MOLECULAR AND CELLULAR BIOLOGY, Nov. 1985, p. 3048–3057
0270-7306/85/113048-10$02.00/0

Vol. 5, No. 11

Simian Virus 40 Minichromosomes Contain Torsionally Strained DNA Molecules

JAMES BARSOUM AND PAUL BERG*

Department of Biochemistry, Stanford University Medical Center, Stanford, California 94305

Received 24 May 1985/Accepted 5 August 1985

Sundin and Varshavsky (J. Mol. Biol. 132:535–546, 1979) found that nearly two-thirds of simian virus 40 (SV40) minichromosomes obtained from nuclei of SV40-infected cells become singly nicked or cleaved across both strands after digestion with staphylococcal nuclease at 0°C. The same treatment of SV40 DNA causes complete digestion rather than the limited cleavages produced in minichromosomal DNA. We have explored this novel behavior of the minichromosome and found that the nuclease sensitivity is dependent upon the topology of the DNA. Thus, if minichromosomes are pretreated with wheat germ DNA topoisomerase I, the minichromosomal DNA is completely resistant to subsequent digestion with staphylococcal nuclease at 0°C. If the minichromosome-associated topoisomerase is removed, virtually all of the minichromosomes are cleaved to nicked or linear structures by the nuclease treatment. The cleavage sites are nonrandomly located; instead they occur at discrete loci throughout the SV40 genome. SV40 minichromosomal DNA is also cleaved to nicked circles and full-length linear fragments after treatment with the single strand-specific endonuclease S1; this cleavage is also inhibited by pretreatment with topoisomerase I. Thus, it may be that the nuclease sensitivity of minichromosomes is due to the transient or permanent unwinding of discrete regions of their DNA. Direct comparisons of the extent of negative supercoiling of native and topoisomerase-treated SV40 minichromosomes revealed that approximately two superhelical turns were removed by the topoisomerase treatment. The loss of these extra negative supercoils from the DNA probably accounts for the resistance of the topoisomerase-treated minichromosomes to the staphylococcal and S1 nucleases. These findings suggest that the DNA in SV40 intranuclear minichromosomes is torsionally strained. The functional significance of this finding is discussed.

Separation of the two strands of DNA during replication and transcription introduces increasing torsional strain in topologically closed duplex DNA. In *Escherichia coli* this torsional strain is alleviated by the balanced action of topoisomerase I and gyrase to maintain the genomic DNA in a negatively supercoiled state (10, 21). Indeed, when the introduction of negative supercoiling into the *E. coli* chromosomal DNA is blocked, replication is stalled and the rates of transcription of several genes are altered (5, 9).

The significance of DNA topology in eucaryotes is less clear. Eucaryotic DNA is packaged into a chromatin structure in which the DNA is wrapped around histone protein cores, forming a nucleosomal fiber. When topologically relaxed DNA is wrapped around the nucleosome core, positive supercoils are introduced. DNA topoisomerase relaxes these positive supercoils, resulting in a net relaxed state of the DNA in the nucleosomal fiber. When histones are dissociated from DNA, the DNA becomes negatively supercoiled. Thus, so long as the nucleosomal fiber is maintained intact, the structure is relaxed and the DNA is not torsionally strained. It is generally believed that the number of negative superhelical turns in topologically constrained DNA is about the same as the number of nucleosomes in the chromatin structure from which the DNA was obtained. Measurements of DNA supercoiling in eucaryotes with trimethylpsoralen intercalation supports the belief that the genome is in a relaxed state, although low amounts of negative superhelicity would not have been detected (30).

Recent reports have suggested that DNA in the eucaryotic genome may exist in a torsionally strained (supercoiled) state, rather than being completely relaxed. Luchnik et al. (20) treated simian virus 40 (SV40) minichromosomes (circular viral DNA associated with cellular histones [33]) with DNA topoisomerase I and found that 2 to 5% of the recovered DNA had become completely relaxed. Since topoisomerase I acts only on supercoiled DNA, they concluded that the DNA in some minichromosomes is topologically supercoiled, probably because the DNA is unconstrained by nucleosomes. Luchnik et al. (20) also noted that the amount and sedimentation behavior of such torsionally strained minichromosomes correlated with the behavior of the transcriptionally active minichromosomes, but their results did not eliminate the possibility that localized or extensive dissociation of some minichromosomes occurred during the isolation or treatment with topoisomerase I.

Ryoji and Worcel (26) have recently examined the packaging of circular DNA into chromatin after injection into *Xenopus laevis* oocytes and observed structures which they term "dynamic chromatin." About half of the newly produced minichromosomes yield fully relaxed DNA after treatment with topoisomerase I. They proposed that the DNA in these minichromosomes is topologically supercoiled due to the presence of an active, energy-driven process (gyrase) and that DNA may be associated with altered nucleosomes that do not constrain the coiling of the DNA.

In this paper, we investigate a phenomenon first described by Sundin and Varshavsky (32). When SV40 minichromosomes are treated with staphylococcal nuclease at 0°C, the digestion pauses after making a single nick or double-stranded cut in the minichromosomal DNA. We have confirmed this finding and shown further that the staphylococcal nuclease sensitivity is abolished by treatment of the minichromosomes with topoisomerase I. Furthermore, treatment of the minichromosomes with topoisomerase I

* Corresponding author.

removes approximately two negative superhelical turns in the minichromosomal DNA. These results suggest that there is a low level of unconstrained negative supercoils (torsional stress) in virtually all minichromosomes. This torsional stress is manifested by the creation of staphylococcal nuclease-sensitive sites at one of many discrete locations in the DNA.

MATERIALS AND METHODS

Animal cells and virus. The passage of the monkey cell line CV-1 and the propagation of SV40 have been described elsewhere (22). COS cells (11) were grown in Dulbecco modified Eagle medium supplemented with 10% newborn calf serum, penicillin, and streptomycin.

Enzymes and reagents. Restriction enzymes were purchased either from New England Biolabs or Bethesda Research Laboratories. Wheat germ DNA topoisomerase I was a gift of J. Champoux and later was obtained from Omega Biotec. *E. coli* DNA gyrase was a gift of N. Cozzarelli. S1 nuclease was from Boehringer Mannheim Biochemicals, and staphylococcal nuclease was obtained from Sigma Chemical Co. [*methyl*-^{3}H]thymidine was obtained from New England Nuclear Corp.

Preparation of SV40 minichromosomes. Newly confluent CV-1 cells in 150-mm plates were infected with SV40 virus at 5 PFU per cell; after 24 h [^{3}H]thymidine (5 μCi/ml) was added to the medium. Minichromosomes were harvested between 38 and 42 h postinfection. After the medium was removed, the cells were washed twice with ice-cold 10 mM Tris hydrochloride (pH 6.8)–0.14 M NaCl. All subsequent operations took place between 0 and 4°C. A 3-ml sample of lysis buffer (0.25% Triton X-100, 10 mM Tris hydrochloride [pH6.8], 10 mM sodium EDTA, 0.5 mM phenylmethylsulfonyl fluoride) was added to each of 10 plates of infected cells. The cells were gently scraped with a rubber policeman and then pelleted at 4,000 × *g* for 5 min in a Sorval centrifuge. The pellet, containing the cell nuclei, was washed once in lysis buffer containing 0.1 M NaCl and then was suspended in 2 ml of extraction buffer (0.25% Triton X-100, 10 mM sodium HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.8], 10 mM sodium EDTA, 0.12 M NaCl, 0.1 mM phenylmethylsulfonyl fluoride) for 10 150-mm plates. The suspension of nuclei was pipetted up and down several times and then rocked for 3 h at 4°C. After the sample was centrifuged for 10 min at 5,000 × *g*, the supernatant was loaded onto a 15 to 30% sucrose gradient (in 10 mM sodium HEPES [pH7.5], 1 mM sodium EDTA, 0.1 M NaCl, 0.1 mM phenylmethylsulfonyl fluoride) and centrifuged in an SW41 rotor for 3 h at 37,000 rpm. Fractions (20 to 25) were collected, either from the bottom or top, and the ^{3}H label was detected by scintillation counting.

