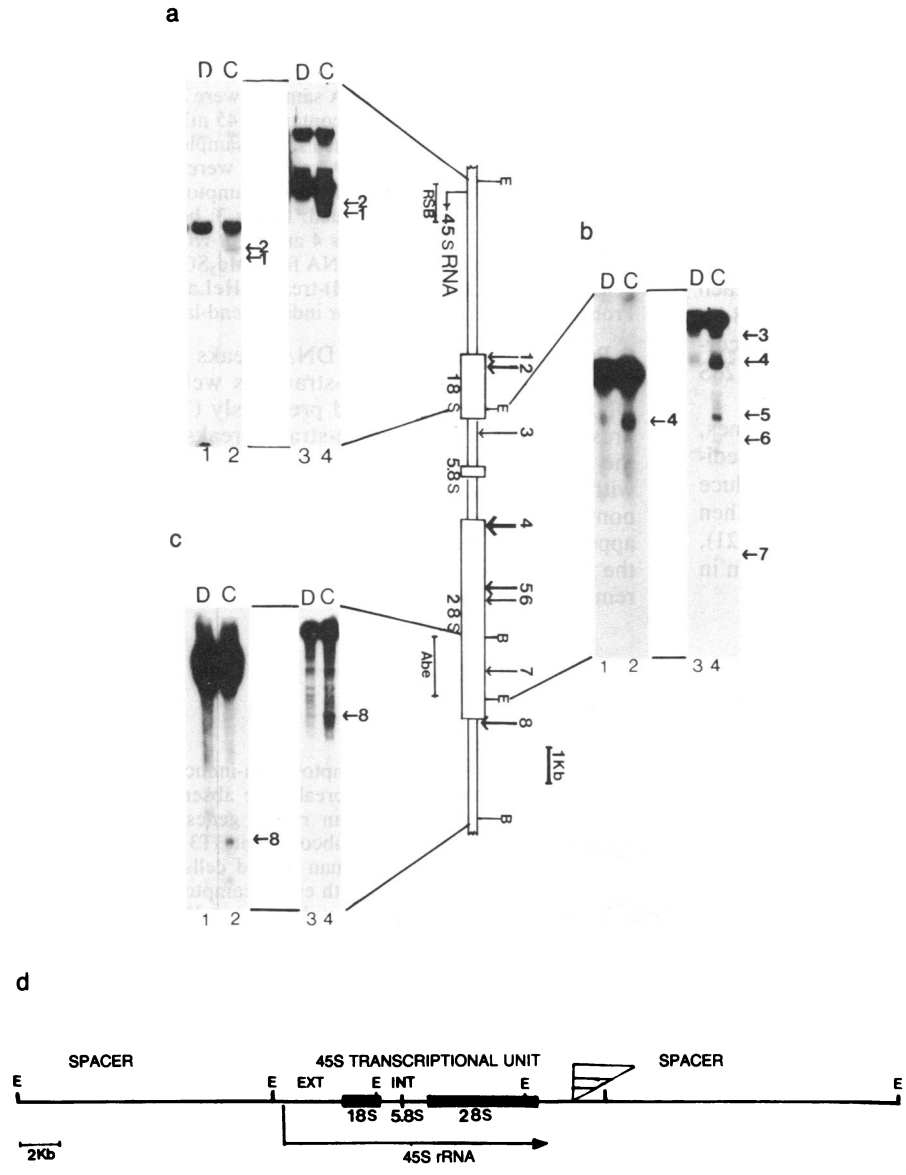

FIG. 1. Localization of camptothecin-induced, topoisomerase I-linked DNA breaks within the transcribed region of human rRNA genes. Logarithmically growing HeLa cells were treated with either 1% Me_2SO or 60 μM camptothecin (in 1% Me_2SO) for 15 min. Cell lysis, DNA isolation, restriction enzyme digestion, and indirect end-labeling were done as described in *Materials and Methods*. DNA was fractionated by both formamide-denaturing (lanes 1 and 2) and nondenaturing (lanes 3 and 4) agarose gels electrophoresis. (*a*) DNA samples were cut with *Eco*RI and probed with the RSB fragment. (*b*) DNA samples were cut with *Eco*RI and probed with the Abe fragment. (*c*) DNA samples were cut with *Bam*HI and probed with the Abe fragment. Numbered arrows indicate the position of camptothecin-induced DNA breaks. Lanes 1 and 3: dimethyl sulfoxide control (D). Lanes 2 and 4: camptothecin-treated (C). (*d*) Map of a human rRNA gene repeat. EXT, external transcribed region; INT, internal transcribed region; E, *Eco*RI; B, *Bam*HI.

