Volume 16 Number 3 1988 Nucleic Acids Research

In vitro transcribed SV40 minichromosomes, as the bulk minichromosomes, have a low level of unconstrained negative supercoils

Mordechai Choder and Yosef Aloni

Department of Genetics, Weizmann Institute of Science, Rehovot 76100, Israel

Received October 22, 1987; Revised and Accepted December 29, 1987

ABSTRACT

The method for detecting the small fraction (1%) of transcriptionally active SV40 minichromosomes, in the presence of the bulk minichromosomes, (14) has been applied to directly analyze the topology of transcribed and non-transcribed minichromosomal DNA. We show here that DNA of both transcribed and non-transcribed minichromosomes have the same number of supercoils which are constrained by nucleosomes. In addition, minichromosomal DNA of both fractions have a low level of unconstrained supercoils (1-2 extra supercoils) which can be relaxed in vitro by topoisomerase I.

INTRODUCTION

The number of revolutions one DNA strand makes about its complement is defined as the "linking number". In closed circular DNA molecules, this number remains constant independent of the environmental conditions. In order to change the linking number of a circular molecule, nicking and closing activities are necessary. The linking number is a mathematical sum of two topological parameters: the helical winding (twist) and the axial winding (supercoiling), of which the former is dependent on environmental conditions (1). In pure circular DNA molecules there is a free equilibrium between the twist and the supercoiling which varies due to response of the twist to changes in environmental conditions. When pure circular DNA molecules, having different linking number (topoisomers), are present in the same environmental conditions (as is the case in gel electrophoresis), they differ only in their supercoiling. Since the electrophoretic mobility of a circular DNA is affected by its superhelicity, different topoisomers have different migration rates in gel electrophoresis. A difference of one supercoil can be detected in gel electrophoresis if the average supercoiling is low enough (2).

In eukaryotes the DNA is complexed with histones to form the well characterized chromatin. It was suggested that, in general, the association with proteins fixes the negative supercoils and leaves no net torsional strain (i.e. DNA supercoils cannot be relaxed by nicking and closing activities). This

conclusion was reached applying two approaches: (i) measurements of psoralen intercalation that is dependent on torsional strain (3), and (ii) measurements of the decrease in linking number as a result of chromatin reconstitution using relaxed circular DNA, histones and topoisomerase I. The latter experiment demonstrated that most, if not all, of the negative supercoiling can be accounted for by binding of the four core nucleosomal histones (4).

Although, in general, the supercoils of the eukaryotic DNA are constrained by the nucleosomal histones, suggestions have been made for involvement of DNA topology in transcription in vivo (5-8). Two main possibilities which are not mutually exclusive can be envisaged: (i) a small portion of the eukaryotic chromosomal DNA is topologically strained so that it is completely relaxable, and (ii) chromosomal DNA, or part of it, is slightly strained having, in addition to the constrained supercoils, an additional level of extra supercoils.

There are reports on the existence of a small fraction (suggested to be the transcriptionally active) in which the DNA is capable of being completely relaxed by nicking and closing activities. Two deals with SV40 minichromosomes (9, 10) and the other with chromatin of 5S ribosomal RNA genes (11). The functional significance of this unconstrained supercoiling has been reviewed (5-8). In contrast to these results, very recently it has been shown that the transcriptionally active SV40 minichromosomal DNA is completely constrained by nucleosomes and does not contain a topoisomerase I relaxable portion (12). The complete absence of topological strain found by these authors is not consistent with previous results, suggesting involvement of DNA topology in regulation of gene expression (5-8). Barsum and Berg (13) and more recently Ambrose et al (10) have reported the existence of a low level of unconstrained negative supercoils in the bulk of the SV40 minichromosomes. In these studies the authors have not directly analyzed the topology of transcriptionally active SV40 minichromosomes.

Using our method that allows a direct structural and topological analyses of the small transcriptionally active fraction of SV40 minichromosomes (14), we show here that, under physiological conditions, the bulk and the transcriptionally active minichromosomes have the same number of constrained supercoils and a low level of unconstrained supercoils (1-2 extra supercoils).

