Cell, Vol. 48, 289–295, January 30, 1987, Copyright © 1987 by Cell Press

Topological Characterization of the Simian Virus 40 Transcription Complex

Bronislawa Petryniak and Leonard C. Lutter
Department of Biological Chemistry
The University of Michigan Medical School
Ann Arbor, Michigan 48109-0606

Summary

We have used sedimentation analysis as well as agarose gel electrophoresis to characterize the topological state of the DNA of the Simian Virus 40 (SV40) transcription complex. We found that the complex DNA contained constrained topological tension, presumably resulting from nucleosome-like structures, but no detectable unconstrained (i.e., relaxable) topological tension. These results contradict previous conclusions that the SV40 transcription complex contains only unconstrained topological tension. Our findings are also the opposite of what has been proposed to be the case for the 5S gene analyzed in Xenopus oocytes. Thus the proposal that expression from the 5S gene is associated with substantial topological tension is not valid for expression from the SV40 late gene.

Introduction

Topological tension has been shown to play an important role in the control of gene expression in prokaryotes (Fisher, 1984; Gellert, 1984). DNA gyrase, a type II topoisomerase, is the enzyme responsible for inserting topological tension into bacterial DNA, and the antibiotic novobiocin is known to inhibit both DNA gyrase and eukaryotic topoisomerase II by inhibiting the ATPase activity of the two enzymes (Fisher, 1984; Gellert, 1984). However, a gyrase-like activity has not yet been detected in eukaryotic cells, nor does there appear to be a substantial amount of unconstrained topological tension in the chromatin of eukaryotic cells (Sinden et al., 1980). Nevertheless, several recent studies employing novobiocin treatment of eukaryotic cells (Ryoji and Worcel, 1984; Villeponteau et al., 1984; Han et al., 1985; Kmiec and Worcel, 1985; North, 1985; Ness et al., 1986; Kmiec et al., 1986b), as well as other approaches (Harland et al., 1983), have led to the proposal that topological tension may also be important for transcription in eukaryotic cells.

Luchnik et al. (1982) have proposed that the SV40 viral transcription complex contains topological tension, and this proposal has been widely cited because of its relevance to eukaryotic transcription (e.g., Ryoji and Worcel, 1984; Villeponteau et al., 1984; Gargiulo et al., 1984; Han et al., 1985; Kmiec et al., 1986a and 1986b) as well as Z-DNA function (e.g., Nordheim and Rich, 1983; Peck and Wang, 1985). Luchnik et al. (1982) find that treatment of isolated SV40 minichromosomes with topoisomerase I results in a small fraction (2%–5%) of those minichromosomes becoming relaxed and losing their histones. They find, furthermore, that the sedimentation behavior of this relaxable fraction correlates with that of the majority of the viral transcription complexes. The viral transcription complex, monitored by in vitro transcription activity, normally sediments faster than the bulk of the viral minichromosomes. Topoisomerase treatment of the gradient fraction containing the transcription complex activity causes the relaxation of a significant portion of the DNA, and resedimentation reveals that this relaxed DNA and the transcription activity both sediment much more slowly than the bulk of the minichromosomes following treatment. Luchnik et al. (1982) interpret these results to mean that the viral complexes that contain in vitro RNA polymerase activity are torsionally strained. They propose that treatment by topoisomerase causes complete relaxation of the transcription complex DNA as well as loss of histones, resulting in the decreased sedimentation rate of the complex.

A question has arisen as to whether such viral complexes, which are characterized by RNA polymerase II extension activity in vitro, are the same complexes as those that produce viral mRNA in the cell. Llopis and Stark (1982) propose that the mRNA-producing complexes, which can be labeled by a short [^{3}H]uridine pulse in vivo, differ from the complexes mentioned above, which are detected by their ability to transcribe in vitro. It has been proposed that the complexes that can be labeled in vitro are precursors to those that are labeled in vivo (Hay et al., 1982), but this precursor–product relationship has not been well established (Llopis and Stark, 1981 and 1982). We shall refer to these two types of complexes as in vitro–extendable and mRNA-producing complexes, respectively.

We describe here results that characterize the topological properties of both types of complexes. We have found that the sedimentation of the mRNA-producing complexes is unaffected by treatment of the complex with either topoisomerase I or deoxyribonuclease I (DNAase I), treatments that would be expected to relax any unconstrained topological tension. We also used agarose gel electrophoresis to analyze directly the topology of the DNA of both the mRNA-producing and the in vitro–extendable transcription complexes after each type of complex was tested with topoisomerase. In neither case did we detect any substantial unconstrained topological tension. These results contradict the conclusions of Luchnik et al. (1982) and render unlikely recent proposals that topological tension is a fundamental requirement for eukaryotic gene expression.

