Enhancer-activated plasmid transcription complexes contain constrained supercoiling

Pedro J.Bonilla^{1,2}, Svend O.Freytag^{1,2} and Leonard C.Lutter^{1*}

¹Molecular Biology Research Program, Henry Ford Hospital, 2799 West Grand Blvd, Detroit, MI

48202 and ²Department of Biological Chemistry, University of Michigan, Ann Arbor, MI 48109, USA

Received March 14, 1991; Revised and Accepted June 5, 1991

ABSTRACT

It has been proposed that transcriptionally active chromatin contains totally unconstrained supercoiling. The results of recent studies have raised the possibility that this topological state is the property of highly transcribed genes. Since the transcription rate of RNA polymerase II genes can be dramatically increased by the presence of an enhancer, we have determined if the transcription complex of an enhancer-activated plasmid contains totally unconstrained supercoils. Following transfection into COS7 cells, the topology of the transcription complex DNA was determined directly by agarose gel electrophoresis. We find that an enhancer-activated plasmid transcription complex is supercoiled, and these supercoils remain following topoisomerase I treatment. Thus the transcribing complexes contain constrained supercoils, and the level of supercoiling suggests a nucleosome-like organization. However, we cannot rule out the possibility that unconstrained supercoils exist in addition to these constrained supercoils in the transcription complex in the cell.

INTRODUCTION

Much of our knowledge regarding the structure of eukaryotic chromatin is derived from studies which utilized material that was largely transcriptionally inactive (1). In contrast, considerably less is known about the structure of chromatin that is in the process of being transcribed. Results from a number of studies have led to the proposal that the DNA topology of active chromatin differs from that of inactive chromatin (1–4).

In contrast to prokaryotes (5,6), the role of supercoiling in transcription in eukaryotes is controversial (5,7,8). The DNA of a eukaryotic cell is supercoiled, but most, if not all, of this superhelical tension is constrained by nucleosomes (9). Since most of the eukaryotic genome is transcriptionally inactive (10), these studies say little about the structure of active chromatin, and it seems logical to conclude that nucleosome-constrained supercoiling is a property of inactive chromatin. The role of supercoiling in eukaryotic transcription has been addressed using

the 5S rRNA gene and SV40 late minichromosome as model systems (11–15). In the case of the 5S rRNA gene, active transcription closely correlates with the presence of totally unconstrained supercoiling, i.e. the supercoiling of the transcribed gene is proposed to be completely relaxable. However, this correlation is entirely based on examination of the topology of bulk chromatin; the structure of the transcribed chromatin was never directly examined. Thus in the 5S rRNA system, a relationship between unconstrained supercoiling and transcription is not well-established. In contrast to the 5S rRNA studies, the topology of transcribing SV40 late minichromosomes has been examined directly. The SV40 late transcription complex was found to contain constrained supercoiling in amounts similar to bulk, nucleosomal SV40 chromatin (15–17). This observation contrasts with a previous finding (12), and raises some concern regarding proposals that promote a causal role for supercoiling in eukaryotic transcription (11,12).

The presence of constrained, nucleosome-like supercoiling in the SV40 late transcription complex could be due to the possibility that this complex is weakly transcribed, and that totally unconstrained supercoiling is a property which is characteristic of very active genes such as the 5S rRNA or enhancer-activated RNA polymerase II genes. This possibility is strengthened by the observation that supercoiling stimulates the expression of transfected plasmids, particularly those which contain an enhancer (18). The SV40 late gene is not substantially activated by an enhancer (19,20), and its promoter lacks a TATA box and requires the GC-boxes for transcription (21,22). Thus, the SV40 late promoter has the features of a typical cellular 'housekeeping' gene promoter, which is weakly transcribed (23). To test whether totally unconstrained supercoiling is a property which is characteristic of genes that are very actively transcribed, we have directly analyzed the topology of an enhancer-activated plasmid transcription complex (enhanced PTC)¹ *in vitro*. Such complexes contain RNA polymerase II molecules which were initiated *in vivo*, but which are active in run-on transcription *in vitro* (15,17). Although an enhancer dramatically stimulated the activity of the PTC both *in vivo* and *in vitro*, the PTC contained constrained supercoiling consistent with a nucleosome-like organization

* To whom correspondence should be addressed

EXPERIMENTAL PROCEDURES

Materials

DNA topoisomerase I was from Promega. All other DNA-modifying enzymes were purchased from Boehringer Mannheim. Radioactive reagents ($[^{14}\text{C}]$ chloramphenicol, $[\alpha^{32}\text{P}]\text{dCTP}$, and $[\alpha^{32}\text{P}]\text{UTP}$) and hybridization transfer membranes were obtained from DuPont-New England Nuclear or ICN Biomedicals. Centricon 30 microconcentrators were from Amicon. Other reagents were obtained from Sigma or Fisher.