In some cases, 5,5′-dithiobis(2-nitrobenzoic acid) was added to all buffers at 0.5 mM to prevent the uncoating of previrions (3). In those preparations where the endogenous topoisomerase was removed from the minichromosomes, the extraction and sucrose gradient centrifugation were performed in the presence of 0.36 M NaCl.

Topoisomerase I and staphylococcal nuclease treatment of minichromosomes. SV40 minichromosomes (5μg of minichromosomal DNA per ml of 10 mM sodium HEPES [pH 7.8]–0.2 M NaCl–0.2 mM sodium EDTA–0.1 mM phenylmethylsulfonyl fluoride) were incubated with 0.5 to 2.0 U of wheat germ DNA topoisomerase I (1 U converts 1 μg of supercoiled pBR322 DNA to the relaxed form in 30 min at 30°C in the above buffer) at 30°C for 30 min. Mock digestions were performed with samples that were kept at 30°C for 30 min in the same buffer. Samples of minichromosome were digested directly with staphylococcal nuclease, or they were adjusted to 0.36 M NaCl and repurified in a 15 to 30% sucrose gradient in 0.36 M NaCl to remove the topoisomerase I. After the topoisomerase I incubation, samples were cooled to 0 to 1°C, and $CaCl_2$ was added to 2 mM. Staphylococcal nuclease (2 to 10 μg/ml) was added, and the incubation proceeded for 15 min at 0°C. Varying the ionic strength between 10 and 240 mM did not affect the extent of nuclease digestion. The reaction was stopped by the addition of EDTA to 15 mM and sodium dodecyl sulfate to 0.2% followed by proteinase K (100 μg/ml) digestion for 30 min at 37°C. Two extractions with phenol-chloroform were performed at 4°C to prevent formation of cruciforms (4). The DNA was precipitated with ethanol and suspended in 0.5X TBE (1X TBE is 89 mM Tris borate [pH 8.3]–2.5 mM sodium EDTA) containing 10% glycerol. Samples of DNA were resolved in a 1.0% agarose–1X TBE, 20-cm horizontal gel with 1X TBE as the buffer at approximately 3 V/cm for 18 to 20 h. DNA was visualized by staining with 1 μg of ethidium bromide per ml and viewing through a 254-nm UV light box and by fluorography (2).

Mapping of the staphylococcal nuclease cleavage sites. After digestion of the SV40 minichromosomes extracted in 0.36 M NaCl as described above, the linear DNA was purified by electrophoresis in low-melting-point agarose gel and elution from the gel (7). The linear DNA was digested in separate samples with *Eco*RI, *Bgl*I, and *Taq*I endonucleases. The digestion products were resolved in agarose gel and transferred to nitrocellulose filters (31). The filters were annealed with the following purified, ^{32}P-nick-translated restriction fragments: (i) 165-base-pair (bp) *Taq*I-*Mbo*II; (ii) 302-bp *Bgl*I-*Kpn*I; (iii) 206-bp *Eco*RI-*Pst*I. The *Eco*RI-*Pst*I fragment was annealed to the *Eco*RI-digested sample, the *Bgl*I-*Kpn*I fragment was annealed to the *Bgl*I digest, and the *Taq*I-*Mbo*II fragment was annealed to the filter containing the *Taq*I digest. Annealing was in 50% formamide, 5X SSPE (1X SSPE is 0.15 M NaCl, 10 mM sodium phosphate, 1 mM sodium EDTA [pH 7.0]), 5X Denhardt solution (1X Denhardt solution is 200 μg of bovine serum albumin [fraction V] per ml, 200 μg of Ficoll per ml, 200 μg of polyvinyl pyrrolidone per ml), and 100 μg of sonicated unlabeled *E. coli* DNA per ml for 16 h at 42°C. Filters were washed six times for 30 minutes each in 0.2% sodium dodecyl sulfate–0.1X SSPE at 52°C and then exposed to XAR-5 film with an intensifying screen at −80°C.

The same procedure was used for the mapping of staphylococcal nuclease cleavage sites of SV40 supercoiled DNA, except that the nuclease concentration was lowered to 0.2 μg/ml and the entire reaction product was used in the subsequent restriction digest.

S1 nuclease digestion of SV40 minichromosomes and DNA. SV40 minichromosomes and supercoiled DNA were digested in 30 mM sodium acetate (pH 4.5)–30 mM NaCl–3 mM $ZnCl_2$ for 15 min at 37°C. S1 nuclease concentrations were 40 to 1,000 U/ml for minichromosomes and 0.2 to 20 U/ml for DNA. The cleavage sites were mapped as described above for staphylococcal nuclease. When the S1 nuclease digestion followed topoisomerase I treatment, the mock- and topoisomerase-treated samples were dialyzed for 6 h into the appropriate buffer for S1 nuclease digestion.

Analysis of DNA topoisomers by two-dimensional gel electrophoresis. DNA was purified from [^{3}H]thymidine-labeled SV40 minichromosomes. Several samples of this DNA were treated with topoisomerase I in the presence of various

concentrations of ethidium bromide (1 to 5 μM) to produce a population of SV40 DNA having between zero (fully relaxed) and the number of superhelical turns in untreated supercoiled DNA (17). One-dimensional analytical gel electrophoresis was used to determine the number of supercoils in each sample. DNA samples containing between 0 and approximately 15 negative supercoils were pooled and used as markers in two-dimensional gel electrophoresis. SV40 minichromosomes were extracted and purified in 0.36 M NaCl. One sample was left untreated, and another was incubated with topoisomerase I under conditions which render the DNA insensitive to staphylococcal nuclease digestion at 0°C. DNA was recovered from these two minichromosome samples; 1 μg of each of these DNA samples was mixed with 0.5 μg of the marker DNA. This material was subjected to two-dimensional gel electrophoresis by the method of Wang et al. (40), with minor modifications. Samples were loaded such that they were run in the same line in the first dimension (separated by 7 cm) rather than side by side so that migration in the second dimension would be comparable. The gel consisted of 0.9% agarose in 1X TBE and was 30 by 30 cm. The first dimension of electrophoresis was at 120 V for 24 h. The gel was soaked in 1X TBE containing 3.0 μM chloroquine for 7 h. The gel was turned 90° with respect to the first dimension of electrophoresis, and second-dimension electrophoresis was in 1X TBE containing 3.0 μM chloroquine (to resolve the more highly negatively supercoiled species) at 120 V for 28 h. DNA was visualized by fluorography.

In some instances, the electrophoresis of topoisomers was in only a single dimension. In these cases, the samples, gel, and electrophoresis buffer contained chloroquine at 1.28, 10, or 30 μM.

RESULTS

Digestion of SV40 minichromosomes by staphylococcal nuclease at 0°C is eliminated by prior treatment with topoisomerase I. Sundin and Varshavsky (32) observed that the DNA in SV40 minichromosomes is cleaved only once with staphylococcal nuclease (micrococcal nuclease) at 0°C; under these conditions of limited digestion the amount of nicking and double-strand cleavages of the minichromosomal DNA was nearly equal. The sites at which the DNA was cleaved appeared to be nonrandomly distributed. Such pausing after a single cleavage in minichromosomal DNA by staphylococcal nuclease was not observed when naked DNA was digested at 0°C or when the SV40 minichromosomes were treated at 37°C. In the latter instance, mono- and oligonucleosomes were the predominant digestion products.