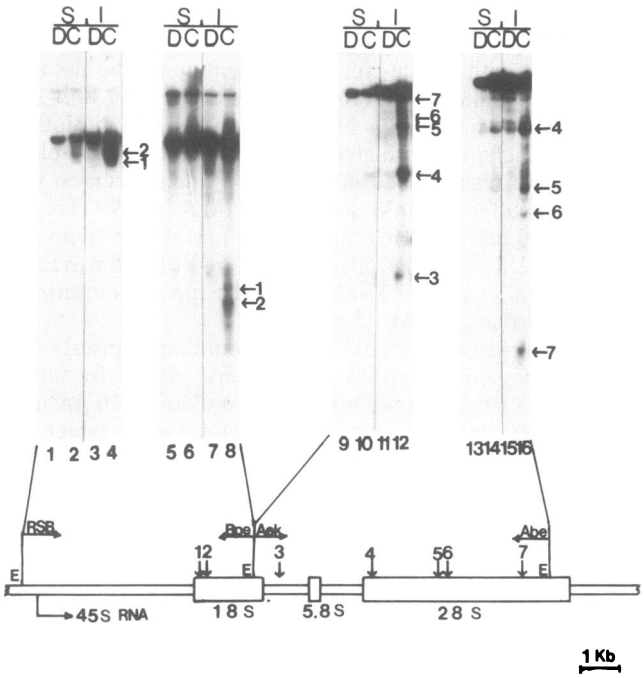

FIG. 2. Camptothecin-induced DNA breaks are protein-linked. HeLa cells were treated with 60 μM camptothecin for 15 min and protein-linked DNA was isolated as described in *Materials and Methods*. DNA recovered from the interface (I) or the aqueous supernatant (S) was digested with *Eco*RI and analyzed by nondenaturing agarose gel electrophoresis. DNA probes used for indirect end-labeling were RSB (lanes 1–4), Bpe (lanes 5–8), Aek (lanes 9–12), and Abe (lanes 13–16). The location of these sequences are indicated at the bottom of the figure. Odd numbered lanes: dimethyl sulfoxide (D) control. Even-numbered lanes: camptothecin-treated (C). E, *Eco*RI.

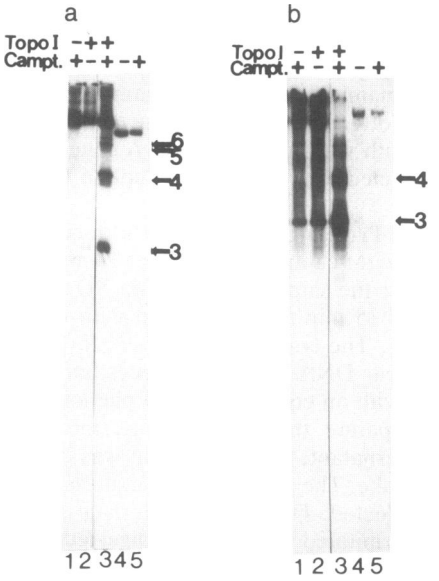

FIG. 3. Camptothecin-induced, topoisomerase I-linked DNA breaks occur at similar sites on human rRNA genes *in vitro* and *in vivo*. Plasmid pA, which contains the DNA fragment located between the two *Eco*RI sites within 18S and 28S rRNA genes, was cut with *Eco*RI restriction enzyme. The DNA was mixed with calf thymus topoisomerase I (1 μg/ml) in the presence or absence of camptothecin (60 μM) at 37°C for 30 min. The reaction was stopped by adding $NaDodSO_4$ to 1% and proteinase K to 400 μg/ml and incubated at 37°C for 1 hr. DNA samples were analyzed by agarose gel electrophoresis in a buffer containing 45 mM Tris base, 45 mM boric acid, and 1 mM EDTA. (*a*) DNA samples were in a neutral buffer before loading. (*b*) DNA samples were denatured in alkali before loading. Lanes 1: control, 60 μM camptothecin added. Lanes 2: control, topoisomerase I added. Lanes 3: both topoisomerase I and camptothecin added. Lanes 4 and 5: *in vivo* samples, cut with *Eco*RI restriction enzyme, of DNA from Me_2SO (1%)-treated HeLa cells and camptothecin (60 μM)-treated HeLa cells, respectively. Probe Aek (Fig. 2) was used for indirect end-labeling.