Cells and Transfections

COS7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum, 2 mM glutamine, 10 mM HEPES, 100 μg streptomycin and 100 U penicillin G per ml (DMN-10) at 37°C in an atmosphere of 92% air and 8% CO_2 . Cells were transfected using the electroporation technique (24). Cells were harvested and washed with cold D-PBS without added MgCl_2 or CaCl_2 . Each individual electroporation was performed in 0.5 ml containing 10^7 cells/ml and 40–60 μg of cesium chloride gradient-purified plasmid DNA. After electroporation, cells were incubated at 4°C for 10 minutes and then plated onto 150 mm dishes containing DMN-10. After incubating overnight, cell monolayers were washed with D-PBS and fresh DMN-10 was added back. Cells were further incubated until harvested for preparation of nuclear extracts, cellular extracts or Hirt supernatants.

DNA Constructs

All plasmids were derived from pmgAS6 (see Figure 1). Plasmid pmgAS6 is similar to pmgAS10 (25) except that the Bam HI site 3' to the CAT gene and the Hind III site in exon 4 of the human argininosuccinate synthetase (AS) gene have been removed. Plasmid pmgAS6 contains the following sequences of the human AS gene: 150 bp of 5'-flanking sequence (AS promoter), all 34 bp of exon 1, approximately 350 bp of intron 1, 1961 bp of intron 3, and 54 bp of exon 4. Plasmid pORAS6 was made by ligation of a 91 bp Nco I/Stu I fragment from pSV2CAT (26), which contains SV40 sequences between nucleotides 5193 and 41, into the unique Nde I restriction site of pmgAS6 using standard techniques. This 91 bp fragment spans the SV40 'core' origin of replication (T-antigen binding site II). The AS promoter in pORAS6 drives the expression of the CAT gene. Plasmid pORAS7 was made by inserting SV40 enhancer sequences (nucleotides 37–275) into the Bam HI site of pORAS6 (25).

Chloramphenicol Acetyltransferase Assays

CAT assays were performed as described (26), except that acetyl-CoA was added to a final concentration of 10 mM. Assays in which different extracts were compared for CAT activity were performed using equivalent amounts of cellular protein. The amount of radioactivity in all acetylated products was determined by liquid scintillation spectroscopy after scraping the corresponding spots identified by autoradiography.

Analysis of Plasmid DNA Copy Number

Two days after transfection, low molecular weight DNA was extracted from COS7 cells by the procedure of Hirt (27). DNA in the Hirt supernatants was digested with Hind III and Dpn I. Hind III linearizes replicated plasmid DNA, while Dpn I digests non-replicated plasmid DNA into restriction fragments of less than 2 kb. DNA samples were electrophoresed in 1% agarose gels in TAE buffer (40 mM Tris-HCl, pH 7.4, 20 mM sodium acetate, 1 mM EDTA). DNA was analyzed by Southern blotting as described before (25) and transfer membranes were hybridized to a Hind III linearized ^{32}P -labeled pORAS7 probe. Radioactive fragments were visualized by autoradiography, excised from the transfer membrane and the amount of radioactivity in each fragment was determined by scintillation spectrophotometry.

Wheat Germ Topoisomerase I Incubations

Nuclear extract (containing PTC) was prepared as described (16,28). For subsequent topoisomerase incubation, the salt concentration of the extract was adjusted by diluting with an equal volume of cold TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and concentrating in a Centricon 30. Topoisomerase reactions were performed in a volume of 100 μl containing 1 mM DTT and 80 U/ml topoisomerase I. Some reactions contained 0.1 mg/ml chloroquine (see text). After incubation at 37°C for 60 minutes, samples were used for *in vitro* transcription assays.