Does the unusual staphylococcal nuclease sensitivity and cleavage pattern reflect the existence of a torsionally strained or otherwise perturbed DNA structure in minichromosomes? Since topoisomerase I removes positive and negative supercoils from DNA, we treated SV40 minichromosomes obtained under physiological salt conditions (0.1 M NaCl) with topoisomerase I to determine whether the staphylococcal nuclease digestion was altered. After staphylococcal nuclease digestion of the minichromosomes [Fig. 1; MC + (mT) + SN] the amount of linear and nicked DNA was increased compared with that in an undigested sample [MC + (mT)]. Even 10-fold-longer exposures of this fluorogram failed to reveal any DNA fragments smaller than full-length linear SV40 DNA in the nuclease-treated samples (data not shown). This finding reproduced the observation reported by Sundin and Varshavsky (32). When the minichromosomes were incubated with topoisomerase I before the staphylococcal nuclease digestion (Fig. 1; MC + T + SN), there was a noticeable reduction in the amount of nicking and double-strand breakages. Note that the DNA isolated from minichromosomes treated with topoisomerase I alone (Fig. 1; MC + T) was not appreciably different from that which was incubated in the absence of topoisomerase [MC + (mT)]. Both contained primarily supercoiled and nicked DNA, although each sample also contained a collection of what appeared to be topoisomers. Since minichromosome preparations similar to ours have been shown to contain topoisomerases I and II (12, 14, 18, 39, 41), the endogenous topoisomerase may be responsible for producing the topoisomer ladder in Fig. 1 [MC + (mT)]. This endogenous topoisomerase may also reduce the sensitivity of the minichromosomes to nicking and cleavage by staphylococcal nuclease.

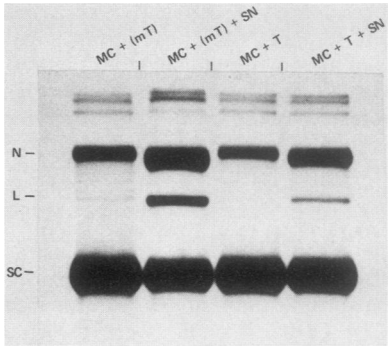

FIG. 1. Treatment of SV40 minichromosomes with DNA topoisomerase I inhibits cleavage of the minichromosomal DNA by staphylococcal nuclease at 0°C. [^{3}H]thymidine-labeled SV40 minichromosomes prepared in 0.1 M NaCl were incubated with or without 0.5 U of wheat germ DNA topoisomerase I per μg of minichromosomal DNA at 30°C. Half of the untreated and topoisomerase I-treated samples were then incubated with 2 μg of staphylococcal nuclease per ml for 15 min at 0°C, and the isolated DNA was electrophoresed in a 1.0% agarose gel. DNA was visualized by fluorography. Abbreviations: MC, DNA from minichromosomes; (mT), minichromosomes incubated at 30°C in the absence of topoisomerase; T, minichromosomes incubated with DNA topoisomerase I; SN, DNA from minichromosomes that had been digested with staphylococcal nuclease; SC, supercoiled DNA; N, nicked DNA; L, full-length linear DNA.

To circumvent this complication, minichromosomes were extracted and purified in either 0.1 or 0.36 M NaCl; it had previously been shown that topoisomerase is removed from chromatin at the higher ionic strength (16). Accordingly, the staphylococcal nuclease sensitivity and the effect of topoisomerase I pretreatment were determined for the two preparations of minichromosomes (Fig. 2). Comparing the two preparations incubated in the absence of added topoisomerase [Fig. 2; MC + (mT)], it is evident that the bulk of the DNA in minichromosomes prepared in high salt is supercoiled and that during the mock topoisomerase incubation no topoisomers are formed; this result differs from that obtained with the low-salt minichromosomes, in which a topoisomer ladder forms. However, the significant feature of this experiment is that about 70% of the DNA in the high-salt minichromosomes became nicked or cleaved to linear DNA after incubation with staphylococcal nuclease [Fig. 2; compare lanes MC with MC + SN and MC + (mT) with MC + (mT) + SN]; however, pretreatment of the high-salt minichromosomes with topoisomerase completely prevented the formation of nicked or linear DNA [compare lanes MC + SN or MC + (mT) + SN with MC + T + SN].

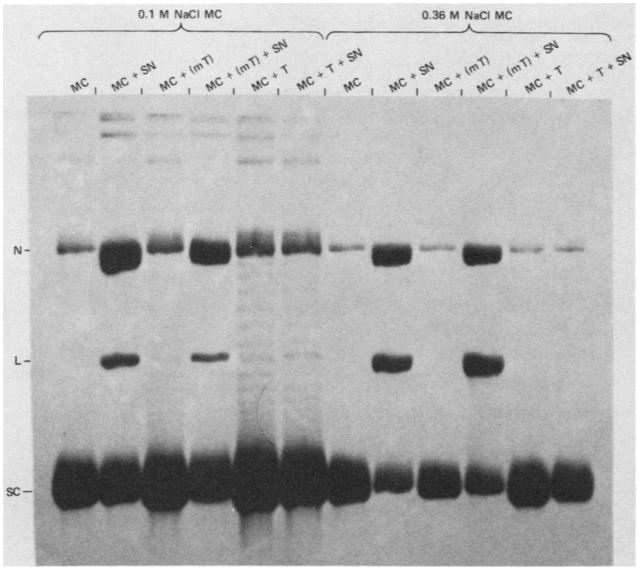

FIG. 2. Action of both endogenous and exogenous DNA topoisomerase I eliminates the staphylococcal nuclease sensitivity of SV40 minichromosomes. SV40 minichromosomes were extracted and purified by sucrose density gradient centrifugation at either 0.1 or 0.36 M NaCl concentration. Both types of minichromosomes were maintained at either 0 or 30°C in the presence or absence of 2.0 U of wheat germ DNA topoisomerase I per μg of minichromosomal DNA (a fourfold higher level of topoisomerase I than that used in Fig. 1). Half of each sample was then digested with 2.0 μg of staphylococcal nuclease per ml for 15 min at 0°C, and the DNA was recovered from the minichromosomes, electrophoresed in an agarose gel, and fluorographed. Abbreviations are as in Fig. 1.

With higher levels of staphylococcal nuclease (7 versus 2 μg/ml in Fig. 2), virtually all of the high-salt minichromosomes were digested to either nicked or linear DNA by staphylococcal nuclease at 0°C.

Topoisomerase I does not directly inhibit staphylococcal nuclease. When the minichromosomes were purified by sucrose gradient sedimentation in 0.36 M NaCl after the topoisomerase I treatment, the minichromosomes were still resistant to staphylococcal nuclease digestion (data not shown). Moreover, topoisomerase I did not inhibit staphylococcal nuclease digestion of SV40 minichromosomal DNA at 37°C. Under these conditions, minichromosomal DNA was digested by staphylococcal nuclease to mono- and oligonucleosomes irrespective of whether the minichromosomes had been treated with topoisomerase.

Since the purification of minichromosomes involves several steps and occurs over approximately 8 h, it is possible that the structure which is cleaved by staphylococcal nuclease at 0°C and is removed by topoisomerase I treatment was formed in vitro during the preparation. This is unlikely because minichromosomes contained in a rapid, whole cell lysate showed the same digestion properties. After the medium was removed, cells were quick chilled and lysed immediately by scraping in lysis buffer (see Materials and Methods) containing divalent cations and no EDTA. Various amounts of staphylococcal nuclease were added; after only 5 min at 0°C, the DNA was purified and fractionated by gel electrophoresis. Here, too, the nuclease digestion stopped after producing a single cut in SV40 DNA with very little degradation apparent beyond this single cleavage. In this experiment as much as 70% of the minichromosomal DNA became nicked or linearized.