tandem repeats in four different chromosomal loci (20). Each repeat is composed of a 31-kilobase (kb) spacer and a 13-kb transcribed region, which codes for the 45S rRNA precursor. The transcribed region contains the 18S, 5.8S, and 28S genes (Fig. 1).

In order to locate topoisomerase I on human rRNA genes, HeLa genes were treated with camptothecin and immediately lysed with a buffer containing $NaDodSO_4$ to induce topoisomerase I-linked DNA breaks; these breaks were then located by the indirect end-labeling method (15, 16, 21), using cloned rRNA gene fragments RSB and Abe shown in Fig. 1 as probes.

Prominent double-strand DNA breaks were observed in these experiments. Double-strand as well as single-strand DNA breaks were observed previously (15, 16, 21). In the present experiments, double-strand breaks were observed at the sites indicated by numbered arrows in Fig. 1, primarily within the transcribed region; no breaks were detected in the nontranscribed spacer region. These double-strand breaks appear within minutes following camptothecin addition to the culture medium and also disappear rapidly upon drug removal (data not shown). In agreement with their being

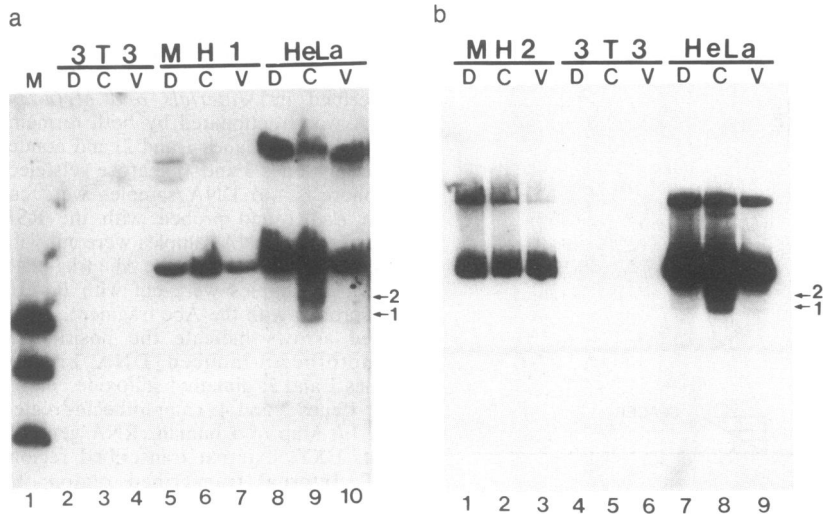

FIG. 4. Camptothecin-induced, topoisomerase I-linked DNA breaks are absent from the coding region of human rRNA genes in mouse–human hybrid cells. Subconfluent 3T3 cells, HeLa cells, and mouse–human hybrid cells (MH1 and MH2) were treated with either camptothecin (60 μM) for 15 min or a topoisomerase II inhibitor, VM-26 (teniposide), for 30 min. DNA was isolated, restricted with *Eco*RI, and probed with RSB. (*a*) Lanes: 1, pBR322 DNA marker (M); 2–4, mouse 3T3 cells; 5–7, mouse–human hybrid cells (MH1); 8–10, HeLa cells. Drug treatment: 1% dimethyl sulfoxide (D), lanes 2, 5, and 8; 60 μM camptothecin (C), lanes 3, 6, and 9; VM-26 (V), lanes 4, 7, and 10. (*B*) A similar experiment using the same controls but with a different mouse–human hybrid cell line, MH2.