Results

Viral Transcription Complex Preparation

A modification of the method of Llopis and Stark (1981) was used to label radioactively and partially purify the viral transcription complexes that are in the process of producing mRNA in vivo. BSC-1 cells were infected with SV40 and incubated with [^{14}C]thymidine from 24 to 45 hr after

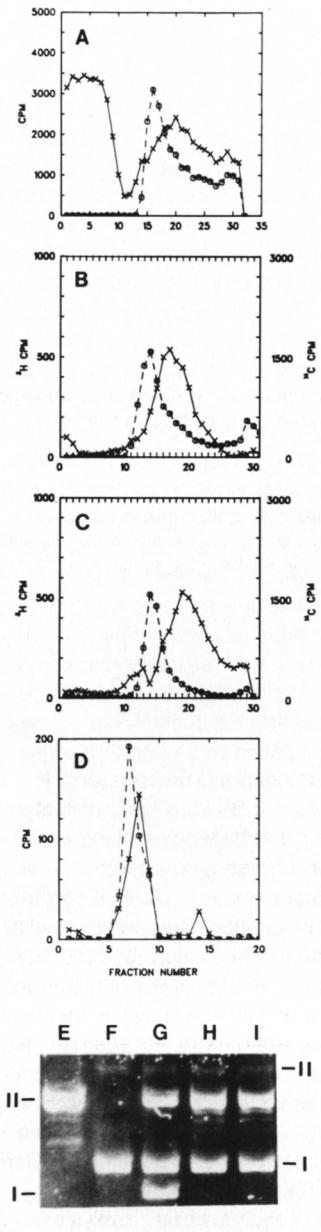

Figure 1. Analysis of Topoisomerase-Treated Viral Transcription Complex

(A): Nuclear extract was prepared from infected cells which were labeled with [^{14}C]thymidine from 24 to 45 hr after infection and with [^{3}H]uridine for 5 min just before harvesting. Extract was sedimented in a 10% isokinetic glycerol gradient for 200 min at 40,000 RPM in an SW40 rotor. The gradient was then fractionated and analyzed for radioactivity (see Experimental Procedures). Sedimentation is from left to right. A typical "90S" pool of the mRNA-producing transcription complex would be fractions 17–27.

(B): An aliquot of the "90S" pool, described in (A) above, was resedimented under conditions described in (A).

(C): An aliquot of the "90S" pool was treated with topoisomerase I for 90 min and then resedimented as in (B).

(D): The pooled ^{3}H peak fraction in (C) was deproteinized and analyzed by centrifugation in Cs_2SO_4 density gradient, with lowest density on the left and highest density on the right. Symbols for A–D: ○, ^{14}C radioactivity (DNA); X, ^{3}H radioactivity (RNA).

(E)–(I): Samples were subjected to electrophoresis in 0.7% agarose. The samples were the following. (E): Purified pBR322 DNA treated with wheat germ topoisomerase I. (F): An aliquot from the 90S pool in Figure 1A. (G): As in (F), plus pBR322 added to 4 μg/ml. (H): As in (G), plus topoisomerase treatment for 20 sec. (I): As in (G), plus topoisomerase treatment for 90 min. The positions of Form I (supercoiled) and Form II (relaxed/nicked circular) DNA are shown for pBR322 (left side) and SV40 (right side).

infection to label viral DNA uniformly. At this point, they were also incubated with [^{3}H]uridine for 5 min to label the nascent RNA of the viral transcription complexes. Nuclei were then prepared and extracted in isotonic buffer and the extract was fractionated by sedimentation in a glycerol gradient.

The profile of such a gradient is shown in Figure 1A. The bulk of the viral minichromosomes represented by the peak of ^{14}C-radioactivity sediments at ~70S, while a somewhat broader peak of ^{3}H radioactivity sediments further down the gradient at ~90S. This ^{3}H-radioactivity peak represents the viral transcription complexes, which presumably sediment more rapidly than the bulk minichromosomes because of the presence of RNA polymerase II, transcription factors, and nascent transcript (see Llopis and Stark, 1982).