SV40 minichromosomal DNA was also sensitive to a single cleavage by S1 nuclease (Fig. 3); the product was predominantly nicked circles with some full-length linear DNA. Gel electrophoresis under denaturing conditions revealed that the nicked circles had single cleavages (data not shown). More significantly, incubation of the minichromosomes with topoisomerase I eliminated the S1 nuclease sensitive structure.

SV40 minichromosomal DNA contains supercoils that are removed by DNA topoisomerase I. The finding that the nuclease-sensitive structure was dissipated by topoisomerase I suggests that the minichromosomal DNA is torsionally strained—that is, the DNA possesses negative supercoils that are not compensated by nucleosomes. By this view, topoisomerase I would remove the extra supercoils, thereby relaxing the minichromosome and making it insensitive to nuclease cleavage. To test for the existence of extra superhelical turns in minichromosomal DNA, the following experiment was performed. SV40 minichromosomes were either untreated or incubated with topoisomerase I under conditions which render the DNA insensitive to staphylococcal nuclease digestion at 0°C. DNA purified from these two samples was mixed with a marker SV40 DNA with between 0 and 15 negative supercoils. The samples were subjected to two-dimensional gel electrophoresis (see Materials and Methods) (40). The presence of chloroquine in the second dimension lowers the negative superhelical density of the DNA and allows the resolution of the most highly negatively supercoiled species (29).

The electrophoretic pattern with the mixture of topoisomers used as markers and the DNA obtained from untreated minichromosomes is shown in Fig. 4. The number and spacing of the marker spots indicate a range of topoisomers with 0 to 14 negative supercoils. The DNA topoisomers contributed by the untreated minichromosomes ranged in the number of supercoils between 18 and 30, with only traces on either side of that distribution. (Each of the topoisomer species would be better resolved in both shorter and longer exposures of the fluorograms.) The peak of intensity corresponded to topoisomers with 24 negative supercoils, and substantial intensities indicate frequent topoisomers with 22 to 26 supercoils; this is precisely the value (24 ± 2) determined for SV40 DNA by Keller (17).

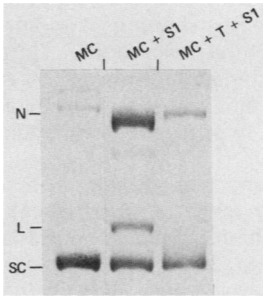

FIG. 3. S1 nuclease digestion of the DNA in SV40 minichromosomes is also blocked by treatment of the minichromosomes with DNA topoisomerase I. SV40 minichromosomes that had been extracted and purified in 0.36 M NaCl were either untreated or incubated with 2.0 U of wheat germ DNA topoisomerase I per μg of minichromosomal DNA. Both untreated and topoisomerase I-treated minichromosomes were dialyzed into S1 nuclease digestion buffer and digested with 100 U of S1 nuclease for 15 min at 37°C. DNA was recovered and electrophoresed in an agarose gel. Abbreviations are as in Fig. 1, plus the following; S1, DNA from minichromosomes that had been digested with S1 nuclease.

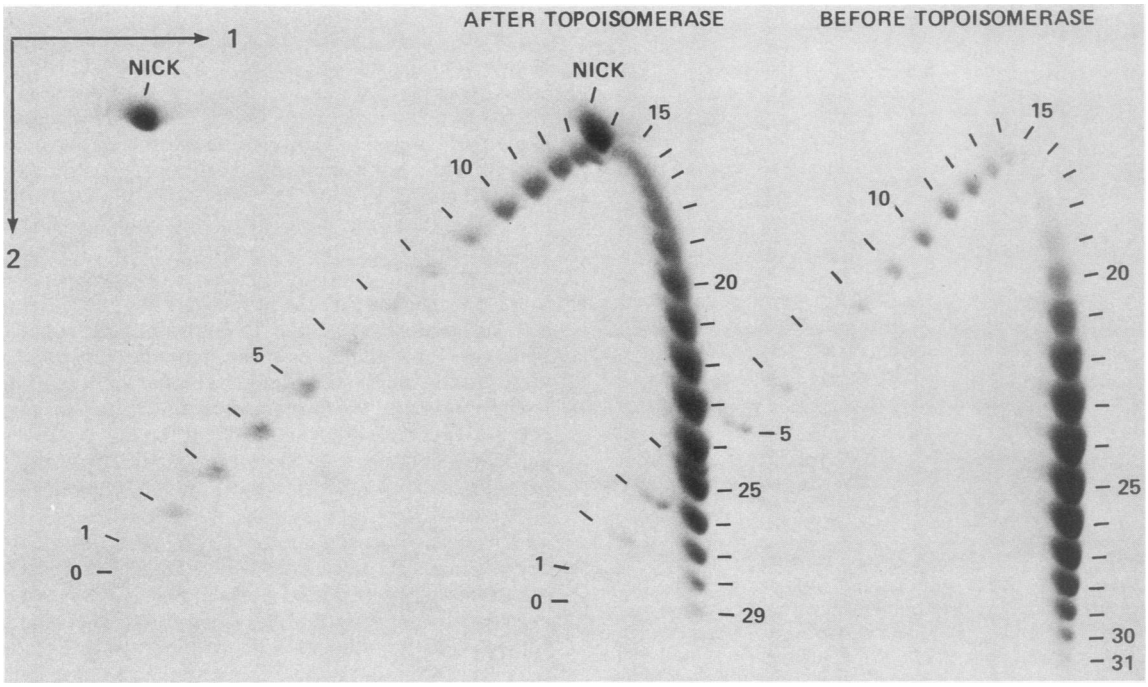

FIG. 4. Two-dimensional gel electrophoretic analysis of topoisomer distributions in minichromosomal DNA after treatment of SV40 minichromosomes with topoisomerase I. SV40 minichromosomes extracted and purified in 0.36 M NaCl were treated with (left) or without (right) topoisomerase I, and the DNA was purified. To create marker samples, samples of SV40 DNA were treated with topoisomerase I in the presence of 1 to 5 μM ethidium bromide. With one-dimensional gel analysis, topoisomer samples with 0 to 15 negative supercoils were pooled and mixed with a twofold excess of the supercoiled DNA recovered from the topoisomerase I-treated and untreated minichromosomes. These samples were electrophoresed in a two-dimensional 0.9% agarose gel. The running buffers were identical in both dimensions, except for the presence of 3.0 μM chloroquine in the second dimension. DNA was visualized by fluorography. The numbers next to the DNA spots refer to the number of negative superhelical turns, determined by counting spots in relation to the nicked spot using several different fluorographic exposures. The arrows indicate the directions of first- and second-dimension electrophoresis.

The DNA obtained from topoisomerase-treated minichromosomes produced a somewhat different population of topoisomers (Fig. 4). In this case the topoisomer arc was shifted in the direction of less-negative supercoils. The most negatively supercoiled molecule visible in the fluorogram is at position 29 compared with 31 in the untreated minichromosomal DNA. Intense spots occur as far up the arc as 16 negative supercoils and perhaps beyond (at that point they overlap with the topoisomer markers). The peak of intensity in this distribution of topoisomers is approximately at 22 negative supercoils. Thus, it appears that treatment of SV40 minichromosomes with topoisomerase I alters the linking number of the DNA, consistent with the removal of about two negative supercoils per DNA molecule.

Figure 5 summarizes the results of a somewhat different comparison of the range of superhelical densities in DNA isolated from minichromosomes before and after treatment with topoisomerase. Here, topoisomers were resolved in one-dimensional gels, which allowed the determination of only the relative number of supercoils. In Fig. 5A, topoisomers were separated in a one-dimensional agarose gel in the presence of a low concentration of chloroquine, which slightly reduced the negative superhelicity of the topoisomers. The results show, first, that the covalently closed SV40 DNA recovered from virus-infected CV-1 cells by the Hirt extraction method (15) has about the same distribution of topoisomers as DNA extracted from untreated minichromosomes; this means that there has been no substantial change in the linking number of the DNA during the isolation of the minichromosomes. More significantly, however, is the finding that treatment of the minichromosomes with topoisomerase results in the loss of the most negatively supercoiled DNA species and a shift toward decreasing negative superhelicity in the entire distribution.