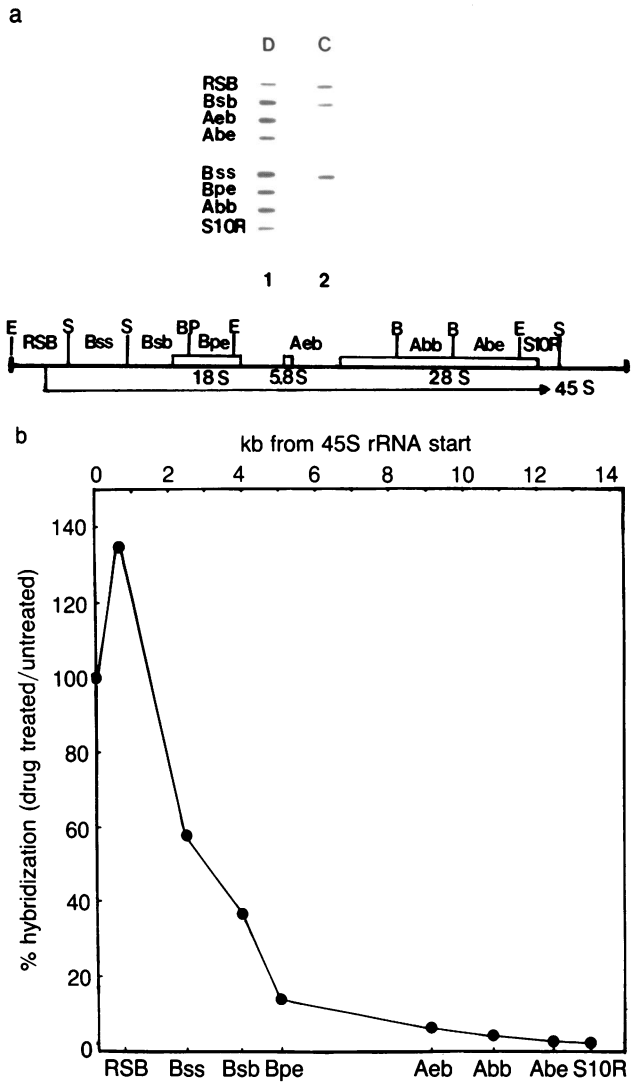

FIG. 5. Camptothecin preferentially reduces nuclear run-on transcripts near the 3′ end of the rRNA transcription unit. (*a*) HeLa cells were treated with either 1% dimethyl sulfoxide (column D) or 60 μM camptothecin in 1% dimethyl sulfoxide (column C) for 30 min. Nuclei were isolated for run-on transcription assays. The ^{32}P-labeled RNA was hybridized to the human rRNA gene fragments immobilized on a GeneScreen membrane. The immobilized probes, which are cloned DNA fragments from different regions of the transcribed unit of human rRNA genes, are diagramatically shown below the autoradiogram. E, *Eco*RI; S, *Sal* I; B, *Bam*HI; P, *Pst* I. (*b*) The results from *a* were quantitated by densitometry. The percent reduction of nuclear run-on transcripts by camptothecin was plotted as a function of the distance from the beginning of the transcription unit. Nuclear run-on transcription in control cells (column D) was taken as 100% for each probe.

associated with covalently bound protein (topoisomerase I), repeated phenol extractions of cell lysates showed that DNA containing such breaks was enriched in the proteinaceous material at the phenol/water interface (Fig. 2).

Using purified calf thymus DNA topoisomerase I, we also mapped cleavage sites on a cloned human rRNA gene fragment *in vitro*. As shown in Fig. 3, the addition of camptothecin to the reaction mixture induced double-strand DNA breaks at sites similar to those observed *in vivo*. In the absence of camptothecin and at higher concentrations of purified topoisomerase I, cleavage at similar places was observed (Fig. 3, lanes 2). These results again support the notion that the *in vivo* cleavage sites induced by camptothecin were topoisomerase I-mediated.

Preferential Association of Topoisomerase I with Actively Transcribed Human rRNA Genes. To test whether the presence of camptothecin-induced, topoisomerase I-linked DNA breaks correlates with the transcriptional state of human rRNA genes, we examined camptothecin-induced DNA breaks in two different mouse–human hybrid cell lines, MH1 and MH2. Although both human and mouse rRNA genes are present in the hybrid cells, only the mouse genes are active, presumably due to the lack of species-specific initiation factor(s) for the human rRNA genes (22). DNA breaks induced by camptothecin in the various cell lines were mapped by use of appropriate cloned probes (Fig. 4). Whereas topoisomerase I-linked DNA breaks were detected in the rRNA genes in HeLa cells, they were not observed in the human rRNA genes in the hybrid cells, suggesting that topoisomerase I is preferentially associated with actively transcribed rRNA genes. Control experiments using mouse rRNA gene probes showed that topoisomerase I-linked DNA breaks mapped within the transcribed unit of mouse rRNA genes in both mouse 3T3 cells and hybrid cells (data not shown).

