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Role of DNA topoisomerase I in the transcription of supercoiled rRNA gene

(DNA relaxation/camptothecin-sensitive transcription/rRNA synthesis)

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ABSTRACT **The fraction DE-B obtained by fractionating an extract from rat mammary adenocarcinoma cells on a DEAE-Sephadex column was used for transcribing linear and supercoiled rRNA gene (rDNA). This fraction, which is known to contain RNA polymerase I and essential transcription factors, also contains DNA topoisomerase I activity. Inhibition of this topoisomerase activity by the selective inhibitor camptothecin markedly diminished transcription of supercoiled rDNA, and at a concentration of 150 μM, camptothecin almost completely inhibited DNA topoisomerase I activity and supercoiled rDNA transcription. Addition of exogenous calf thymus DNA topoisomerase I to the sample containing the drug restored the ability of the extract to transcribe supercoiled rDNA. Camptothecin, even at a concentration of 500 μM, had no significant effect on the transcription of linear rDNA. These studies show that relaxation of supercoiled rDNA by DNA topoisomerase I is essential for its transcription. The preferential inhibition of rRNA synthesis *in vivo* following treatment with camptothecin is probably due to selective camptothecin inhibition of DNA topoisomerase I activity.**

There has been considerable interest in understanding the role of supercoiled DNA in DNA replication and transcription (for a review, see ref. 1). Reports that supercoiling of cloned, circular prokaryotic chromosomes can activate the promoters of various genes *in vitro* (2, 3) have prompted studies to explore whether there is a similar mechanism in eukaryotic gene transcription. Several studies indicate that DNA supercoiling may have profound effects on gene expression in eukaryotes (3–7). Supercoiled DNA can be readily relaxed by DNA topoisomerases I and II, the enzymes that induce transient single-strand and double-strand breaks in DNA, respectively (1). DNA topoisomerase I is closely associated with the nucleolus (8, 9). However, it is not known whether relaxation of supercoiled rRNA gene (rDNA) by the type I DNA topoisomerase is obligatory to its transcription. We have been able to obtain a partially purified fraction from rat mammary adenocarcinoma cells that can accurately transcribe cloned rat rDNA (10). This fraction contains RNA polymerase I and a few accessory polypeptides, one of which has been subsequently identified as poly(ADP-ribose) polymerase (11). A similar fraction has also been obtained from nuclear extracts of normal rat liver and a rat hepatoma (12). We now report that this fraction contains DNA topoisomerase I that can control transcription of supercoiled rDNA.

METHODS

***In Vitro* Transcription Assay.** *In vitro* transcription was performed essentially as described (10). The whole cell extract derived from rat mammary adenocarcinoma cells was fractionated on a DEAE-Sephadex column, and the fraction designated DE-B that contains RNA polymerase I and essential transcription factors (10) was used in the assay. Supercoiled or *Xho* I-linearized plasmid (pB7-2.0) that consists of rat rDNA from position −167 to position +2000 (10) was used as template. The reaction was carried out for 30 min at 30°C in a total volume of 25 μl.

S1 Nuclease Mapping. Unlabeled RNA from supercoiled DNA was prepared essentially as described (10, 11). The transcripts were treated with RNase-free DNase I at 300 μg/ml, incubated with the 5′-end-labeled *Sal* I–*Xho* I fragment of rat rDNA (extending from position −167 to position +635) for 15 min at 80°C followed by incubation in 80% (vol/vol) formamide, 28 mM Pipes (pH 6.4), 0.4 M NaCl, 1 mM EDTA at 58°C for 12 hr. The sample was diluted with S1 nuclease buffer (250 mM NaCl/30 mM sodium acetate, pH 4.5/1 mM $ZnCl_2$) and treated with 150 units of S1 nuclease at 37°C for 30 min. The S1 nuclease-resistant hybrid was then recovered by ethanol precipitation. The samples were subjected to electrophoresis on 4% polyacrylamide/8 M urea gel along with suitable markers.