The change in the linking number after topoisomerase treatment was quantitated by gel electrophoresis of the DNA topoisomers in high enough levels of chloroquine to induce positive supercoiling in all but nicked DNA (Fig. 5B). Thus, in these gels, topoisomers with increasing numbers of negative supercoils are less positively supercoiled by the chloroquine and consequently migrate more slowly. Conversely, molecules that are less negatively supercoiled move more rapidly during electrophoresis in this level of chloroquine. The densitometer tracing of this gel (Fig. 5C) shows that the peak of maximal intensity has shifted down by two supercoils, as has the entire pattern including the least negatively supercoiled species. Thus, in this experiment also, topoisomerase I treatment of SV40 minichromosomes appears to remove approximately two negative superhelical turns from the DNA.

Mapping the sites of staphylococcal and S1 nuclease cleavage in minichromosomal DNA. Are the staphylococcal and S1 nuclease-sensitive sites in minichromosomal DNA localized to a specific region of the molecules, or do they occur at random locations? To answer that question we mapped the cleavage sites produced in minichromosomal DNA by staphylococcal nuclease at 0°C according to the protocol outlined in the legend to Fig. 6 and described in detail in Materials and Methods. After the staphylococcal nuclease digestion,

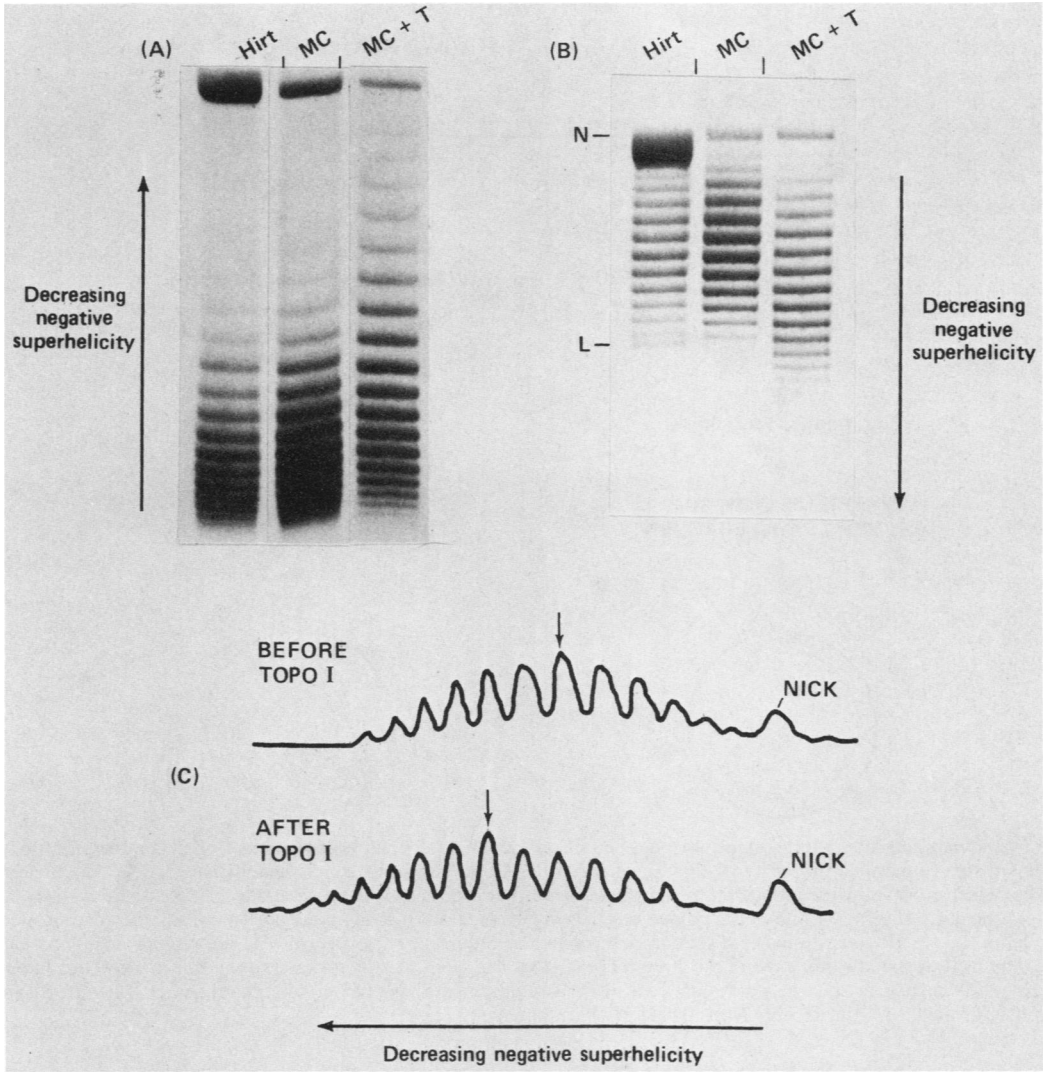

FIG. 5. Topoisomerase I treatment removes approximately two negative supercoils from SV40 minichromosomes. SV40 minichromosomes were extracted and purified in 0.36 M NaCl. One-half of the minichromosomes were treated with wheat germ DNA topoisomerase I, and the others were untreated. DNA was then purified from these minichromosomes. A Hirt extract (15) of SV40-infected CV-1 cells was also performed, and DNA was purified by CsCl gradient centrifugation. (A) DNA samples were run in an 0.9% agarose gel containing 1.28 μM chloroquine to decrease the superhelicity of samples. Abbreviations are as in Fig. 1, plus the following: Hirt: DNA extracted by the Hirt procedure. (B) Samples similar those in (A), but from a different experiment, were electrophoresed in an agarose gel containing 30 μM chloroquine to positively supercoil the DNA. (C) A densitometer tracing of samples MC (upper tracing) and MC + T (lower tracing) in gel (B). Top to bottom in the gels is shown as right to left in the tracings. Arrows indicate the maximum peaks of intensity. The direction of topoisomers having decreasing negative superhelicity is shown in all panels.

during which almost all of the supercoiled DNA had been cleaved once, the linear DNA was isolated by gel electrophoresis and digested with endonucleases that cleave SV40 DNA once: *Eco*RI, *Taq*I, and *Bgl*I. After gel electrophoresis of the individual digests, the samples were transferred to nitrocellulose (31) and annealed with appropriate ^{32}P-labeled probes; each probe spanned a short region beginning at the chosen restriction site and extending in one direction (Fig. 6). The size of the fragments hybridizing with each probe measures the distance, in one direction, from the unique restriction endonuclease cleavage site to the different staphylococcal nuclease cleavage sites. A similar procedure was used to map the S1 nuclease cleavages in minichromosomal DNA.