Topoisomerase Treatment Causes Relaxation of a Small Fraction of the Minichromosome DNA but Does Not Cause a Shift in Sedimentation of the Transcription Complex

Luchnik et al. (1982) found that topoisomerase I treatment of the 90S minichromosome fraction resulted in the relaxation of a significant proportion of the minichromosome DNA. We treated the 90S fraction with topoisomerase I and then analyzed the topoisomer distribution of the extracted DNA by two-dimensional agarose gel electrophoresis. Figure 2A shows that, for the unincubated sample, the bulk of the closed circular DNA is negatively supercoiled, but Figure 2B shows that the DNA from the minichromosomes that had been incubated with topoisomerase I contain a small fraction that is substantially relaxed. We also observe significant relaxation when the sample is simply incubated without exogenously added topoisomerase (data not shown), indicating that endogenous topoisomerase can also produce the relaxation. Thus our preparation of SV40 minichromosomes also contains a topoisomerase I–relaxable fraction similar to that originally described by Luchnik et al. (1982) and also seen by Barsoum and Berg (1985; cf. their Figure 2).

Luchnik et al. (1982) found that the sedimentation constant of the in vitro–extendable viral transcription complex drops dramatically when the gradient-isolated complex preparation is treated with topoisomerase I. We analyzed the mRNA-producing complex to determine if it responded similarly. Fractions containing the bulk of the ^{3}H radioactivity were pooled from the 90S region of the gradient in Figure 1A and an aliquot of this pool was incubated with purified wheat germ topoisomerase I. The fraction was then resedimented in a glycerol gradient, and the profiles are shown in Figures 1B and 1C. Here it can be seen that the incubation with topoisomerase has little if any effect on the sedimentation of this ^{3}H-radioactivity peak, which represents the mRNA-producing viral tran-

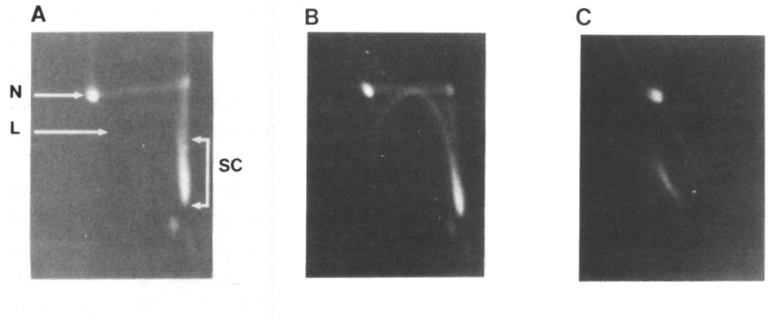

Figure 2. Two-Dimensional Gel Electrophoresis of Topoisomerase-Treated Minichromosome DNA

The 90S fraction was treated with or without topoisomerase I, after which the DNA was extracted and analyzed by two-dimensional gel electrophoresis. First dimension is from left to right and was carried out in TBE buffer plus 0.2 μM chloroquine. Second dimension was from top to bottom and was carried out in TBE plus 1.3 μM chloroquine.

(A): DNA extracted from 90S minichromosomes that had not been treated with topoisomerase I.

(B): DNA extracted from 90S minichromosomes that had been treated with topoisomerase I.

(C): Hirt-extracted SV40 DNA that had been treated with topoisomerase I under conditions identical to that used in (B) above.

In (A), N indicates nicked circular SV40 DNA, L indicates linear SV40 DNA, and SC indicates the range of the bulk of the negatively supercoiled SV40 DNA. In (B), the series of spots forming an arc rising from the top of the supercoiled DNA represents a series of topoisomers. The linking numbers of the topoisomers become larger as one proceeds counterclockwise around the arc.

scription complex. Similar results were obtained when purified calf thymus topoisomerase I (BRL) was used.

To ensure that the wheat germ topoisomerase was indeed functioning when added to the 90S fraction, a parallel incubation was carried out in which we added to the 90S fraction purified supercoiled pBR322 DNA in an amount equivalent to that of the SV40 DNA in the sample. Aliquots were removed at various incubation times and the DNA analyzed by electrophoresis in an agarose gel. The gel results in Figure 1E-1I show that the disappearance of the supercoiled pBR322 DNA is complete within the first 20 sec of incubation. The presence of relaxed topoisomers of pBR322 indicates that this disappearance is due to topoisomerase relaxation rather than endogenous nicking activity. Since the incubation carried out in Figure 1C was for 90 min, the topoisomerase activity present there is greater than 300 times that which would be needed to relax the equivalent amount of purified SV40 DNA present in the sample.

Finally, Figure 1D shows Cs_2SO_4 density gradient analysis of the pooled peak of ^3H radioactivity from Figure 1C. Here it can be seen that 90% of the ^3H radioactivity bands at a hybrid density slightly below that of pure DNA (see also Llopis and Stark, 1982). This demonstrates that almost all of the 90S ^3H radioactivity in Figure 1C represents the viral transcription complex and not free RNA.