Topoisomerase Assay. Topoisomerase I was assayed by determining the extent of relaxation of supercoiled rDNA or supercoiled pBR322 under transcription conditions (10). The reaction was stopped by addition of 50 mM Tris·HCl (pH 7.4), 10 mM EDTA, 1% $NaDodSO_4$, and proteinase K at 750 μg/ml and incubated for 2 hr at 37°C. The reaction mixture was extracted with phenol twice and precipitated with ethanol. Pellets were resuspended in 5 μl of 20 mM Tris·HCl, pH 7.6/1 mM EDTA/10 mM NaCl. To this 5 μl of dye was added, and the samples were electrophoresed on 1% agarose gel for 6 hr at 30 V and analyzed after staining with ethidium bromide.

Quantitation of Transcripts. The amounts of transcripts produced in the presence and absence of camptothecin were quantitated by analysis of the autoradiograms with a Zenith soft laser scanning densitometer.

RESULTS

The Fraction that Contains RNA Polymerase I and Essential Transcription Factors also Contains DNA Topoisomerase I Activity. We first determined whether the fraction eluting with RNA polymerase I from the DEAE-Sephadex column (fraction DE-B) contained adequate amounts of topoisomerase activity. This fraction has been shown (10) to contain the transcription factors including RNA polymerase I that are essential for accurate transcription of rat rDNA. DNA topoisomerase activity associated with this fraction was assayed by using different amounts of the plasmid containing the rat rDNA insert from position −167 to position +2000 (10) (Fig. 1). ATP was included at a concentration of 0.5–1 mM to optimize DNA topoisomerase II activity (13), if present.

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Abbreviation: rDNA, rRNA gene.

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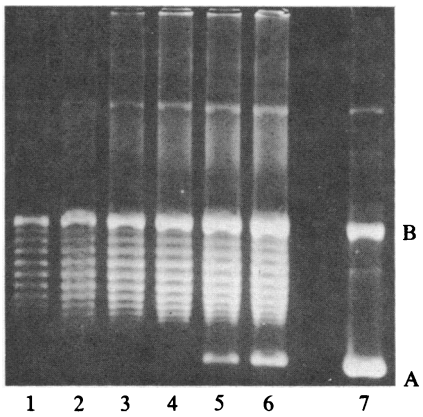

FIG. 1. Effect of various amounts of DNA on DNA topoisomerase activity. Lanes: 1, 0.1 μg of DNA; 2, 0.2 μg of DNA; 3, 0.4 μg of DNA; 4, 0.6 μg of DNA; 5, 0.8 μg of DNA; 6, 1 μg of DNA; 7, 0.6 μg of DNA incubated in the absence of fraction DE-B. Bands A and B correspond to supercoiled and open, circular DNA, respectively.

Untreated DNA consisted of two major fractions, closed, circular form I and open, circular form II. Incubation at 30°C for 30 min converted the supercoiled DNA into relaxed form resulting in topoisomers with different linking numbers, which demonstrated the presence of active topoisomerase activity in fraction DE-B. The topoisomerase activity increased with larger amounts of DNA in the reaction mixture, reaching a maximum at 0.6 μg per reaction mixture. Higher concentrations of DNA resulted in reduced conversion of supercoiled DNA into the topoisomers (see Fig. 1, lanes 5 and 6).

We then investigated whether DNA topoisomerase I was associated with fraction DE-B. To test this possibility, the effect of camptothecin, a specific inhibitor of topoisomerase (14), was included in the assay. This drug, at a concentration of 150 μM, completely abolished the DNA topoisomerase I activity (Fig. 2, compare lanes 4 and 5 with lanes 6 and 7, respectively). Dimethyl sulfoxide used for dissolving camptothecin had no significant effect on the enzyme activity (compare lanes 2 and 3 with lanes 6 and 7, respectively).

Relaxation of Supercoiled rDNA by DNA Topoisomerase I Is Essential for its Transcription. We then tested whether DNA topoisomerase I is required for transcription of supercoiled rDNA. For these studies, supercoiled plasmids containing the rDNA insert were used in the transcription assay. The transcript corresponding to the first 635 nucleotides was analyzed by hybridizing the transcript to a 5′-labeled rDNA insert cleaved with *Xho* I, which truncates the rDNA 635 nucleotides downstream of the initiation site (15). If transcription were to occur at nucleotide +1, the S1-nuclease-protected hybrid must be 635 nucleotides long. As shown in Fig. 3, the transcript was indeed 635 nucleotides long.