Figure 7A shows the array of fragments produced by *Eco*RI or *Bgl*I endonuclease digestion of the linear DNA produced either by staphylococcal nuclease digestion of minichromosomal DNA at 0°C or by S1 nuclease. Note that there are discrete fragments and not a continuous smear in each double digest. This indicates that the staphylococcal and S1 nuclease cleavages in minichromosomal DNA do not occur at random sites. Rather, there are a substantial number of preferred cleavage sites, but generally only one per minichromosome. The preferred sites of staphylococcal and S1 nuclease cleavages in minichromosomes are summarized in Fig. 7C. They are clearly distributed around the entire SV40 DNA; in some, although not all, instances the staphylococcal and S1 nuclease cleavage sites coincide. The size of the most intense bands indicates that many of the nuclease cleavages of minichromosomal DNA occur in the early region of the genome. Interestingly, the exposed nonnucleosomal region (28, 35, 36), which has been localized to

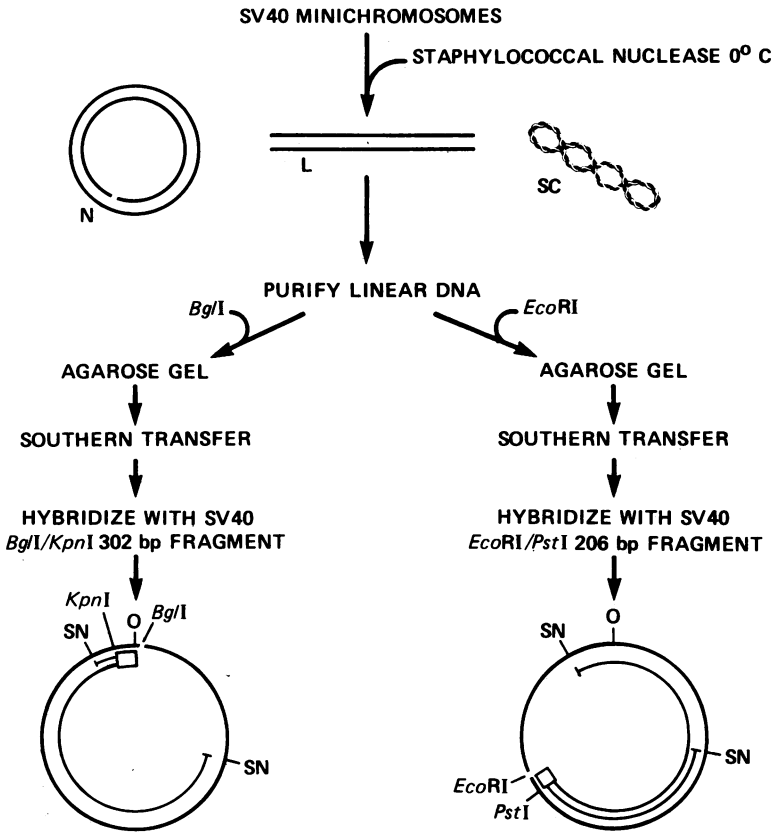

FIG. 6. Strategy for mapping the sites of staphylococcal nuclease cleavage of minichromosomal DNA. Double-stranded staphylococcal nuclease cleavage sites were mapped by purifying linearized SV40 DNA and digesting with a restriction enzyme which cleaves once in the SV40 genome. The digest products were electrophoresed, transferred to a nitrocellulose filter, and hybridized with a short ^{32}P-labeled DNA fragment which has one of its ends at the site of the unique restriction cleavage. In this way the staphylococcal nuclease sites could be mapped by their distance from the restriction site in the direction determined by the small DNA probe. The 302-bp *BglI-KpnI* SV40 DNA fragment was hybridized to the *BglI*-digested samples, and the 206-bp *EcoRI-PstI* fragment was hybridized to the *EcoRI*-digested samples. Along with the two restriction enzyme digests shown, staphylococcal nuclease-linearized SV40 DNA was also digested with *TaqI*, and the resulting digestion pattern was probed with the 165-bp *TaqI-MboII* DNA fragment.

the region between the beginnings of the early and late regions (36) in about 20% of intranuclear minichromosomes (27, 38), is not particularly sensitive to staphylococcal or S1 nuclease cleavage.

Figures 7B and C summarize the results obtained after parallel digestions of supercoiled SV40 DNA with staphylococcal and S1 nucleases followed by *EcoRI* and *BglI* endonuclease digestion. Single cleavages of supercoiled DNA occur at only a limited number of locations with S1 nuclease, a point noted previously (1); however, staphylococcal nuclease makes multiple cleavages in supercoiled SV40 DNA. With either nuclease, most of the cleavages occur in the early region; S1 nuclease cleavages occur predominately at the locations described earlier (1), whereas staphylococcal nuclease cuts are more widespread.

The data summarized in Fig. 7C indicate that several regions seem to be preferred sites for staphylococcal nuclease cleavage of torsionally strained minichromosomes and for S1 nuclease cleavage of torsionally strained minichromosomes and free supercoiled SV40 DNA (regions A, B, C, D, E, and F). Regions G and H contain sites at which both staphylococcal and S1 nucleases cleave minichromosomal DNA, but not free supercoiled DNA. This being the case, it may be that the staphylococcal and S1 nuclease cleavage sites in minichromosomes occur in regions that are unwound or have some otherwise unusual secondary structure (e.g., cruciforms) induced by the additional supercoiling that is removable by topoisomerase I.

DISCUSSION

We have found that the DNA in SV40 minichromosomes appears to be torsionally strained rather than relaxed. This strain derives from approximately two negative supercoils which are not constrained by nucleosomes and can be relaxed by DNA topoisomerase I treatment. Staphylococcal nuclease cleaves the DNA in SV40 minichromosomes only once at 0°C; however, this occurs only when torsional strain exists. Thus, the staphylococcal nuclease sensitivity of nucleosomal DNA is lost when the minichromosomes are treated with topoisomerase I. The sites at which staphylococcal nuclease cleaves the DNA are nonrandom, but they occur at various sites throughout the SV40 genome. Since the single strand-specific S1 nuclease also cleaves the DNA of torsionally strained minichromosomes, often at the same regions cleaved by staphylococcal nuclease, it seems likely that the torsional strain causes specific loci in the genome to become underwound relative to relaxed B-form DNA and the remainder of the genome. Some of the sites at which staphylococcal and S1 nucleases cleave minichromosomal DNA overlap with sites at which S1 nuclease cleaves