MATERIALS AND METHODS

CV1 cells were grown, infected with 776-SV40 virus and labeled with (^{3}H)methyl thymidine as described (15). Minichromosomes were extracted from nuclei of SV40 infected cells at 42-48 hrs post infection. Two extraction procedures were used:

(i) procedure A: extraction with hypotonic buffer at 28°C was carried out as detailed previously (14). Procedure B: Triton X-100 extraction in the presence of 0.36M NaCl at 4°C was performed as described (13). This salt concentration is known to remove the endogenous topoisomerases' activities but not histones (13-16). Nuclear extracts were loaded on 5-30% (w/v) sucrose gradient containing buffers as described in the Figure Legends. Centrifugation was carried out in a Beckman SW56 rotor at 50000 r.p.m. for 40 min (procedure A) or for 80 min (procedure B), at 2°C. Fractions containing the minichromosomes (~75S in procedure A, ~60S in procedure B) were pooled.

Viral transcriptional complexes (VTC) were obtained by deproteinization of minichromosome by Sarkosyl followed by sedimentation through a sucrose gradient as detailed (14). The 23S fractions containing the VTC peak were pooled. For in vitro RNA transcription elongation partially purified minichromosomes or VTC were adjusted to 2mM $MnCl_2$, 0.15M $(NH_4)_2$ SO_4, 0.3mM each of ATP, CTP and GTP, 1μM of (α-^{32}P)UTP (400 Ci/mmole, Amersham) and 1% Sarkosyl or 0.5mg/ml heparin as indicated in the Figure Legends. Incubations were carried out at 30°C for 15 min and terminated by addition of EDTA to 15mM.

DNA topoisomers and topoisomers of ternary complexes were analyzed on a vertical (17 x 15 x 0.3 cm) agarose gel in 50mM Tris phosphate pH 7.2 containing chloroquine phosphate (Teva, Israel) (2) in concentrations as indicated in the Figure Legends. Electrophoresis was at room temperature (20-24°C) at 2-4 v/cm. The buffer was continuously recirculated between the compartments at a rate of 2.75 ml/minute. The gel was stained with 1mg/ml Et. Br. in electrophoresis buffer (without chloroquine) for 1 hr and UV irradiated at 254nm to nick the DNA in order to avoid biased staining of closed circular DNA having different superhelical densities (2). The gel was further stained with 0.5mg/ml Et. Br. in electrophoresis buffer for several hrs, destained in electrophoresis buffer for 0.5-1 hr and photographed over UV light using 665 Polariod film (positive negative) or 667 Polariod film. The gel was then dried under vacuum at room temperature or, as indicated, fluorographed (17) using ethanol as the solvent and exposed to x-ray films (Kodak XAR-5). Spectrometric scanning of the negatives of the 665 Polaroid films and the autoradiograms were done using Beckman DU-8 spectrophotometer at 500 nm.

RESULTS

Analysis of transcribed and non-transcribed SV40 molecules by gel electrophoresis

We have previously developed a method for detecting the small fraction of

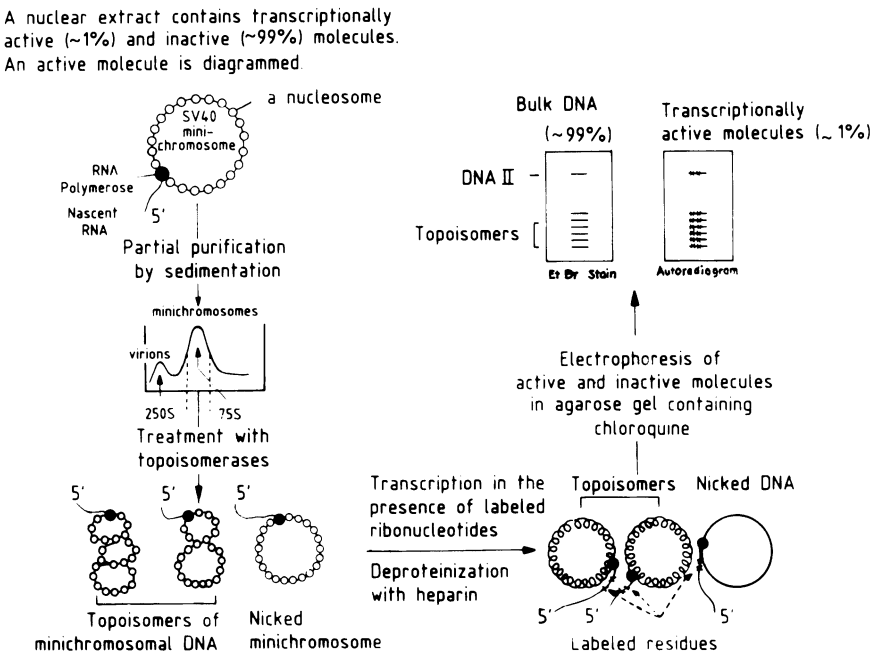

Figure 1: General experimental design for analyzing topological state of circular DNA molecules originated from both transcriptionally active and transcriptionally inactive SV40 minichromosomes (see text for details).