In Vitro Transcription Assay

This assay is a modification of previously described procedures (14,15,28). Samples of concentrated nuclear extract were used for *in vitro* RNA extension in a final volume of 150 μl of a reaction mixture containing 69 mM NaCl, 2.5 mM KCl, 30 mM Tris-HCl (pH 7.9), 1 mM EDTA, 0.1 mM PMSF, 100 mM $(\text{NH}_4)_2\text{SO}_4$, 3 mM MnCl_2 , 1.7 mM DTT, 0.5 mM each of CTP/GTP/ATP, 1 μM $[\alpha^{32}\text{P}]\text{UTP}$ (650 Ci/mmol), and 0.2 mM aurintricarboxylic acid (ATA)¹ or 0.5 mg/ml heparin. RNA extension reactions were incubated for 15 minutes at 30°C, and stopped by adding EDTA to a final concentration of 20 mM. Unincorporated radioactive nucleotide was removed by centrifugation through two successive 1 ml Sephadex G-50 spin columns equilibrated with TE and 0.2 mM ATA. Sample volumes were reduced by evaporation in Speed-Vac Concentrator prior to electrophoresis.

Electrophoresis in Agarose Gels

One-tenth volume of sample buffer (30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol) was added to each sample, and samples were subjected to electrophoresis in a 30 cm gel of 1% agarose in TBE (89 mM Tris-HCl, pH 8.3, 89 mM boric acid, 2 mM EDTA). Some gels contained 0.75 μM chloroquine as indicated in the text. Electrophoresis was for 16 hours at 2 V/cm. Gels were stained in 0.1 $\mu\text{g}/\text{ml}$ ethidium bromide, photographed under ultraviolet light and dried. Dried gels were exposed to Kodak X-Omat AR or X-Omat RP film at -70°C .

RESULTS

The Human Argininosuccinate Synthetase Promoter is Activated by the SV40 Enhancer

To determine the effect of an enhancer on the topology of a transcription complex, we have used a plasmids which feature the argininosuccinate synthetase (AS)¹ promoter. The AS

¹ The abbreviations used are: AS, argininosuccinate synthetase; ATA, aurintricarboxylic acid; bp, base pairs; CAT, chloramphenicol acetyltransferase; D-PBS, Dulbecco's phosphate buffered saline; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; kb, kilobase pair; PMSF, phenylmethylsulfonyl fluoride; PTC, plasmid transcription complex; VTC, SV40 viral transcription complex.

promoter is subject to enhancement by the SV40 enhancer (25). The plasmids used in these studies are shown schematically in Figure 1. The parent plasmid, pmgAS6, contains the human argininosuccinate synthetase (AS) promoter driving expression of the CAT gene. Into this plasmid was cloned the 'core' SV40 origin of replication creating pORAS6, and the SV40 enhancer was cloned into pORAS6 creating pORAS7. The AS promoter was used instead of the SV40 early promoter to avoid repression by T-antigen when expressed in COS7 cells. Plasmids were introduced by electroporation into COS7 cells, and two days later, CAT activity and plasmid DNA copy number were determined.

The results of one such experiment are shown in Figure 2. Here it can be seen that there is a substantially greater amount of CAT activity produced by transfection with pORAS7 (Figure 2A, lane d) than with pORAS6 (lane c), even though similar amounts of plasmid DNA are present in the cells (Figure 2B, lanes c and d). This indicates that the presence of the enhancer results in greater expression from the AS promoter. This is confirmed by the quantitative results of CAT activity normalized to DNA copy number presented in Table I. The SV40 enhancer produces a 38-fold increase in expression when the CAT gene is driven by the AS promoter and 15-fold when driven by the SV40 early promoter. Expression from pSV2CAT was one order of magnitude less than that from pORAS7, perhaps due to the autorepression effect of T-antigen in COS7 cells.

The In Vitro Transcription Assay Reflects the Enhancer Effect

The topology of the DNA of a viral transcription complex in a eukaryotic cell can be determined by agarose gel electrophoresis of the purified RNA-DNA hybrid of the transcription complex (16). A recent version of this procedure involves the analysis of the ternary transcription complex (15,17,28,29). The ternary complex is prepared from the chromatin-organized transcription complex by treatment with sarkosyl, heparin, or aurintricarboxylic acid (ATA)¹. These various agents remove the histones and most of the chromosomal proteins but leave nascent RNA and engaged RNA polymerase II in a form that allows transcriptional elongation *in vitro*. One advantage of

analysis of the ternary complex is that the topology of transcription complex DNA can be assessed due to the fact that individual topoisomers can be resolved (15,28,29). This in turn allows detection of changes in the linking number of transcription DNA of the equivalent of less than 0.5 topological turns (15).