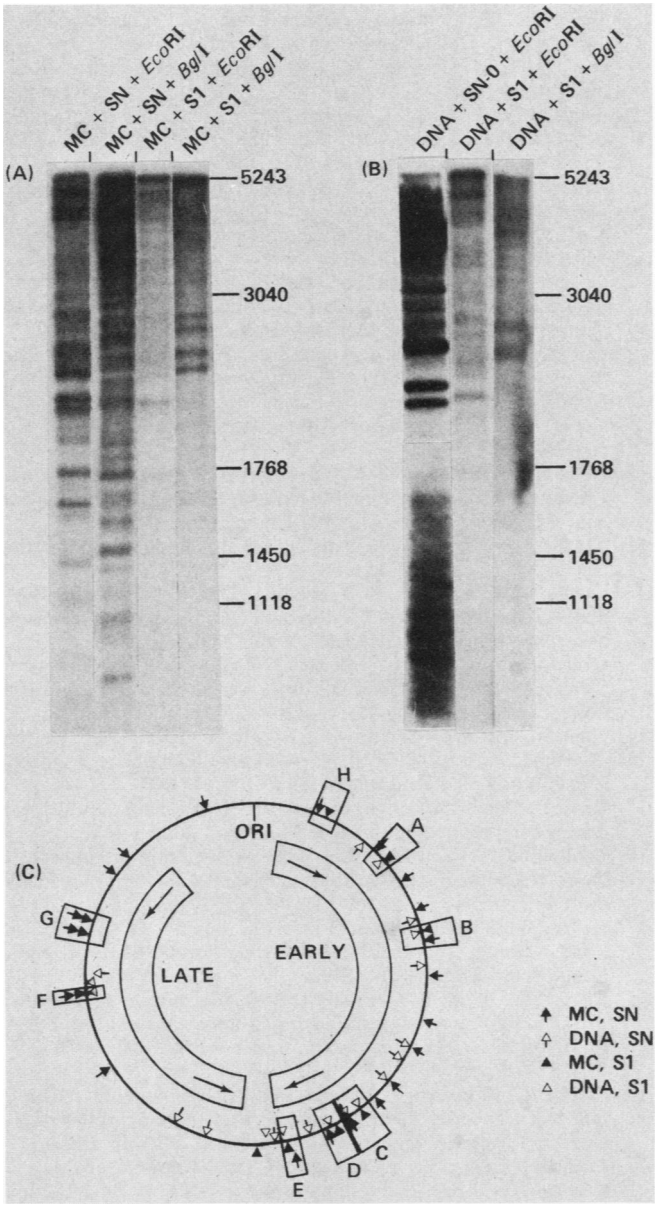

FIG. 7. Mapping the sites of staphylococcal and S1 nuclease cleavages in SV40 minichromosomal DNA and in free supercoiled SV40 DNA. (A) SV40 minichromosomes were digested with either staphylococcal or S1 nuclease. Full-length linear SV40 DNA was purified and digested with either *Eco*RI or *Bgl*I. The digest products were electrophoresed in an agarose gel, transferred to nitrocellulose filters, and hybridized with a ^{32}P-labeled DNA fragment which maps to a position immediately adjacent to the single-cut restriction enzyme used (Fig. 6). Tracks from the resulting autoradiograms are shown. Numbers to the right of panel A denote the positions of DNA size markers. (B) Free supercoiled SV40 DNA was digested with 0.2 μg of staphylococcal nuclease per ml at 0°C for 15 min (SN-0) or with 10 U of S1 nuclease. The total reaction product of the staphylococcal nuclease digestion was treated with *Eco*RI, and full-length linear SV40 DNA was purified from the S1 nuclease digestion and treated with either *Eco*RI or *Bgl*I. These samples were electrophoresed, transferred, and hybridized as described above. All of the lanes in (A) and (B) were derived from one gel. The lanes were reordered and different exposures were used to equalize total intensities. The lane DNA + SN−0 + *Eco*RI is a composite of two different exposures to reveal the smaller fragments seen only upon longer exposure. Abbreviations are as in Fig. 1 and 3. (C) The positions of the nuclease cleavage sites, as determined from the autoradiograms in (A) and (B) and several other experiments with *Taq*I as well as *Eco*RI and *Bgl*I restriction digests are shown in relation to the SV40 genome. The approximate boundaries of the early and late regions and the direction of transcription are indicated. Symbols: ↑, staphylococcal nuclease cleavage sites in minichromosomes at 0°C; ↑, staphylococcal nuclease cleavage sites in supercoiled DNA; ▲, S1 nuclease cleavage sites in minichromosomes; △, S1 nuclease cleavage sites in supercoiled DNA. The boxes A through H indicate regions in which staphylococcal and S1 nuclease cleavage sites in minichromosomes are in close proximity.

supercoiled SV40 DNA. This suggests that the DNA in torsionally strained minichromosomes may absorb the torsional strain by unwinding the DNA duplex in specific preferred loci. However, some of the staphylococcal nuclease cleavage sites in minichromosomes are not cut by S1 nuclease, nor are they staphylococcal nuclease cleavage sites in supercoiled DNA. It may be, therefore, that DNA-protein interactions play a role in the creation of some of the nuclease-sensitive sites.

Since the additional negative supercoils can be removed by topoisomerase I, they may take the form of a writhing of the DNA. DNA in the core nucleosome is not likely to be free to writhe, whereas the linker DNA between nucleosomes may be far more topologically pliable. If two negative supercoils were distributed over the entire 5,243 bp of SV40 DNA, the net superhelical density of the minichromosome would be only −0.004. However, if these two supercoils were confined to a single DNA segment between adjacent nucleosomes (an average of 70 bp for SV40) the superhelical density in that one region would be −0.299. This density appears to be unusually high; therefore, the supercoils may reside in longer internucleosomal segments, or the torsional stress may be relieved by local denaturation.

In some experiments, virtually all of the minichromosomal DNA can be cleaved once, and only once, by the nuclease. This cutting can be completely relieved by pretreatment with topoisomerase I. Therefore, it is likely that the extra supercoiling is present in all minichromosomes in vivo. If this is so, one wonders why topoisomerases I and II, which are present in nuclei, do not remove the torsional strain. A possible explanation is that the extra supercoils are not susceptible or available to topoisomerase in vivo because of protein interactions or DNA folding that is lost in vitro. Another possibility is that supercoiling of minichromosomal DNA is constantly being maintained by an active supercoiling activity.

The loss of nucleosomes in the exposed region of the SV40 minichromosome, which spans about 400 bp between the beginning of the early and late regions, could account for the two unconstrained negative supercoils in minichromosomes. An argument against this view is the fact that only approximately 20% of minichromosomes possess the nucleosome gap (27, 28; unpublished data), but all of the minichromosomes display a topology-dependent nuclease cleavage.

Another possible explanation to account for the torsional strain stemming from two superhelical turns per DNA molecule is that they are a consequence of higher-order folding of the SV40 minichromosome. For example, it is possible to devise a model of higher-order folding in which the formation of a solenoid structure could introduce two to three negative supercoils (6). An argument against this view is that both compact minichromosomes, such as those which exist

at high ionic strengths in the presence of histone H1 (24, 34), and extended minichromosomes, which occur in low-ionic-strength solutions, are cleaved equally efficiently by staphylococcal nuclease at 0°C.

Rather than assuming that the extra negative superhelical turns result from the loss of nucleosomes or from the presence of higher-order folding, we have considered the possibility that the additional unconstrained supercoils result from the activity of an as yet undiscovered mammalian DNA gyrase. Although evidence for the existence of a mammalian DNA gyrase is elusive, we have found that *E. coli* DNA gyrase can introduce additional negative supercoils into SV40 minichromosomes which had been treated with topoisomerase I and also partially restore the sensitivity to staphylococcal nuclease at 0°C (data not shown). We have also observed, in preliminary experiments, that treatment of SV40-infected cells with the type II topoisomerase inhibitor novobiocin decreases the sensitivity of the minichromosomal DNA to staphylococcal nuclease cleavage; however, the loss in nuclease sensitivity is never complete and only occurs at high novobiocin concentrations. In this connection, Villeponteau et al. (37) have recently found that an actively transcribed chicken β-globin gene in chromatin is sensitive to cleavage by DNase I and that this sensitivity is lost after novobiocin treatment of the cells. They propose that the active chromatin structure is maintained by an ATP-dependent, novobiocin-sensitive supercoiling activity.

At present, the function of the torsional strain in minichromosomes is obscure. Although virtually all minichromosomes possess a torsionally strained genome, only about 1% of SV40 minichromosomes appear to be transcriptionally active (8, 13, 19). Therefore, it is unlikely that torsional strain is a feature peculiar to only those minichromosomes engaged in transcription. Perhaps the extra supercoiling is necessary for the production of templates capable of being transcribed or replicated. Two negative supercoils unconstrained by nucleosomes could favor both replication and transcription, since approximately 21 bp of duplex DNA could be completely unwound without introducing additional strain in the remainder of the molecule in the form of positive supercoiling.

This small degree of unconstrained DNA supercoiling may not be unique to extrachromosomal DNA circles. Since chromosomes are organized into large, topologically closed DNA loops (25) and transcriptional units appear to be precisely arranged with regard to the positioning of a loop on the chromosome scaffold (23), it is possible that gene expression could be regulated, in part, by selective alteration of the DNA topology in specific chromosomal loops.

ACKNOWLEDGMENTS

We thank J. Champoux and N. Cozzarelli for generous gifts of, respectively, wheat germ DNA topoisomerase I and *E. coli* DNA gyrase. We also thank D. Brutlag for helpful discussions.

J.B. is supported by a Public Health Service postdoctoral fellowship from the National Institutes of Health.

This research was funded by Public Health Service grants GM-13235 and CA-31928 from the National Institutes of Health.