To summarize the results to this point, the data in Figure 1D demonstrate that the ^3H radioactivity represents intact mRNA-producing viral transcription complexes. Treatment with demonstrably active topoisomerase I does not result in a significant change in the sedimentation of this ^3H radioactivity (Figures 1B, 1C, 1H, and 1I), even though such a treatment does cause relaxation of a small fraction of total minichromosome DNA (Figure 2).

Nicking of the DNA of the Transcription Complex Does Not Cause a Shift in Sedimentation

We also employed DNAase I nicking as an alternative to topoisomerase for assaying for topological tension in the transcription complex. A DNAase I–produced nick in the DNA of the transcription complex would also be expected to release topological tension, and the advantage of this approach is that the bulk of the minichromosomes in the sample can be nicked and shown to be so by subsequent electrophoretic analysis in an agarose gel. Since transcriptionally active cellular chromatin is more sensitive to DNAase I digestion than is bulk chromatin (reviewed in Reeves, 1984), it is reasonable to assume that the transcription complex DNA has been nicked if the majority of the bulk minichromosome DNA is nicked.

The results of such a nicking analysis are shown in Figure 3. Here it can be seen that DNAase I treatment sufficient to nick all of the supercoiled DNA of the bulk minichromosome population (Figure 3C) has virtually no effect on the sedimentation of the transcription complex. We conclude from the results of both the topoisomerase analysis and the DNAase I analysis that the in vivo–labeled, mRNA-producing viral transcription complexes are not torsionally strained, at least not in such a way that relaxation of this strain causes a gross structural change like that which occurs in the in vitro–extendable complexes studied by Luchnik et al. (1982).

Both the mRNA-Producing and In Vitro–Extendable Transcription Complexes Contain Constrained, but No Unconstrained, Topological Tension

What we have shown up to this point is that mRNA-producing transcription complexes do not change their sedimentation properties as a result of topoisomerase I

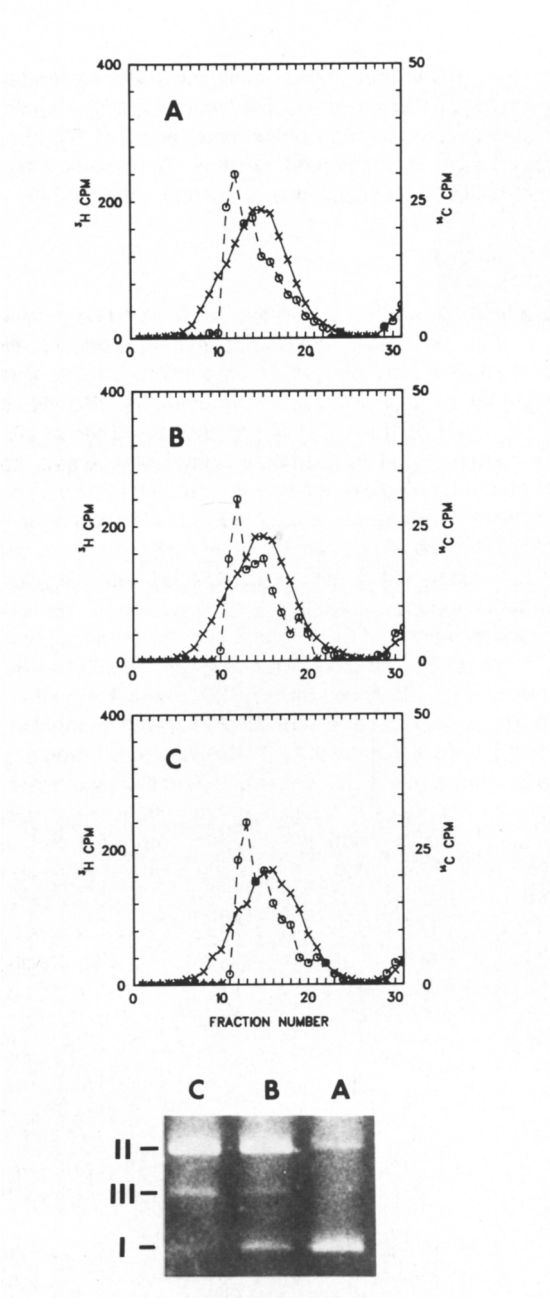

Figure 3. Treatment of Transcription Complexes by DNAase I

Aliquots of the pooled 90S fraction from a preparation similar to that shown in Figure 1A were incubated with 0 ng/ml (A), 4 ng/ml (B), or 40 ng/ml (C) DNAase I for 1 min at 28°C. Resedimentation in glycerol gradients was carried out as in Figure 1B, while electrophoresis in an agarose gel was performed as in Figure 1E. The positions of Form I (supercoiled), Form II (nicked/relaxed circle), and Form III (linear) SV40 DNA are indicated on the left side of the agarose gel. O, [^{14}C]thymidine label (DNA); ×, [^{3}H]uridine label (RNA).