We have employed a modification of this methodology to examine the topology of the PTC. COS7 cells were transfected by electroporation, and two days later cells were harvested and nuclear extract was prepared. Extracts containing PTCs were added to an *in vitro* transcription mix, and *in vivo*-initiated complexes were allowed to extend in the presence of [³²P]UTP. Also included in the reaction were heparin or ATA, agents which inhibit topoisomerase (not shown) and remove histones from the transcription complex to yield the ternary complex. The resulting ternary complexes were analyzed by agarose gel electrophoresis.

Shown in Figure 3 is a representative autoradiograph of a dried agarose gel. In this gel system, the ternary complex as well as bare plasmid DNA exhibit increasing mobility with an increasing level of negative supercoiling. The ternary complex of pORAS7

Figure 1. Diagram of plasmid constructs. Plasmid pmgAS6 was used to prepare pORAS6 and pORAS7 (see details in Experimental Procedures). Solid boxes represent exons from the human AS gene. The transcription initiation site is indicated by the arrow. A fragment containing the SV40 'core' origin of replication was cloned into the Nde I site of pmgAS6 to produce pORAS6. A fragment containing SV40 enhancer sequences was cloned into the Bam HI site of pORAS6 to produce pORAS7. The figure is not drawn to scale. B, Bam HI; E, Eco RI; H, Hind III; N, Nde I.

Figure 2. DNA copy number and CAT expression of transfected DNA in COS7 cells. COS7 cells were transfected using the electroporation protocol. Two days after transfection, Hirt supernatants (A) or cellular extracts for CAT assays (B) were prepared. (A) Southern blot analysis of Dpn I/Hind III treated Hirt supernatants from transfected COS7 cells. Transfer membranes were hybridized to a Hind III linearized ³²P-labeled pORAS7 probe. Lane a, pA10CAT; lane b, pSV2CAT; lane c, pORAS6; and lane d, pORAS7. Numbers on the left indicate fragment size in kb. Virtually all of the DNA present at this time has been replicated, as indicated by the absence of the Dpn I digestion products (2 kb). (B) CAT Assay. Samples were spotted onto TLC plates at the origin (O) and subjected to ascending chromatography. The mobilities of [¹⁴C] chloramphenicol (C) and the acetylated forms of [¹⁴C] chloramphenicol (AC) are indicated. Lanes a-d in B correspond those in A.

(marked SC at the right) has lower mobility than that of the bare supercoiled pORAS7 DNA (marked with the arrow). This reduced mobility, or 'bandshift', is similar to that observed for the SV40 ternary complex (15,28), and is most likely due to the presence of RNA polymerase II and nascent RNA on the DNA template. These results demonstrate that a supercoiled ternary complex of the PTC can be visualized using procedures similar to those used to visualize the ternary complex of the SV40 viral transcription complex (VTC) in its various topological forms.

In addition to being able to assess the topology of this complex, this system of analysis can also be used to demonstrate transcriptional enhancement (Table 2). COS7 cells were transfected with either pORAS6 (enhancer minus) or with pORAS7 (enhancer plus) and the amount of radioactivity in the ternary complex in the agarose gel was determined for the two

Figure 3. Agarose gel electrophoresis of the Plasmid Transcription Complex. A sample of nuclear extract from COS7 cells transfected with pORAS7 was used for extension *in vitro* in a transcription reaction containing ATA and [32 P]UTP. The plasmid transcription complex (PTC) was visualized by agarose gel electrophoresis and autoradiography. Lane a, 14 C-labeled SV40 DNA shown as a mobility marker. The mobilities of the nicked (N) and supercoiled (SC) forms are indicated at the left. Lane b, *in vitro* extension products from pORAS7 nuclear extract. At the right are indicated the mobilities of the nicked (N) and supercoiled (SC) forms of the pORAS7 PTC, while the arrow indicates the mobility of bare supercoiled pORAS7 DNA determined from the ethidium-bromide stained gel.

plasmids. The data in Table 2 show that the presence of the enhancer produced an average of 17-fold more transcriptional activity in the ternary complex. This degree of enhancement *in vitro* by the SV40 enhancer is comparable to the 38-fold stimulation of CAT activity produced *in vivo* (see Table 1). Therefore, the *in vitro* RNA extension assay reflects the strong activation of transcription displayed by enhancers.

The Effect of Topoisomerase on the Topology and Activity of the PTC

As discussed previously, different lines of evidence suggest that totally unconstrained supercoiling is a property of genes transcribed at a high rate. If an enhancer-activated PTC possesses totally unconstrained supercoils, incubation of the PTC with topoisomerase I should relax this tension by causing a change in the linking number, and this in turn should result in the ternary complex migrating at the nicked/relaxed mobility during agarose gel electrophoresis.