Distance-Dependent Inhibition of Nuclear Run-On rRNA Transcription by Camptothecin. Treatment of HeLa cells with camptothecin results in rapid inhibition of [^{3}H]uridine incorporation and severe inhibition of the 45S rRNA precursor synthesis (data not shown). To test whether camptothecin inhibits initiation or elongation of RNA transcription, the nuclear run-on assay (19) was used to monitor the distribution of RNA polymerase molecules within the transcription unit of human rRNA genes. In this assay, nuclear transcripts that had been initiated prior to the isolation of nuclei were labeled by the addition of α-^{32}P-labeled triphosphates to the nuclei. Samples of the labeled RNA were hybridized to various cloned rRNA gene fragments immobilized on membrane filters (Fig. 5*a*). The treatment of HeLa cells with camptothecin causes a graded reduction of nuclear run-on transcripts along the transcription unit: transcripts near the immediate 5′ end of the transcription unit are minimally affected, whereas transcripts near the 3′ end of the transcription unit are almost completely abolished. The intensities of these bands were quantitated by densitometry (Fig. 5*b*). The graded decrease of the nuclear run-on transcripts toward the 3′ end of the transcription unit signifies a parallel decrease of the RNA polymerase density toward the 3′ end of the transcription unit. There is a slight stimulation of nuclear run-on transcription near the immediate 5′ end of the gene (Fig. 5*b*). This stimulation becomes more pronounced when rRNA transcription slows down (e.g., in confluent HeLa cells or confluent NIH 3T3 cells) (data not shown).

DISCUSSION

Using camptothecin to trap covalent complexes between DNA and topoisomerase I, we found that the enzyme is present within the transcribed region of rRNA genes in HeLa cells but is absent in the nontranscribed spacer region in the same cells or in inactive human rRNA genes in mouse–human hybrid cells. These results, together with previous findings (5–12, 15, 16), suggest that DNA topoisomerase I is functionally associated with genes being actively transcribed by either RNA polymerase I or RNA polymerase II.

Camptothecin is a reversible inhibitor of RNA synthesis in mammalian cells and is known to inhibit the synthesis of high molecular weight RNAs such as the 45S rRNA precursor and heterogeneous nuclear RNAs (23, 24). Inhibition of rat rRNA synthesis in a partially purified system by the drug was reported recently (25). Experiments on the effect of camptothecin on nuclear run-on rRNA transcription were carried out. Whereas the amounts of nuclear run-on tran-

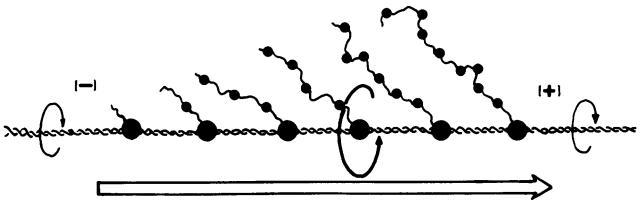

FIG. 6. Illustration of rotation of the RNA polymerase ensemble around the DNA.

scripts at the beginning of the transcription unit of human rRNA genes were little affected, they were sharply reduced toward the end of the transcription unit. This result is consistent with camptothecin being a specific inhibitor of the elongation step of RNA transcription.

There are two plausible explanations for such an inhibitory effect of camptothecin. One is that the movement of the transcribing RNA polymerase is physically blocked by the camptothecin-trapped topoisomerase I–DNA abortive complex. As the transcribing polymerase moves along the template, the probability of encountering such a roadblock is proportional to the distance it travels; therefore, the density of polymerase molecules is expected to decrease toward the 3′ end of the transcription unit.