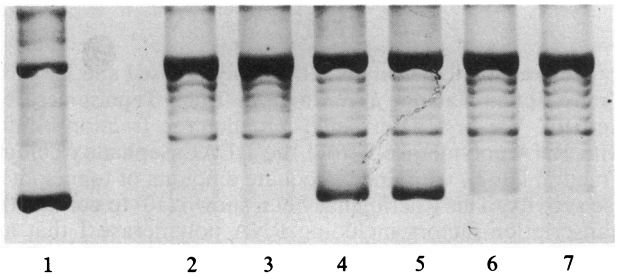

FIG. 2. Effect of camptothecin on DNA topoisomerase I activity. Each sample contained 0.2 μg of DNA. Lanes: 1, control DNA incubated in the absence of fraction DE-B; 2, 7.5 μl of fraction DE-B; 3, 10 μl of fraction DE-B; 4, 7.5 μl of fraction DE-B and 150 μM camptothecin; 5, 10 μl of fraction DE-B and 150 μM camptothecin; 6, 7.5 μl of fraction DE-B and 1.5 μl of dimethyl sulfoxide used for dissolving the drug; 7, 10 μl of fraction DE-B and 1.5 μl of dimethyl sulfoxide.

If relaxation of supercoiled rDNA is essential for its transcription, then inhibition of this reaction by the specific inhibitor camptothecin (14) should block transcription. Three different concentrations of camptothecin were included in the transcription assay (lanes 2–4). Control samples contained appropriate amounts of dimethyl sulfoxide used for dissolving camptothecin (lanes 5–7). The inhibition of transcription was evident at a concentration of 75 μM. At 150 μM of the drug, transcription was inhibited as much as 95% (compare lanes 4 and 7), as determined by densitometric scanning of the autoradiogram. The solvent had only minimal effect on transcription (compare lanes 5–7 with lane 1).

We then investigated the specificity of the camptothecin-induced inhibition of supercoiled rDNA transcription. For this purpose, the effect of this drug on transcription of linear rDNA was analyzed (Fig. 4). Even at a concentration as high as 500 μM, this drug had no effect on the transcription of *Xho* I-cleaved rDNA when compared to the appropriate control samples (compare lanes 8 and 9).

The involvement of DNA topoisomerase I in transcription of supercoiled rDNA was substantiated by determining if the camptothecin-induced inhibition of transcription could be restored by exogenous DNA topoisomerase I. For this purpose, highly purified DNA topoisomerase I was added to samples containing camptothecin, and the effect of such supplementation on transcription was compared with that of the control sample containing only camptothecin (Fig. 5). At 100 μM inhibitor concentration, the amount of rDNA tran-