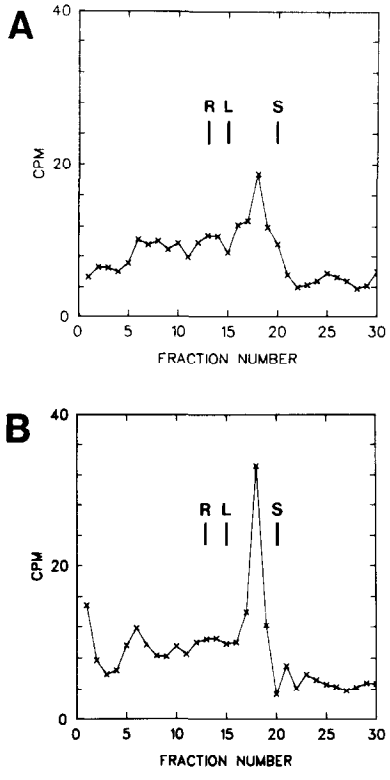

Figure 4. Direct Electrophoretic Analysis of the Topology of In Vivo–Labeled Transcription Complex DNA

An aliquot of the 90S fraction from [^{3}H]uridine pulse-labeled infected cells was treated with or without topoisomerase I. Proteins were removed and unduplexed nascent RNA was digested away with ribonucleases. The DNA:RNA hybrids were then fractionated by electrophoresis in 0.7% agarose and the gel sliced and counted. The samples were DNA from: (A) minichromosomes that were not incubated; (B) minichromosomes that were incubated with topoisomerase I. R designates the mobility in the same channel of ethidium bromide–stained relaxed SV40 DNA, S designates that of supercoiled DNA, and L designates that of linear DNA.

treatment, a behavior that contrasts with that of the in vitro–extendable complexes of Luchnik et al. (1982). However, this lack of change in sedimentation does not in itself necessarily mean that the mRNA-producing complex contains no relaxable tension, because the sedimentation change seen by Luchnik et al. (1982) was apparently the result of relaxation causing loss of most proteins of the complex, which in turn caused the sedimentation change. It is possible that our complex contains tension that can be relaxed, but the relaxation does not cause protein loss and the resultant sedimentation shift.

To investigate this possibility, we analyzed the transcription complex DNA:RNA hybrid by agarose gel electrophoresis to determine *directly* if there was a substantial topological change in the transcription complex DNA as a result of topoisomerase treatment. Thus the [^{3}H]uridine-labeled 90S fraction was treated with or without topoisomerase I and the nucleic acid purified. This sample was treated with ribonuclease to remove all attached RNA save that duplexed with the DNA. The samples were then subjected to electrophoresis in an agarose gel, after which the gel was sliced and counted. The results in Figure 4A show that the transcription complex DNA has a slightly lower mobility than Form I (supercoiled) DNA, demonstrating that the DNA of the transcription complex is supercoiled but not quite as much as the bulk DNA. This indicates that the linking number of transcription complex DNA is similar to that of bulk minichromosome DNA, but shows a slightly lower level of supercoiling due to the

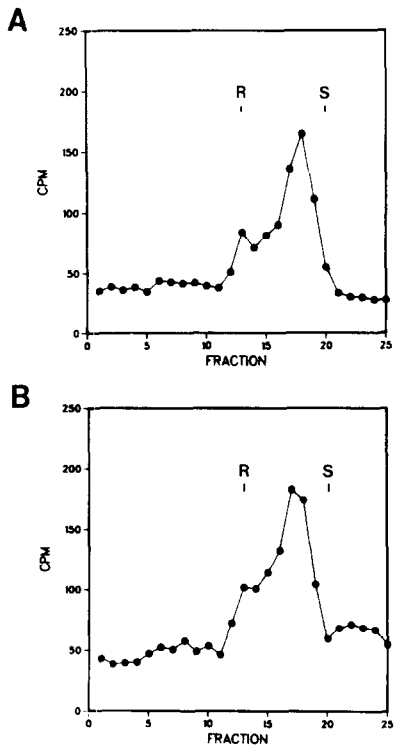

Figure 5. Direct Electrophoretic Analysis of the Topology of In Vitro–Extendable Transcription Complex DNA

The aliquot of 90S chromosome fraction was treated with or without topoisomerase I, after which nascent RNA was extended in vitro using [^{3}H]UTP. Samples were processed and fractionated by electrophoresis in a 0.7% agarose gel as described in Figure 4. The samples were DNA from: (A) minichromosomes that were not incubated, (B) Minichromosomes that were incubated with topoisomerase I. R designates the mobility in the same channel of ethidium bromide–stained relaxed SV40 DNA. S designates the position of supercoiled SV40 DNA.

relaxation caused by the presence of the short remaining fragment of ^{3}H-labeled RNA duplexed with the transcription complex DNA (see also Birkenmeier et al., 1977). This similarity in linking numbers provides the first demonstration that the mRNA-producing transcription complex is most likely organized in nucleosomes or in nucleosome-like structures.