Such was not the case for pORAS7 (Figure 4). Portions of nuclear extract from pORAS7-transfected cells were incubated either with or without added wheat germ topoisomerase I. After the incubation period, both samples were extended and the resulting labeled PTCs were analyzed by agarose gel electrophoresis. Lanes b and c correspond to pORAS7 nuclear extract incubated in the absence or presence of added topoisomerase, respectively. If the PTC contained quantitatively relaxable supercoiling, the sample treated with topoisomerase would be expected to migrate at nicked/relaxed mobility (marked N for the PTC) in Figure 4. The results clearly demonstrate that topoisomerase treatment had no detectable effect on the mobility or activity of the PTC. In contrast, addition of 1 μ g of supercoiled bare pORAS7 DNA to nuclear extract containing topoisomerase resulted in the complete relaxation of the added DNA (data not shown). Furthermore, the intensity of the PTC band in lane b is about the same as that in lane c, indicating that the topoisomerase had little effect on transcription activity. These results show that the enhancer-containing PTC does not possess totally unconstrained supercoiling.

An additional control was performed to ensure that topoisomerase would be able to relax unconstrained supercoiling if it were present in the PTC (Figure 5). Unconstrained positive supercoiling was introduced into the PTC by the addition of chloroquine to the nuclear extract (15), after which it was determined if topoisomerase would change the linking number of the PTC. To detect changes in the linking number of the PTC,

Table 1. Normalization of CAT Activity as a function of DNA Copy Number

Plasmid	14 C-acetylated chloramphenicol (cpm) ^a		32 P in Hirt DNA (cpm) ^a		Normalized CAT Activity 14 C/ 32 P		Fold Enhancement
	A ^b	B ^b	A	B	A	B	
pA10CAT	15,663	4,683	10,788	9,837	1.5	0.5	15 $\pm$ 9 ^c
pSV2CAT	34,542	18,740	3,699	1,629	9.3	11.5	
pORAS6	19,155	34,799	5,627	8,627	3.4	4.0	38 $\pm$ 11
pORAS7	763,606	732,386	4,570	6,667	167	110	

^aRadioactivity was quantitated as described in the legend to Figure 2.

^bA and B refer to the results of two different experiments.

^cThis value was determined by dividing the normalized CAT activity of the enhancer-containing plasmid by that of the non-enhancer-containing plasmid. Shown is the average value for experiments A and B.

the topology of the ternary complexes was analyzed using agarose gel electrophoresis in the presence of a low level of chloroquine. If topoisomerase is able to act on the PTC, then such treatment should relax the positive supercoiling induced by chloroquine, which would result in an increase in the mobility (increased level of negative supercoiling) of the ternary complex when analyzed in an agarose gel containing 0.75 μ M chloroquine. Figure 5 shows densitometer tracings of lanes of the autoradiograph from such an experiment. The tracings for the sample of nuclear extract incubated without topoisomerase (Figure 5, curve A) and with topoisomerase (curve B) each reveal a broad peak composed of a series of small peaks. These small peaks represent the individual negatively supercoiled topoisomers of the PTC, now distinguishable because of the presence of chloroquine in the gel. Electrophoresis is from right to left and higher mobility (i.e. toward the left) corresponds to higher negative supercoiling. The center of the distribution of these small peaks represents the mean linking number of the PTC. The fact that the center of the distribution of the sample incubated without topoisomerase (curve A) coincides with that of the sample incubated with topoisomerase (curve B) indicates that the added topoisomerase had caused no relaxation of supercoils, demonstrating that these supercoils are

Figure 4. Topoisomerase treatment of nuclear extract does not cause relaxation of the PTC. COS7 cells were transfected with pORAS7, and two days after transfection nuclear extract was prepared. Samples of nuclear extract were treated with or without wheat germ topoisomerase I, after which samples were analyzed as in Figure 3. Lane a, 14 C-labeled SV40 DNA. Lanes b and c, *in vitro* extension products of extracts which were treated with (+) or without (–) topoisomerase.

constrained. Curve C demonstrates that the topoisomerase can in fact detect unconstrained tension in the PTC, because incubation of nuclear extract with topoisomerase and chloroquine caused the PTC to become much more highly supercoiled, i.e. it migrates with higher mobility on the gel. This observation that the topoisomerase incubation can relax unconstrained tension in the PTC was confirmed by the finding that a temperature shift in the presence of topoisomerase (15) also causes the expected change in PTC topology (not shown). To summarize, the lack of relaxation of the PTC coupled with the demonstration that unconstrained PTC supercoils can be relaxed show that the enhanced PTC contains constrained negative supercoils.