SV40 minichromosomes active in transcription (about 1%) and for analyzing their chromatin structure (14). The present approach for analyzing the topology of the actively transcribed minichromosomes is based on this method, with some variations, as illustrated in Figure 1: SV40 minichromosomes are leached out of nuclei of infected cells using either detergentless hypotonic buffer (14) or Triton X-100 containing hypertonic buffer (13). Minichromosomes are partially purified by sedimentation through a sucrose gradient and are treated with topoisomerases. The topoisomerases treated minichromosomes are then incubated in transcription mixture containing either Sarkosyl or heparin and labeled ribonucleoside triphosphate. Addition of Sarkosyl results in inactivation of topoisomerases and in removal of most of the minichromosomal proteins but not of the RNA polymerase II. Under these conditions, RNA polymerase II remains active in transcription elongation, but not initiation (14, 18-20). The in vivo preinitiated nascent RNA chains are elongated, in this step, and become labeled. Heparin has similar effects on DNA proteins interactions and on inactivation of topoisomerases as Sarkosyl but leads to a better resolution of the topoisomers on chloroquine containing agarose gel (unpublished observation).

The mixture containing ternary complexes of RNA, DNA and RNA polymerase II (RNA-DNA-polymerase), originating from the in vivo transcriptionally active minichromosomes, and the naked DNA, originating from the bulk transcriptionally inactive minichromosomes is then fractionated by electrophoresis in agarose gel, or agarose gel containing chloroquine which allows the resolution of topoisomers (2). The bulk DNA is identified by staining the gel with ethidium bromide (Et. Br.) and the ternary complexes originating from the transcriptionally active molecules by exposing the gel to an X-ray film. It is possible to detect distinct topoisomers of RNA-DNA-polymerase II complexes because the length of the associated nascent RNA does not affect the electrophoretic mobility of the complexes. Moreover, we have shown that during in vitro transcription of supercoiled SV40 ternary complexes the elongated nascent RNA is actively displaced from the template leaving only about 20 nt at the 3' end of the elongated RNA hydrogen bonded to the template strand in the transcription bubble. As a result, a constant region (within $\pm$ 1 bp) remains unwound during transcription elongation (Choder and Aloni, unpublished). This, and the observation that the topoisomer bands remain in phase with the Et.Br. stained bands (see below) exclude the possibility of D-loop formation between the template and transcript which may also alter the topology of the template.

Transcribed and non-transcribed SV40 minichromosomal DNA do not have a high level of relaxable supercoils which can be detected by electrophoresis in regular agarose gel

To investigate the topological dynamics of the transcriptionally active SV40 minichromosomes we examined whether this fraction can be relaxed in vitro by the endogenous topoisomerases or by exogenously added topoisomerase I. SV40 minichromosomes were leached out of nuclei of infected cells and partially purified by sedimentation through a sucrose gradient. The minichromosomes were then mixed with naked supercoiled plasmid DNA, that served as an internal control for ensuring relaxation activity. The relaxation of the two DNAs by both the endogenous topoisomerases and the exogenously added topoisomerase I was analyzed on a regular agarose gel.

Figure 2 shows that DNA originating from both the bulk SV40 minichromosomes, identified by ethidium bromide staining (Et. Br. stain) (Figure 2a), and from the transcriptionally active fraction, identified by autoradiography (Figure 2b), show no detectable relaxation by the endogenous topoisomerases (Type I and Type II - see 21; 22) (Figure 2, lanes 2), or by the addition of topoisomerase I (Figure 2, lanes 3). Since histone H1 was suggested to be involved in the repression of transcriptionally active chromatin (8), we