When the DNA of the topoisomerase-treated transcription complex is analyzed, it can be seen (Figure 4B) that it has virtually the same mobility as that of the untreated complex DNA, indicating that the topoisomerase treatment caused no substantial change in the topology of the transcription complex DNA. This in turn demonstrates that the mRNA-producing transcription complex DNA contains no substantial relaxable topological tension. Thus the lack of a change in sedimentation constant of the complex (Figure 1B and 1C) is not simply due to the retention of proteins while the complex was relaxed; in fact, there was no relaxation of topological tension in the DNA of the transcription complex as a result of topoisomerase treatment.

Such a direct topological analysis was also performed on the DNA of the in vitro–extendable transcription complex, the complex similar to that studied by Luchnik et al. (1982). The results shown in Figure 5 are similar to those for the mRNA-producing complex: the in vitro–extendable complex contains nucleosome-like constrained topological tension (confirming Birkenmeier et al., 1977), but no substantial unconstrained tension. This result directly contradicts the conclusions of Luchnik et al. (1982).

Discussion

We have shown that neither topoisomerase I nor DNAase I treatments cause a significant shift in the sedimentation of the mRNA-producing viral transcription complex. Direct analysis by agarose gel electrophoresis also demonstrates that both the mRNA-producing and the in vitro–elongatable viral transcription complexes contain constrained topological tension to an extent that would suggest that both are organized in nucleosomes or nucleosome-like structures. However, neither type of complex contains substantial unconstrained (relaxable) topological tension.

These results directly contradict the conclusions of Luchnik et al. (1982). We find no evidence that the SV40 transcription complex contains substantial amounts of relaxable supercoils. We do observe, as did Luchnik et al. (1982), a small fraction of the bulk minichromosome population that is relaxable, but we question the importance of this fraction since it can simply result from minichromosome dissociation during preparation and topoisomerase treatment. Relevant to this point, we have observed an increase in the amount of this relaxable fraction as a result of freezing and thawing of the minichromosome preparation. Our results of the direct gel analysis also preclude the possibility that we see no relaxable tension in the complex because the complex had already relaxed during the preparation: the gel analysis shows that the transcription complex DNA is supercoiled, but that this supercoiling is constrained (i.e., nucleosome-like and unrelaxable), not unconstrained. Finally, it should be pointed out that the study of Luchnik et al. (1982) is a correlative one, whereas our study analyzes directly the transcription complex DNA topology.

A number of studies cite Luchnik et al. (1982) in their various proposals for a connection between topological tension and eukaryotic transcription. Several papers have interpreted the results of experiments employing novobiocin, an inhibitor of bacterial DNA gyrase and eukaryotic topoisomerase II, to mean that topological tension may modulate eukaryotic transcription (Ryoji and Worcel, 1984; Kmiec and Worcel, 1985; Kmiec et al., 1986b; Villeponteau et al., 1984; Han et al., 1985; Ness et al., 1986). However, such interpretations must be made with caution because of the high levels of novobiocin used and the fact that novobiocin is not highly specific for topoisomerase II but rather can affect a number of cellular processes (Wright et al., 1981; Gottesfeld, 1986; Cotten et al., 1986).

Work from Worcel's laboratory (Ryoji and Worcel, 1984; Kmiec et al., 1986a and 1986b) on the Xenopus 5S gene has led to the proposal that transcription from an injected plasmid carrying the 5S gene requires the action of a cellular gyrase activity to convert the plasmid into a "dynamic" (i.e., relaxable) chromatin structure, a structure

that is proposed to be the template for 5S RNA transcription. The results that we present here indicate that the SV40 transcription complex differs profoundly from the 5S plasmid system with respect to topological characteristics. Worcel's group proposes that the 5S transcription complex DNA contains relaxable (i.e., "dynamic," or unconstrained) supercoils only and no unrelaxable supercoils. We find that the situation for SV40 is just the opposite: we observe that the SV40 transcription complex contains unrelaxable (i.e., constrained, or nucleosome-like) supercoils only and no relaxable supercoils.