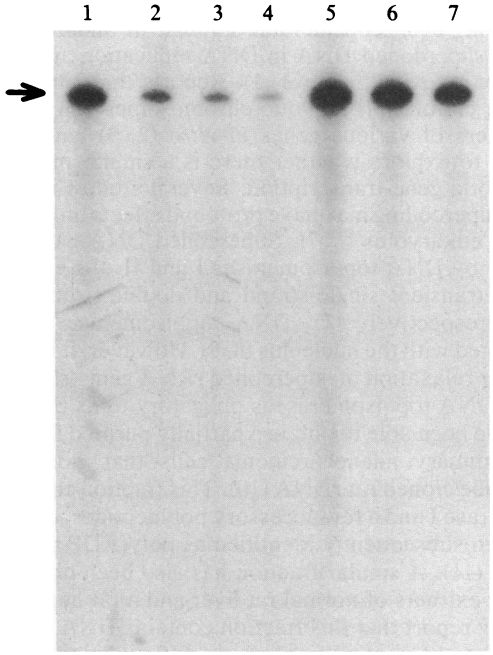

FIG. 3. Autoradiogram of transcript obtained from supercoiled DNA. RNA was synthesized *in vitro* from 250 ng of DNA; the transcripts derived from four reaction mixtures were combined and hybridized to a 5′-end-labeled *Sal* I–*Xho* I fragment. The S1 nuclease-resistant hybrid was analyzed on 4% acrylamide/8 M urea gel. Lane 1, control sample wtihout dimethyl sulfoxide. Lanes 2, 3, and 4, transcripts derived from samples incubated in the presence of 75 μM, 125 μM, and 150 μM camptothecin, respectively. Lanes 5, 6, and 7, transcripts obtained in the presence of dimethyl sulfoxide used in volumes corresponding to those used in samples 2, 3, and 4, respectively. Arrow corresponds to 635-nucleotide product.

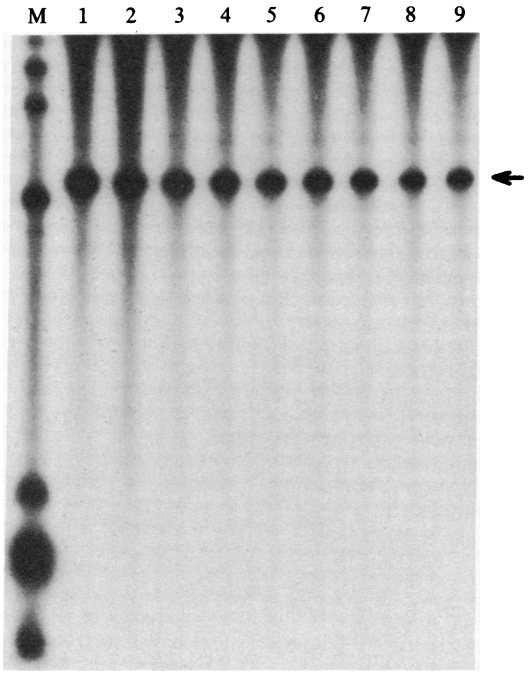

FIG. 4. Effect of camptothecin on transcription of pB7-2.0 linearized with *Xho* I. The linearized DNA (350 ng) was transcribed using 15 μg of fraction DE-B in the absence or presence of camptothecin. Lane M, molecular weight markers (ϕX174 replicative form DNA digested with *Hae* III). Lane 1, transcript in the absence of dimethyl sulfoxide and camptothecin. Lanes 3, 5, 7, and 9, transcription obtained in the presence of 150, 300, 400, and 500 μM of camptothecin, respectively. Lanes 2, 4, 6, and 8, transcripts obtained in the absence of camptothecin, but in the presence of appropriate volumes of dimethyl sulfoxide used in samples 3, 5, 7, and 9, respectively. The arrow indicates 635-nucleotide transcript.

script was only 26% of the control sample, as determined by densitometric scanning. Addition of exogenous topoisomerase I to the sample containing 100 μM of the drug (lane 3) restored the transcription (lane 2) to almost the control level (lane 1).

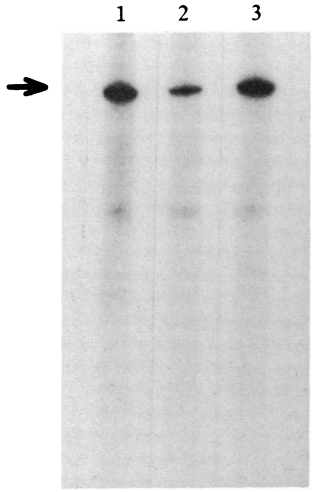

FIG. 5. Effect of purified DNA topoisomerase I on the camptothecin-inhibited transcription of supercoiled rDNA. Transcription of supercoiled pB7-2.0 was carried out. Plasmid (250 ng) was incubated in the absence (lane 1), in the presence of 100 μM camptothecin (lane 2), or of 100 μM camptothecin plus 10 units of purified calf thymus DNA topoisomerase I (Bethesda Research Laboratories) (lane 3). Sample used for each lane was derived by combining four reaction mixtures. The arrow corresponds to 635-nucleotide product.