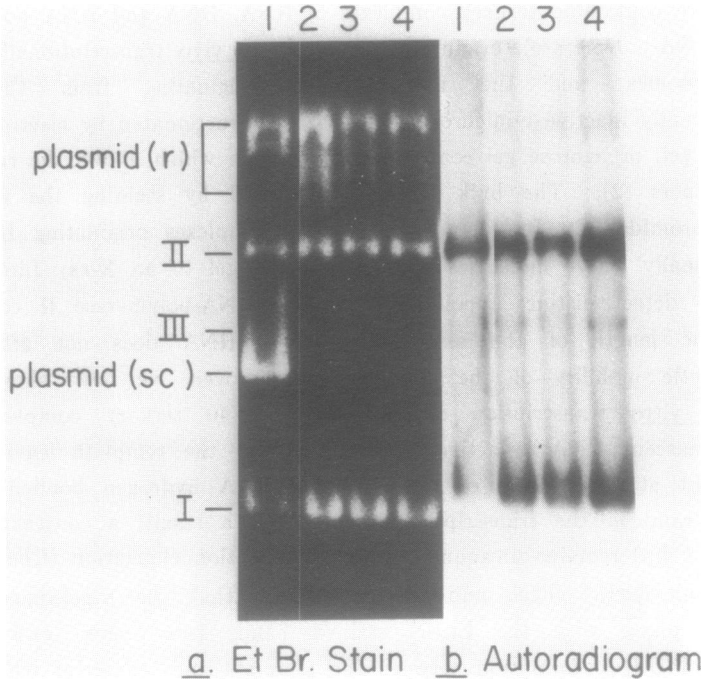

Figure 2: Electrophoretic mobility, in agarose gel, of viral DNA and VTC extracted from minichromosomes which had been treated with nicking closing activities.

For outline of the experiment follow Figure 1. A nuclear extract of SV40 infected cells was prepared by the hypotonic buffer procedure (see text). SV40 minichromosomes were partially purified from the extract by sedimentation through a sucrose gradient containing 50 mM Tris-HCl pH 7.9, 0.1 M NaCl, 1 mM $MgCl_2$, 1 mM DTT (lanes 1-3) or through a similar gradient as above but containing 0.6 M NaCl (lanes 4). The fractions containing the minichromosomes were pooled. In lane 4 the pooled fractions were dialysed against 50 mM Tris HCl pH 7.9, 0.1M NaCl,1 mM $MgCl_2$ 15% sucrose. The minichromosomes were mixed with naked supercoiled plasmid DNA of 7400 bp's and were adjusted in 20μl to 5 mM $MgCl_2$. In lanes 1 Sarkosyl was added to 1% to prevent the endogenous nicking closing activity and it serves as a control. In lanes 2 the endogenous nicking closing activity is measured. In lanes 3 and 4, 10 units of calf thymus topoisomerase I (BRL) were added. The four fractions were incubated at 30°C for 1 hr. Sarkosyl was then adjusted to 1% and RNA was elongated as detailed in the text. Addition of Sarkosyl inactivated the topoisomerases and removed most of the proteins. Samples were electrophoresed in 1.2% agarose gel and the gel was stained, photographed under UV light and exposed to X-ray film. I, II and III depict the position of closed circular form I, nicked circular form II and linear form III of SV40 DNA, respectively. Plasmid(sc) and plasmid(r) depict the position of the supercoiled and relaxed plasmid DNA, respectively.

studied the possibility that during leaching of viral minichromosomes from nuclei of infected cells, molecules of histone H1 are assembled with the active minichromosomes in vitro and thus prevent the possible relaxation of the DNA. For this purpose the minichromosomes were washed with 0.6 M NaCl prior to addition of topoisomerase I to remove H1 from the minichromosomes (23). Again, no detectable relaxation by exogenously added topoisomerase I was observed (Figure 2, lanes 4).

In contrast to the minichromosomal DNA, the naked plasmid DNA was completely relaxed (Figure 2, lanes 2-4), indicating the existence of an efficient nicking-closing activity. Notice that form I DNA of the ternary complexes, originating from the transcriptionally active minichromosomes, migrated slower than form I DNA of the bulk minichromosomes (Figure 2) (see below). These results contradict those of Luchnik et al. (9) and are in agreement with those of Petryniak and Lutter (12).