The differences between these two studies may derive from the particular gene studied (5S plasmid vs. SV40 viral genome), the type of polymerase (RNA polymerase III vs. RNA polymerase II), or the host/chromatin assembly system (injection into oocyte vs. viral infection of cultured cells). However, it should be pointed out that all of the 5S data is correlative; a direct topological analysis of *transcribed* DNA, such as the one we have performed here for SV40, has not been reported for the 5S plasmid system. Whatever the explanation for the difference between the two systems, our results with SV40 make it clear that the proposal that topological tension is closely associated with eukaryotic transcription (Ryoji and Worcel, 1984) is not valid for *all* forms of eukaryotic transcription.

Experimental Procedures

Cells and Virus

BSC-1 cells were used for infections with SV40 to obtain SV40 minichromosomes. The cells were grown in 100 mm culture plates containing Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine; penicillin and streptomycin; and 5% fetal calf serum. When the cells were at approximately 80% confluency, they were infected with 10 pfu/cell of SV40 strain 776 using DMEM supplemented as above, except that 2% fetal calf serum was used instead of 5%. Infections were carried out at 37°C for 45 hr.

Labeling of Cells and Preparation of Nuclear Extracts

Viral minichromosomes were labeled by addition of [2-^{14}C]thymidine, 4 μCi/10 ml (ICN) to the medium from about 24 to 45 hr following infection. At 45 hr after infection, the medium was removed and the cells were pulse-labeled by addition of 0.5 ml of [5-^{3}H]uridine (1 mCi/ml, 54 Ci/mmol [Amersham]) plus 1.5 ml DMEM/2% fetal calf serum per plate. The plates were incubated for 5 min at 37°C and then placed on ice.

Nuclear extract was prepared according to the method of Llopis and Stark (1981), which involves an isotonic isolation of nuclei followed by an isotonic extraction of intact nuclei as described below. Following pulse labeling, each plate was washed twice with cold TD buffer (137 mM KCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 50 mM Tris-HCl [pH 7.4]), after which the cells were scraped into TD buffer (1 ml/100 mm plate). Cells were then pelleted by centrifugation for 5 min at 1500 × g, after which they were resuspended in ice-cold TD plus 0.5% NP-40 and 0.2 mM PMSF. Nuclei were pelleted by centrifugation as above and resuspended in ice-cold TD buffer plus 0.2 mM PMSF but without the Na_2HPO_4 (200 μl/plate). The nuclei were then treated with 30 strokes in a Dounce homogenizer (Wheaton, type A pestle) and the resulting suspension was incubated at 4°C for 2 hr with occasional agitation. The suspension was then centrifuged for 10 min at 17,000 × g. The nuclear extract supernatant was fractionated by sedimentation in a glycerol gradient. Hybridization analyses of the "90S" region material (see Figure 1A) employing an M13 phage vector into which was cloned the early or late region of SV40 indicated that virtually all of the ^{3}H radioactivity represents SV40 sequences, while base hydrolysis demonstrated that the ^{3}H radioactivity is in RNA (data not shown).

Glycerol Gradient Fractionation of Nuclear Extracts

A 10% isokinetic glycerol gradient (in .1 mM Na_2EDTA, 10 mM Tris-HCl [pH 7.9]) (McCarty et al., 1974) was used to partially purify the viral transcription complexes from the bulk of the viral minichromosomes as well as from free proteins in the nuclear extract. The nuclear extract described above was layered onto a 12 ml gradient and subjected to ultracentrifugation at 40,000 rpm for 200 min at 4°C using an SW40 rotor. Gradients were fractionated through an ISCO gradient analyzer (Model 184) and 10-drop fractions were collected. A 25 μl aliquot from each fraction was then added to 4 ml ACS scintillation fluid (Amersham) and analyzed by scintillation spectrometry. Resedimentation was performed on 15% isokinetic gradients. Construction of isokinetic gradients was as described in McCarty et al., 1974.

Equilibrium Density Gradient Analysis

The sample to be analyzed (0.1–0.5 ml) was adjusted to 1% in sodium dodecyl sulfate (SDS) and layered onto a Cs_2SO_4 step gradient in a 5.1 ml polyallomer Quick-seal VTi65 centrifuge tube (Beckman). The step gradient consisted of four 1.2 ml steps of Cs_2SO_4 solutions of 1.17 g/ml, 1.36 g/ml, 1.61 g/ml, and 1.73 g/ml, respectively, in 10 mM sodium phosphate (pH 7.1), 1 mM EDTA. Tubes were then centrifuged at 22°C for 4½ hr at 65,000 rpm in a Beckman VTi65 rotor. The gradient was fractionated using an ISCO gradient fractionator (Model 184), and 8-drop fractions were collected and spotted onto Whatman 3MM filters. Filters were washed twice in 5% trichloroacetic acid and once in 95% ethanol, dried, added to 10 ml OCS scintillation fluid (Amersham), and analyzed for radioactivity by scintillation spectrometry.