The approximate level of negative supercoiling can also be estimated from the data in Figure 5 because a rough estimate of the supercoil density of a DNA molecular can be determined from the concentration of chloroquine required to cause the topoisomers to migrate as a well-separated, symmetrical distribution of bands. The PTC is represented by a relatively symmetrical distribution of resolved topoisomers in this gel (curves A and B) which contains 0.75 μ M chloroquine. Analysis of the SV40 VTC population (C. Nicita and L. Lutter, in preparation) has demonstrated that VTC topoisomers also migrate as a symmetrical and well-separated distribution of bands when analyzed in the presence of 0.75 μ M chloroquine; however, analysis of the SV40 VTC in the presence of 0.2 μ M or 1.5 μ M chloroquine results in the VTC topoisomers being compressed in asymmetric distributions at the supercoiled or the relaxed ends of the fractionation range, respectively. Thus, in a gel containing 0.75 μ M chloroquine, both the PTC (Figure 5, curves A and B) and SV40 VTC (C. Nicita and L. Lutter, in preparation) show similar well-separated topoisomer distributions, not distributions compressed toward the highly supercoiled or relaxed ends of the fractionation range; this indicates that the PTC contains constrained negative supercoils in an amount similar to that found in the SV40 VTC. The SV40 VTC in turn contains a supercoil density which is similar to that of bulk minichromosome DNA (C. Nicita and L. Lutter, in preparation), or $\sigma \approx -0.05$ (30).

One final feature of the procedure is illustrated by curve D in Figure 5. Here a sample of nuclear extract was incubated with 0.1 mg/ml chloroquine but in the absence of added exogenous topoisomerase. The ternary complex here comigrates with that in curve c, demonstrating that a chloroquine-induced linking number change has occurred in the PTC even in the absence of added topoisomerase. Although a previous study reported that the endogenous topoisomerase is not active on SV40 minichromosomes (31), the result in curve D indicates that our nuclear extract contains an endogenous topoisomerase which is

Table 2. PTC Activities of pORAS6 and pORAS7

Plasmid	PTC ^a (cpm)		DNA ^b (cpm)		Normalized Transcription Activity ($\times 10^3$) ^c		Average	Relative Activation ^d
	A ^e	B	A	B	A	B		
pORAS6	12,742	391	3,110,687	106,981	4.10	3.65	3.9 $\pm$ 0.2	1.00 $\pm$ 0.05
pORAS7	15,129	3,131	249,483	42,182	60.64	74.23	67 $\pm$ 7	17 $\pm$ 3

^aPTC activity was determined by scintillation counting of the areas from dried gels containing the 32 P-labeled PTC.

^bThe amount of DNA was determined by scintillation counting of the amount of a 32 P-labeled pORAS6 probe hybridized to a sample of nuclear extract after transfer onto GeneScreen Plus membranes.

^cNormalized Transcription Activity = (PTC c.p.m.)/DNA c.p.m.)

^dRelative Activation of each plasmid was determined relative to that of the wild type human AS promoter in pORAS6.

^eA and B are the results of two different experiments.

Figure 5. Agarose gel electrophoresis in the presence of chloroquine. Samples of nuclear extract from pORAS7-transfected cells were treated with wheat germ topoisomerase I and 0.1 mg/ml chloroquine as indicated. *In vitro* RNA extension was performed as in Figure 4, except that ATA was replaced by heparin. The resulting ternary complexes were then analyzed by agarose gel electrophoresis in the presence of 0.75 μ M chloroquine followed by processing as in Figure 4. At this low chloroquine concentration all topoisomers are still negatively supercoiled. Shown are densitometer scans of the lanes of the autoradiograph, with electrophoresis from right to left. For clarity, scans are displaced vertically, and scan C is broken at its crossover point with scan D. A higher level of negative supercoiling results in higher mobility, i.e. more negatively supercoiled topoisomers migrate further to the left. Resolved individual topoisomers of the PTC (in C and D) are indicated by the inverted arrowheads at the top of the Figure. A., sample which was incubated without added topoisomerase; B., sample which was incubated with added topoisomerase; C., sample which was incubated with added topoisomerase in the presence of 0.1 mg/ml chloroquine. The peak of this chloroquine-incubated sample is narrower (more compressed) than those in A or B because it is running at the position of maximum supercoiling for this gel, i.e. its component topoisomers are not resolved; D., sample which was incubated with no added topoisomerase in the presence of 0.1 mg/ml chloroquine.

able to act on the PTC to relax unconstrained tension. This in turn means that our assay cannot detect unconstrained supercoiling which may have been present in addition to the constrained supercoiling we observe. Thus it remains possible that unconstrained supercoiling may be present in addition to the constrained supercoiling when the complex is in the cell.