A low level of unconstrained supercoils can be detected in SV40 minichromosomes by electrophoresis in chloroquine containing agarose gels

In order to examine minor changes in superhelicity which cannot be detected by a regular agarose gel, as shown in Figure 2, we analyzed the minichromosomal DNA in gels containing the intercalator chloroquine. Chloroquine reduces the twist of the DNA and thereby lowers its negative superhelical density. The reduced superhelicity allows the separation of (otherwise) highly negative supercoiled topoisomers (2). Two different concentrations of chloroquine were used, which result either in reduced negative supercoiled molecules (Figure 3a and b), or in positively supercoiled molecules (Figure 3c and d).

Figure 3 (Et. Br. stains) shows that, under physiological salt concentration, topoisomerase I relieved 1-2 supercoils from the bulk of the SV40 minichromosome. This result is in agreement with that of Barsum and Berg (2). To our surprise, the fraction of the actively transcribed minichromosomes, detected in the autoradiograms of Figure 3, behaved no differently from the bulk and was also relaxed by 1-2 supercoils. After removing the proteins by Sarkosyl, the naked DNA, originating from the bulk of the minichromosomes, as well as the (almost) naked transcription complexes, originating from the transcriptionally active minichromosomes, were completely relaxed under the same conditions (Figure 3c).

Notice that, like form I DNA in Figure 2, the topoisomers, originating from the actively transcribed DNA, migrated somewhat slower than those of the bulk

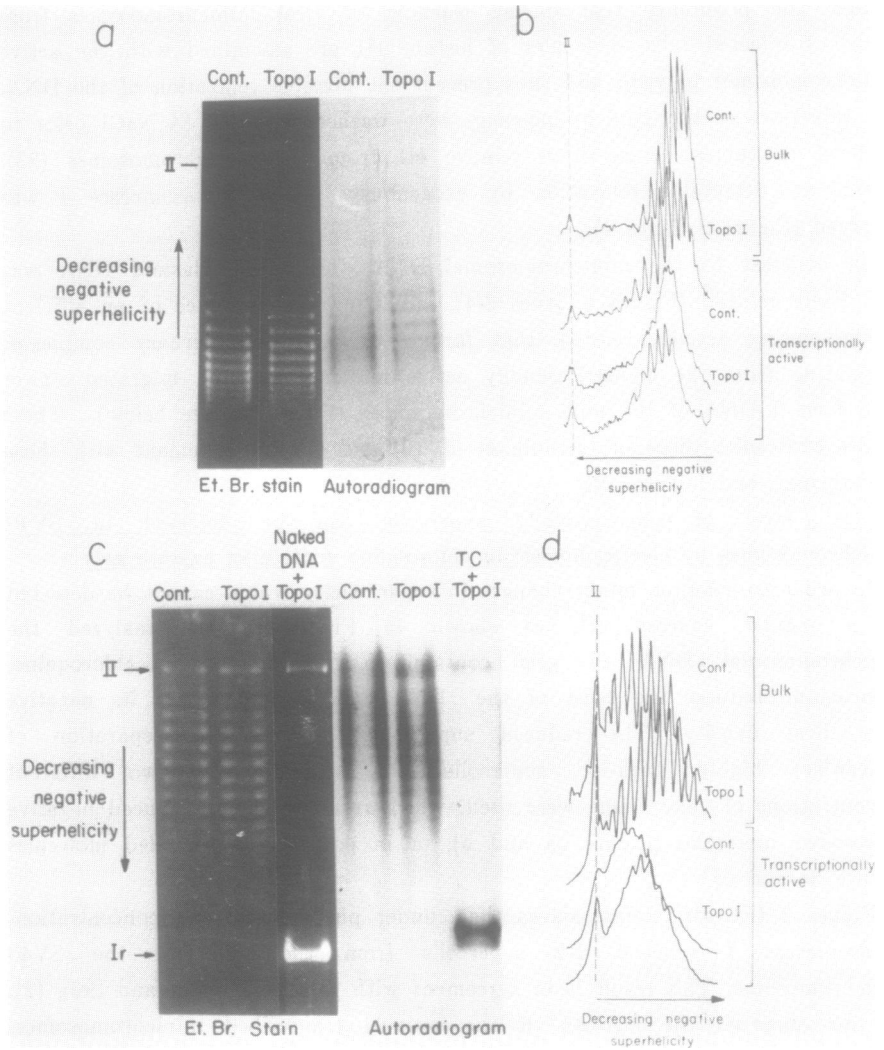

Figure 3 Electrophoretic mobility, in chloroquine containing agarose gels, of viral DNA and VTC extracted from minichromosomes which had been treated with topoisomerase I.