DISCUSSION

The present studies have shown that a fraction designated DE-B that contains RNA polymerase I and essential rDNA transcription factors is also capable of relaxing supercoiled DNA. The inhibition of transcription of supercoiled rDNA, but not of linear rDNA, by camptothecin and the restoration of camptothecin-inhibition by purified DNA topoisomerase I demonstrate that relaxation of supercoiled rDNA by this enzyme is essential for its transcription. We have used an alternate source of highly purified DNA topoisomerase I (generously provided to us by Leroy Liu) and obtained similar results. It may be argued that a stimulating factor contaminating DNA topoisomerase I preparations is responsible for the restoration of rDNA transcription. This possibility can be ruled out since addition of purified DNA topoisomerase I to the control samples (in the absence of camptothecin) does not stimulate rDNA transcription (data not shown). Camptothecin at concentrations used to block DNA topoisomerase I activity did not inhibit RNA polymerase I activity (data not shown), which further attests to the specificity of the action of camptothecin. The requirement for DNA topoisomerase I in accurate transcription of supercoiled rDNA could explain the preferential inhibition of rRNA synthesis observed *in vivo* after treatment of cells with camptothecin (16–18). The concentration of DNA topoisomerase I in the nucleolar fraction, as demonstrated by indirect immunofluorescence (8) and enrichment of topoisomerase I-like protein near the 5′ and 3′ ends of *Tetrahymena* rDNA transcription unit (19) suggest that type I DNA topoisomerase is an integral part of rDNA transcription machinery. We have been able to obtain a highly purified transcriptionally active RNA polymerase I fraction that exhibits DNA topoisomerase I activity (S.T.J. and S.D., unpublished data). These observations are consistent with a potential role for DNA topoisomerase I in rDNA transcription.

It is conceivable that the DNA topoisomerase I-camptothecin adduct on supercoiled DNA may prevent movement of RNA polymerase I and the accessory factors along the template. Since transcription from linear rDNA proceeds unabated in presence of camptothecin, the above explanation cannot account for the complete sensitivity of the supercoiled rDNA transcription to the drug. Although the present studies focused on the role of DNA topoisomerase I in rDNA transcription, rRNA synthesis may also be modulated by DNA topoisomerase II. However, by virtue of the nucleolar enrichment of DNA topoisomerase I, alteration in rDNA topology by this enzyme may be the primary mechanism by which the expression of supercoiled rDNA is controlled.

The probable relationship of DNA topology in gene expression has been addressed by other investigators. Pruitt and Reeder (20) have shown that efficient trancription of injected ribosomal gene plasmids requires a covalently closed, circular template whereas transcription of endogenous ribosomal genes continues irrespective of its topology. Supercoiled DNAs containing enhancers have been shown to produce higher levels of RNA polymerase II-directed transcripts than linearized DNA following transfection (21). However, the exact topology of the transcriptionally active templates after transfection or microinjection and the role of DNA topoisomerases in transcription have not been investigated.

A 174-base-pair (bp) enhancer element in the 5′-flanking nontranscribed rat rDNA spacer region has been characterized in our laboratory (A. Dixit, L.C.G., and S.T.J., unpublished data). We have now compared *in vitro* transcription efficiency of a plasmid containing the rDNA enhancer and core promoter to that of a plasmid without the enhancer (L.C.G. and S.T.J., unpublished data). These studies have shown that the enhancer action of the 174-bp element persisted when supercoiled rDNA was used instead of linear

rDNA and that camptothecin-induced inhibition of relaxation of the supercoiled rDNA containing the enhancer prevented its transcription. These data further suggest that supercoiling alone does not mimic the effect of the enhancer and the relaxation of supercoiled DNA with or without enhancer is essential for its transcription.

It is plausible that supercoils are required for the formation of a stable complex between DNA and transcription factors or of initiation complex and that only the elongation reaction requires relaxation of the template. However, since linear rDNA templates can also be transcribed *in vivo* or *in vitro* and are known to form stable initiation complex, one needs to speculate that the transcription complex formed with supercoiled DNA has an extended stability. This could be due to association of the latter template with specific transcription factors, which may allow several rounds of initiation of transcription. Further studies are needed to test this possibility.

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