Topoisomerase Incubation

A 1.0 ml aliquot of the 90S pool of ^{3}H radioactivity was adjusted to 50 mM Tris-HCl (pH 7.9), 50 mM NaCl, 1 mM EDTA, and wheat germ topoisomerase I (Promega Biotec) was added to 25 U/ml. The sample was incubated for 90 min at 37°C, after which 0.35 ml of 10 mM Tris (pH 7.9), 0.1 mM EDTA was added. The sample was then analyzed by sedimentation in a glycerol gradient or a Cs_2SO_4 density gradient. When the sample was to be analyzed by electrophoresis in an agarose gel, pBR322 plasmid was added to 4 μg/ml. Aliquots of 20 μl were removed at the appropriate times and made 0.1% in SDS.

DNAase I Digestion

A 0.5 ml aliquot of the 90S pool of ^{3}H radioactivity was adjusted to 1 mM EDTA, 1 mM DTT, and to the appropriate concentration of RNAase-free DNAase I (Amersham). The sample was then incubated for 2 min at 28°C, followed by addition of $MgCl_2$ to 5 mM to start the digestion. After incubation for 1 min at 28°C, EDTA was added to 10 mM and the sample cooled on ice. A 20 μl aliquot was removed and analyzed by electrophoresis by an agarose gel, and the remaining sample was analyzed by sedimentation in a glycerol gradient or a Cs_2SO_4 density gradient.

Electrophoresis in Agarose Gels

For one-dimensional electrophoresis, one-tenth volume of agarose gel sample buffer (1% SDS, 0.01% Bromphenol blue) was added to a sample from the 90S pool and subjected to electrophoresis in 0.7% agarose (BRL) in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA). Electrophoresis was for 3 hr at 2 V/cm, after which time the gel was stained in 1 μg/ml ethidium bromide and photographed through an orange filter under ultraviolet light.

Two-dimensional electrophoresis (Figure 2) was carried out according to a procedure similar to that used by Wang et al. (1982). First-dimension electrophoresis was carried out in TBE buffer plus 0.2 μM chloroquine, while second-dimension electrophoresis was carried out in TBE buffer plus 1.3 μM chloroquine. The inclusion of 0.2 μM chloroquine in the first dimension causes the relaxed topoisomers to be slightly positively supercoiled (see Figure 2C).

RNAase Treatment of Transcriptional Complex DNA

The 90S fraction (from 3 plates) that had been treated with or without topoisomerase I was made 0.5% SDS and 65 μg/ml proteinase, and incubated for 1 hr at 37°C, after which it was precipitated by addition of 2.5 vol of ethanol and incubation at −70°C for 20 min. Following centrifugation (10 min at 10,000 rpm, Sorvall HB-4), the pellet was washed

with 70% ethanol, dried briefly, resuspended in 0.1/ml 2× SSC, and digested with .25 U Ribonuclease T1 and 5 μg pancreatic ribonuclease for 1 hr at room temperature. The sample was again treated with SDS and Proteinase K, and ethanol precipitated as described above. The pellet was resuspended in 0.1 ml TE buffer. An aliquot was subjected to electrophoresis in a 0.7% agarose gel (see above), after which it was stained with ethidium bromide and photographed to locate the SV40 DNA. Individual channels were then sliced with a Hoeffer gel slicer (Model SL-280). Two slice portions were placed in a scintillation vial, 0.5 ml of 0.3 M HCl was added to each vial, and the vials were heated in a boiling water bath for 10 min. After cooling, 4 ml of aqueous counting fluid (Safety-Solve, Research Products International) was added to each vial and the vial was counted.

In Vitro Transcription Extension

A typical in vitro extension reaction (50 μl) contained 35 μl 90S fraction, 74 mM Tris (pH 7.9), 140 mM NaCl, 148 mM $(NH_4)_2SO_4$, 2.1 mM, 1 mM DTT, 470 μM ATP, 470 μM GTP, 470 μM, 9 μM UTP, and 5 μCi [^{3}H]UTP. [^{3}H]UTP. The sample was incubated for 30 min at 30°C. SDS and Proteinase K were then added and the sample was treated with RNAase as described above.

Acknowledgments

We would like to thank Dr. J. Jendrisak for providing purified wheat germ topoisomerase I. This investigation was supported by grants from the American Cancer Society (MV185), The University of Michigan Cancer Research Institute, and The University of Michigan Biomedical Research Council.

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Received September 10, 1986; revised October 29, 1986.

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