For the outline of the experiment follow Figure 1. SV40 minichromosomes were extracted from the infected cells using procedure B (13). The minichromosomes were partially purified by sedimentation through a sucrose gradient containing 10 mM Hepes-NaOH pH 7.5, 0.36 M NaCl 1 mM DTT. Viral transcription complexes (VTC) were prepared as detailed in the text. Minichromosomes in 25 μl were adjusted to 0.15 M NaCl 2 mM EDTA 10 mM Hepes NaOH pH 7.7, 0.5 mM DTT. Topoisomerase I was added (15 units to minichromosome, 5 units to naked DNA and no enzyme to control). Incubation at 30°C was carried out for 1.5 hrs followed by the addition of heparin to 0.5 mg/ml. Five mM $MgCl_2$ were then added to neutralize the chelating activity

of EDTA. Transcription buffer was added and incubation was allowed to proceed at 30°C for 15 min. Samples were electrophoresed at 2.5 volts/cm in 1.2% agarose gel containing either 2.8 μg/ml (a and b) or 30 μg/ml (c and d) of chloroquine phosphate. In (a) and (c) the gel was stained with 1 μg/ml Et. Br. and photographed under UV light, using 665 Polaroid film (positive negative). The gel was then dried and exposed to x-ray film as in Figure 2. In (b) and (d) the Et. Br. stains (Bulk) and the autoradiograms (transcriptionally active) were scanned. The positions of the nicked molecules (form II DNA) are aligned by the dotted line designated as II. Ir depicts the position of the closed circular relaxed SV40 DNA. The direction of topoisomers having decreasing superhelicity is indicated by arrows. Notice that due to the difference in the chloroquine concentration the direction of migration is inverted in a and b as compared to c and d.

DNA whether they were negatively- (Figure 3a and b), or positively-supercoiled (Figure 3c and d). Since the slower migration of the transcribed topoisomers, as compared to the bulk, occurs in both directions it cannot result from a difference in superhelicity between the transcribed and non-transcribed minichromosomes. The slower migration of the transcribed topoisomers presumably reflects their retardation on the gel due to the presence of the RNA polymerase.

DISCUSSION

We conclude that the transcriptionally active SV40 minichromosomes have the same number of constrained supercoils as the bulk minichromosomes. In addition, both fractions have 1-2 superhelical turns which can be relaxed in vitro by topoisomerase I in the presence of physiological salt concentration. The identity in the number of supercoils is consistent with the notion that transcriptionally active SV40 minichromosomes are organized in nucleosomes, or nucleosome-like structure.

The low level of relaxable supercoils is in contrast to Luchnick et al (9), suggestion of a complete relaxability of the negative supercoils in transcriptionally active SV40 minichromosomes. Using their exact protocol we failed to reproduce this result. We believe that they have used impure topoisomerases. The complete relaxable (termed "dynamic") chromatin observed in 5S rRNA genes (11) may represent a different organization of genes heavily transcribed by RNA polymerase III.

The two negative extra supercoils unconstrained by nucleosomes, were assumed to compensate for the tension that otherwise would have been produced during transcription due to the RNA polymerase that unwinds the DNA by ~2 helical turns (13). Based on this assumption the resultant chromosomal DNA of the actively transcribed molecules should therefore be

relaxed. Our surprising finding that both fractions have the same number of extra supercoils may have two main possible explanations: (i) transcription is initiated in a minor fraction of minichromosomes having, before initiation, a higher number of supercoils, as compared to the bulk. These extra unconstrained supercoils are relieved upon the formation of the open promoter complex which unwinds the DNA, and during transcription elongation when the unwinding is constantly retained (24, Choder and Aloni unpublished). Consequently, the superhelical density of the resultant transcribed molecule is reduced to the same level as in the bulk. Notice that removal of the polymerase(s) and the RNA transcript(s) from the ternary complex should result in DNA molecules having an increased negative supercoils. (ii) The local and constant unwinding caused by the polymerase during all phases of transcription relieves the unconstrained supercoils. This relatively relaxed state is immediately sensed by a nicking closing activity (a combination of gyrase-like and relaxation activities) that actively maintains a constant superhelical density. This activity, not discriminating between various fractions of chromatin, thereby brings the transcribed molecules to the same superhelical density, but not necessarily to the same distribution, as in the bulk.