DISCUSSION

The goal of this study was to test the possibility that totally unconstrained supercoiling is a characteristic property of genes that are very actively transcribed, such as enhancer-activated RNA polymerase II-transcribed genes. Our investigation was prompted by two observations: 1) a high rate of transcription of the *Xenopus* 5S rRNA gene has been correlated with the presence of totally unconstrained supercoiling ('dynamic' chromatin), and 2) supercoiling stimulates the expression of transfected plasmids, particularly those which contain an enhancer. The proposed role of supercoiling in eukaryotic transcription is consistent with a large body of evidence demonstrating that supercoiling has a dramatic effect on transcription in prokaryotes.

Our studies show that an enhancer-activated PTC is highly supercoiled, but the supercoils are constrained. This conclusion is based on the finding that the supercoiled state of the enhanced PTC was not significantly affected by treatment with

topoisomerase I *in vitro*. Control experiments involving incubation and analysis in the presence of chloroquine demonstrated that the absence of relaxation of the PTC was neither a result of inactivation of the topoisomerase nor an inability of our experimental protocol to detect substantial amounts of unconstrained supercoiling in the PTC. These experiments also indicated that the PTC is supercoiled to an extent similar to that of the SV40 VTC and minichromosome. Based on these results, we conclude that the enhancer-activated PTC has constrained topological tension similar to bulk chromatin. However, while we found no evidence *in vitro* for substantial unconstrained supercoiling, we cannot rule out the possibility that the PTC had unconstrained supercoiling *in vivo* in addition to the constrained supercoiling we observed *in vitro*, and this extra tension was relaxed during preparation of the nuclear extract. The demonstrated topoisomerase activity in our nuclear extract (Figure 5, trace D) would preclude maintenance of unconstrained supercoiling during preparation. Furthermore, our studies do not address the possible role of supercoiling in chromatin assembly (32,33).

The results presented here are similar to those observed with SV40 late transcription complexes (15,16), but differ significantly from studies using the 5S rRNA gene (11). In the latter case, high transcription rate correlated with the presence of totally unconstrained supercoiling. This difference might be explained on the basis that one study examined an RNA polymerase III-transcribed gene (5S rRNA gene) while the others analyzed an RNA polymerase II-transcribed gene (SV40 late gene and pORAS7). Alternatively, since we do not know the absolute rate of transcription of pORAS7, it is possible that the absolute transcription rate of the 5S rRNA gene in oocytes is considerably greater than that of the enhancer-activated PTC, and that totally unconstrained supercoiling in RNA polymerase II-transcribed genes can only be observed when transcription rates approach those of RNA polymerase III-transcribed genes. However, it should be pointed out that an enhancer-activated immunoglobulin gene is transcribed at a rate similar to that of the 5S rRNA gene *in vivo* (34). Nevertheless, it is clear that RNA polymerase II transcription, whether or not it is enhancer-activated, does not require the presence of totally unconstrained supercoiling.

The methodology described here offers several advantages over other methods used to analyze the structure of active chromatin. First, transcription is occurring from DNA templates that were replicated and assembled *in vivo*. Thus, the structure of the PTC is likely to closely resemble that of native cellular chromatin. Second, instead of correlating the rate of transcription with topology of bulk chromatin, this method examines the topology of the actual structure that is producing the mRNA transcript. This is an important distinction, since most of the bulk chromatin (cellular, viral, or plasmid) is believed to be inactive (10,35). Thus when using other methods, it is not certain that the properties of the transcriptionally active fraction are the same as the bulk (inactive) fraction, and conclusions drawn from such indirect studies are open to interpretation. The methodology employed here avoids this uncertainty by characterizing on the transcribing molecules.

ACKNOWLEDGEMENTS

This work was supported by grants from the American Cancer Society (MV-185, to L.C.L.), National Institutes of Health (CA51748, to S.O.F.), and Michigan Minority Fellowship (to P.J.B.).