LITERATURE CITED

1. **Beard, P., J. F. Morrow, and P. Berg.** 1973. Cleavage of circular, superhelical simian virus 40 DNA to a linear duplex by S1 nuclease. J. Virol. **12**:1303–1313.
2. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for ^{3}H-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–92.
3. **Boyce, F., O. Sundin, J. Barsoum, and A. Varshavsky.** 1982. New way to isolate simian virus 40 nucleoprotein complexes from infected cells: use of a thiol-specific reagent. J. Virol. **42**:292–296.
4. **Courney, A. J., and J. C. Wang.** 1983. Cruciform formation in a negatively supercoiled DNA may be kinetically forbidden under physiological conditions. Cell **33**:817–829.
5. **Cozzarelli, N. R.** 1980. DNA gyrase and the supercoiling of DNA. Science **207**:953–960.
6. **Crick, F. H. C.** 1976. Linking numbers and nucleosomes. Proc. Natl. Acad. Sci. USA **73**:2639–2643.
7. **Crouse, G. F., A. Frischauf, and H. Lehrach.** 1983. An integrated and simplified approach to cloning into plasmids and single-stranded phages. Methods Enzymol. **101**:78–89.
8. **Gariglio, P., R. Llopis, P. Oudet, and P. Chambon.** 1979. The template of the isolated native simian virus 40 transcriptional complexes is a minichromosome. J. Mol. Biol. **131**:75–105.
9. **Gellert, M.** 1981. DNA topoisomerases. Annu. Rev. Biochem. **50**:879–910.
10. **Gellert, M., R. Menzel, K. Mizuchi, M. H. O'Dea, and D. I. Friedman.** 1982. Regulation of DNA supercoiling in *Escherichia coli*. Cold Spring Harbor Symp. Quant. Biol. **47**:763–767.
11. **Gluzman, Y.** 1981. SV40-transformed simian cells support the replication of early SV40 mutants. Cell **23**:175–182.
12. **Goulie, B. B., Y. Pigiet, C. B. Breaux, M. R. Krauss, C. R. King, and R. M. Benbow.** 1981. Polyoma virus minichromosomes: associated enzyme activities. J. Virol. **38**:826–832.
13. **Green, M. H., and T. L. Brooks.** 1976. Isolation of two forms of SV40 nucleoprotein containing RNA polymerase from infected monkey cells. Virology **72**:110–120.
14. **Hamelin, C., and M. Yaniv.** 1979. Nicking-closing enzyme is associated with SV40 DNA *in vivo* as a sodium dodecyl sulfate-resistant complex. Nucleic Acids Res. **7**:679–687.
15. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
16. **Javaherian, K., and L. F. Liu.** 1983. Association of eukaryotic DNA topoisomerase I with nucleosomes and chromosomal proteins. Nucleic Acids Res. **11**:461–472.
17. **Keller, W.** 1975. Determination of the number of superhelical turns in simian virus 40 DNA by gel electrophoresis. Proc. Natl. Acad. Sci. USA **72**:4876–4880.
18. **Krauss, M. R., B. B. Gourlie, M. L. Bayne, and R. M. Benbow.** 1984. Polyomavirus minichromosomes: associated DNA topoisomerase II and DNA ligase activities. J. Virol. **49**:333–342.
19. **Llopis, R., F. Perrin, F. Bellard, and P. Gariglio.** 1981. Quantitation of transcribing native simian virus 40 minichromosomes extracted from CV-1 cells late in infection. J. Virol. **38**:82–90.
20. **Luchnik, A. N., V. V. Bakayev, I. B. Zbarskey, and G. P. Georgiev.** 1982. Elastic torsional strain in DNA within a fraction of SV40 minichromosomes: relation to transcriptionally active chromatin. EMBO J. **1**:1353–1358.
21. **Menzel, R., and M. Gellert.** 1983. Regulation of the genes for *E. coli* DNA gyrase: homeostatic control of DNA supercoiling. Cell **34**:105–113.
22. **Mertz, J. E., and P. Berg.** 1974. Defective simian virus genomes: isolation and growth of individual clones. Virology **62**:112–124.
23. **Mirkovitch, J., M. E. Mirault, and U. K. Laemmli.** 1984. Organization of the higher-order chromatin loop: specific DNA attachment sites on nuclear scaffold. Cell **39**:223–232.
24. **Muller, V., H. Zentgraf, I. Eicken, and W. Keller.** 1978. Higher order structure of simian virus 40 chromatin. Science **201**:406–415.
25. **Paulsen, J. R., and U. K. Laemmli.** 1977. The structure of histone-depleted metaphase chromosomes. Cell **12**:817–828.
26. **Ryoji, M., and A. Worcel.** 1984. Chromatin assembly in Xenopus oocytes: *in vivo* studies. Cell **37**:21–32.
27. **Saragosti, S., G. Moyne, and M. Yaniv.** 1980. Absence of nucleosomes in a fraction of SV40 chromatin between the origins of replication and the region coding for the late leader RNA. Cell **20**:65–73.
28. **Scott, W. A., and D. J. Wigmore.** 1978. Sites in simian virus 40

chromatin which are preferentially cleaved by endonucleases. Cell **15**:1511–1518.
29. **Shure, M., D. E. Pulleyblank, and J. Vinograd.** 1977. The problem of eukaryotic and prokaryotic DNA packaging and in *in vivo* conformation posed by superhelix density heterogeneity. Nucleic Acids Res. **4**:1183–1205.
30. **Sinden, R. R., J. O. Carlson, and D. E. Pettijohn.** 1980. Torsional tension in the DNA double helix measured with trimethylpsoralen in living *E. coli* cells; analogous measurements in insect and human cells. Cell **21**:773–783.
31. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
32. **Sundin, O., and A. Varshavsky.** 1979. Staphylococcal nuclease makes a single non-random cut in the simian virus 40 minichromosome. J. Mol. Biol. **132**:535–546.
33. **Tooze, J.** ed. 1981. DNA tumor viruses, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **Varshavsky, A. J., V. V. Bakayer, S. A. Nedospasov, and G. P. Georgiev.** 1978. On the structure of eukaryotic, prokaryotic and viral chromatin. Cold Spring Harbor Symp. Quant. Biol. **42**:457–473.
35. **Varshavsky, A. J., O. H. Sundin, and M. J. Bohn.** 1978. SV40 viral minichromosome: preferential exposure of the origin of replication as probed by restriction endonucleases. Nucleic Acids Res. **5**:3469–3477.
36. **Varshavsky, A. J., O. Sundin, and M. Bohn.** 1979. A stretch of "late" SV40 viral DNA about 400 bp long which includes the origin of replication is specifically exposed in SV40 minichromosomes. Cell **16**:453–466.
37. **Villeponteau, B., M. Lundell, and H. Martinson.** 1984. Torsional stress promotes the DNase I sensitivity of active genes. Cell **39**:469–478.
38. **Waldeck, W., B. Fohring, K. Chowdhury, P. Gruss, and G. Sauer.** 1978. Origin of DNA replication in papovavirus chromatin is recognized by endogenous endonuclease. Proc. Natl. Acad. Sci. USA **75**:5964–5968.
39. **Waldeck, W., H. Zentgraf, and G. Sauer.** 1982. *In vivo* formation of multimeric DNA structures mediated by purified simian virus 40 chromatin. Proc. Natl. Acad. Sci. USA **79**:1027–1031.
40. **Wang, J. C., L. J. Peck, and K. Becherer.** 1982. DNA supercoiling and its effects on DNA structure and function. Cold Spring Harbor Symp. Quant. Biol. **47**:85–91.
41. **Young, L. S., and J. J. Champoux.** 1978. Interaction of the DNA untwisting enzyme with SV40 nucleoprotein complex. Nucleic Acids Res. **5**:623–635.