Barsum and Berg (13) have suggested for the bulk SV40 minichromosomes that the extra superhelical turns are not necessarily evenly distributed over the entire SV40 DNA. Accordingly, the core nucleosomal DNA is likely to be completely constrained, so that the additional negative supercoils are distributed in linkers. We would like to extend this suggestion to the actively transcribed minichromosomal DNA. Although, based on the present data we cannot conclude on the topological distribution in vivo, we would like to suggest that upon formation of the nucleosome-free region (gap) (15; 25) which is a prerequisite for transcription-initiation as we have previously shown, using a similar analytical method (14), a significant portion of the extra supercoils spontaneously concentrate in the gap. According to this suggestion the gap may be considered as a sink for the extra supercoils. The localization of the extra supercoils may also depend on specific attachment (at least at two sites) of the minichromosome to the nuclear matrix (26). All the above possibilities may lead to a locally high superhelical density which is differently distributed in the transcriptionally active minichromosomes as compared to the bulk. This could be sufficient to activate the formation of non B forms of DNA found in many DNase I hypersensitive regions (8), that are positively correlated with the presence of the nucleosome-free region (14).

ACKNOWLEDGEMENTS

We thank Ruchama Leiserowitz for technical assistance. This research was supported by a research grant CA 14995 from the United States Public Health Service and in part by the Leo and Julia Forcheimer Center for Molecular Genetics and The Henry Gutwirth Fund.

REFERENCES

1. Crick F. H. C. (1976) Proc. Natl. Acad. Sci. USA 73, 2639-2643.
2. Shure, M., Pulleyblank, D. E. and Vinograd, J. (1977) Nucleic Acids Res. 4, 1183-1205.
3. Sinden, R. R., Carlson, J. O. and Pettijohn, D. E. (1980) Cell 21, 773-783.
4. Germond, J. E., Hirt, B., Oudet, P Gross-Bellard, M and Chambon, P. (1975) Proc. Natl. Acad. Sci. USA 72, 1843-1847.
5. Lilley, D. M. J. (1983) Nature 305, 276-277.
6. North, G. (1985) Nature 316, 394-395.
7. Wang, J. C. (1985) Anal. Rev. Biochem. 54, 665-699.
8. Weintraub, H. (1985) Cell 42, 705-711.
9. Luchnik, A. N., Bakayev, V. V., Zbarsky, I. B. and Georgiev, G. P. (1982) EMBO J, 1, 1353-1358.
10. Ambrose, C., McLaughlin, R. and Bina, M. (1987) Nucleic Acid Res. 15, 3703-3721.
11. Ryoji, M. and Worcel, A. (1984) Cell 37, 21-32.
12. Petryniak, B and Lutter, L. C. (1987) Cell 48, 289-295.
13. Barsoum, J. and Berg, P. (1985) Mol. Cell. Biol. 5, 3048-3057.
14. Choder, M., Bratosin, S. and Aloni, Y. (1984) EMBO J. 3, 2929-2936.
15. Jakobovits, E. B., Bratosin, S. and Aloni, Y. (1980) 285, 263-265.
16. Javaherian, K. and Liu, L. F. (1983) Nucleic Acids Res. 11, 461-472.
17. Laskey, R. A. and Mills, A. D. (1975) Eur. J. Biochem. 56, 335-341.
18. Gariglio, P., Buss, J. and Green, M. A. (1974) FEBS Lett. 44, 330-333.
19. Gariglio, P. and Mousset, S. (1975) FEBS Lett. 56, 149-155.
20. Laub, O. and Aloni, Y. (1976) Virology 75, 346-354.
21. Keller, W., Muller, U., Eicken, I., Wendel, J. and Zentgraf, H. (1978) Cold Spring Harbor Symp. Quant. Biol. 42, 227-244.
22. Waldeck, W. ,Theobald, M. and Zentgraf, H. (1983) EMBO J. 2, 1255-1261.
23. Muller, U., Zentgraf, H., Eicken, I. and Keller, W. (1978) Science 201, 406-415.
24. Gamper, H. B. and Hearst, J. E. (1982) Cell 29, 81-90.
25. Saragosti, S. Moyne, G. and Yaniv, M. (1980) Cell 20, 65-73.
26. Abulafia R., Ben-Ze'ev, A., Hay, N. and Aloni, Y. (1984) J. Mol. Biol. 172, 476-487.