REFERENCES

1. van Holde, K.E. (1989). Springer Series in Molecular Biology: Chromatin, Springer-Verlag New York Inc., Secaucus, New Jersey, USA; Berlin, West Germany.
2. Reeves, R. (1984) *Biochim. Biophys. Acta* 782, 343–393.
3. Gross, D.S. and Garrard, W.T. (1988) *Annu. Rev. Biochem.* 57, 159–197.
4. Eissenberg, J.C., Cartwright, I.L., Thomas, G.H. and Elgin, S.C. (1985) *Annu. Rev. Genet.* 19, 485–536.
5. Fisher, L.M. (1984) *Nature* 307, 686–687.
6. Wang, J.C. (1985) *Annu. Rev. Biochem.* 54, 665–697.
7. North, G. (1985) *Nature* 316, 394–395.
8. Futcher, B. (1988) *Trends. Genet.* 4, 271–272.
9. Sinden, R.R., Carlson, J.O. and Pettijohn, D.E. (1980) *Cell* 21, 773–783.
10. Lewin, B. (1980). *Gene Expression 2: Eucaryotic Chromosomes*, pp. 694–727, Wiley, New York.
11. Ryoji, M. and Worcel, A. (1984) *Cell* 37, 21–32.
12. Luchnik, A.N., Bakayev, V.V., Zbarsky, I.B. and Georgiev, G.P. (1982) *EMBO. J.* 1, 1353–1358.
13. Luchnik, A.N., Bakayev, V.V., Yugai, A.A., Zbarsky, I.B. and Georgiev, G.P. (1985) *Nucleic. Acids. Res.* 13, 1135–1149.
14. Hadlock, K.G., Quasney, M.W. and Lutter, L.C. (1987) *J. Biol. Chem.* 262, 15527–15537.
15. Lutter, L.C. (1989) *Proc. Natl. Acad. Sci. USA* 86, 8712–8716.
16. Petryniak, B. and Lutter, L.C. (1987) *Cell* 48, 289–295.
17. Choder, M. and Aloni, Y. (1988) *Nucleic. Acids. Res.* 16, 895–905.
18. Weintraub, H., Cheng, P.F. and Conrad, K. (1986) *Cell* 46, 115–122.
19. Shaw, P.E., Bohmann, D. and Sergeant, A. (1985) *EMBO J.* 4, 3247–3252.
20. Ernoul-Lange, M., Omilli, F., O'Reilly, D.R. and May, E. (1987) *J. Virol.* 61, 167–176.
21. Hartzell, S.W., Byrne, B.J. and Subramanian, K.N. (1984) *Proc. Natl. Acad. Sci. U. S. A.* 81, 23–27.
22. Fromm, M. and Berg, P. (1982) *J. Mol. Appl. Genet.* 1, 457–481.
23. Gardiner-Garden, M. and Frommer, M. (1987) *J. Mol. Biol.* 196, 261–282.
24. Potter, H., Weir, L. and Leder, P. (1984) *Proc. Natl. Acad. Sci. U. S. A.* 81, 7161–7165.
25. Boyce, F.M., Anderson, G.M., Rusk, C.D. and Freytag, S.O. (1986) *Mol. Cell Biol.* 6, 1244–1252.
26. Gorman, C.M., Moffat, L.F. and Howard, B.H. (1982) *Mol. Cell Biol.* 2, 1044–1051.
27. Hirt, B. (1967) *J. Mol. Biol.* 26, 365–369.
28. Hadlock, K.G. and Lutter, L.C. (1990) *J. Mol. Biol.* 215, 53–65.
29. Choder, M., Bratosin, S. and Aloni, Y. (1984) *EMBO. J.* 3, 2929–2936.
30. Keller, W. (1975) *Proc. Natl. Acad. Sci. U. S. A.* 72, 4876–4880.
31. Ambrose, C., McLaughlin, R. and Bina, M. (1987) *Nucl. Acids Res.* 15, 3703–3721.
32. Tabuchi, H. and Hirose, S. (1988) *J. Biol. Chem.* 263, 15282–15287.
33. Weintraub, H. (1988) *Proc. Natl. Acad. Sci. U. S. A.* 85, 5819–5823.
34. Schibler, U., Marcu, K.B. and Perry, R.P. (1978) *Cell* 15, 1495–1509.
35. Llopis, R., Perrin, F., Bellard, F. and Gariglio, P. (1981) *J. Virol.* 38, 82–90.