An alternative explanation is that as the length of a transcript increases, it becomes increasingly dependent on the presence of a "swivel" in the DNA (2, 4, 16). The basic idea is illustrated in Fig. 6. As a polymerase starts transcription, the right-handed geometry of the DNA requires that the enzyme rotate around the DNA. As the polymerase advances, the size of the nascent RNA chain increases and hence the frictional force on the rotating transcription ensemble (including the polymerase, the nascent RNA, and the proteins associated with the RNA) increases. This force drives the positive supercoiling of the DNA in front of the polymerase and the negative supercoiling of the DNA behind it. In the absence of a topoisomerase to relax the supercoiled regions, the movement of the polymerase may be gradually impeded as the degrees of supercoiling become progressively higher. (For the possibility of additional topological constraints, see ref. 4.)

At present, we cannot distinguish between these two possibilities. It is possible that both mechanisms may contribute to the observed inhibitory effect of camptothecin on RNA transcription. Further studies are necessary to establish more precisely the role of topoisomerase I in transcription.

We thank Drs. Annette Bodley and Bret Jessee for their critical reading of the manuscript. This work was supported by U.S. Public Health Service Grants GM27731 and CA39962 to L.F.L. and U.S. Public Health Service Grant GM24544 and an American Cancer Society grant to J.C.W.

1. Maaloe, O. & Kjeldgaard, N. Q. (1966) *Control of Macromolecular Synthesis* (Benjamin, New York).
2. Wang, J. C. (1973) in *DNA Synthesis In Vitro*, eds. Wells, R. D. & Inman, R. B. (University Park Press, Baltimore), pp. 163–174.
3. Gamper, H. B. & Hearst, J. E. (1982) *Cell* **29**, 81–90.
4. Liu, L. F. & Wang, J. C. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 7024–7027.
5. Wang, J. C. (1985) *Annu. Rev. Biochem.* **54**, 665–697.
6. Fleischmann, G., Pflufelder, G., Steiner, E. K., Javaherian, K., Howard, G. C., Wang, J. C. & Elgin, S. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 6958–6962.
7. Gilmour, D. S., Pflufelder, G., Wang, J. C. & Lis, J. T. (1986) *Cell* **44**, 401–407.
8. Muller, M. T., Pfund, W. P., Mehta, V. B. & Trask, D. K. (1985) *EMBO J.* **4**, 1237–1243.
9. Higashinakagawa, T., Wahn, H. & Reeder, R. H. (1977) *Dev. Biol.* **55**, 375–386.
10. Bonven, B. J., Gocke, E. & Westergaard, O. (1985) *Cell* **41**, 541–551.
11. Uemura, T. & Yanagida, M. (1986) *EMBO J.* **5**, 1003–1010.
12. Brill, S. J., DiNardo, S., Voelkel-Meiman, K. & Sternglanz, R. (1987) *Nature (London)* **326**, 414–416.
13. Chen, G. L., Yang, L., Rowe, T., Halligan, B. D., Tewey, K. M. & Liu, L. F. (1984) *J. Biol. Chem.* **259**, 13560–13566.
14. Hsiang, Y.-H., Hertzberg, R., Hecht, S. & Liu, L. F. (1985) *J. Biol. Chem.* **260**, 14873–14878.
15. Gilmour, D. S. & Elgin, S. C. R. (1987) *Mol. Cell Biol.* **7**, 141–148.
16. Stewart, A. F. & Schutz, G. (1987) *Cell* **50**, 1109–1117.
17. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 202–203, 382–286, and 473.
18. Feinberg, A. P. & Vogelstein, B. (1983) *Anal. Chem.* **132**, 6–13.
19. Greenberg, M. E. & Ziff, E. B. (1984) *Nature (London)* **311**, 433–437.
20. Wilson, G. N. (1982) in *The Cell Nucleus*, eds. Wilson, F. E. & Stafford, D. W. (Academic, New York), pp. 287–318.
21. Yang, L., Rowe, T. C. & Liu, L. F. (1985) *Cancer Res.* **45**, 5872–5876.
22. Miesfeld, R., Sollner-Webb, B., Croce, C. & Arnheim, N. (1984) *Mol. Cell. Biol.* **4**, 1306–1312.
23. Abelson, H. T. & Penman, S. (1972) *Nature (London) New Biol.* **237**, 144–146.
24. Wu, R. S., Kumar, A. & Warner, J. R. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 3009–3014.
25. Garg, L. C., DiAngelo, D. & Jacob, S. T. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 3185–3188.