DNA supercoiling of recombinant plasmids in mammalian cells

(DNA transfection/replication/COS cells/chloroquine/agarose gel/blot hybridization)

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ABSTRACT We have used chloroquine/agarose gel electrophoresis and a blot-hybridization technique to study the modulation of superhelicity of extrachromosomal DNA in mammalian cells. The high sensitivity of the procedure has allowed us to measure the change in the specific linking difference or superhedral density (σ) of a plasmid, psvoa1p3d, after its introduction into COS-7 cells by DNA transfection. Because the molecular weight of psvoa1p3d is approximately the same as that of simian virus 40 (SV40) DNA, the latter can be used as a standard for estimating the average linking difference or number of superhelical turns (τ) of psvoa1p3d after separation of the different supercoiled species on chloroquine/agarose gels. It was found that transfection of monkey cells with either fully supercoiled psvoa1p3d isolated from bacteria (τ = −27 ± 1, σ = −0.051) or its relaxed form after treatment with DNA topoisomerase I yields psvoa1p3d samples of the same τ and σ values of −20 ± 1 and −0.038, respectively. The difference between the τ values of psvoa1p3d and SV40 in COS-7 cells, in which both plasmids undergo rounds of replication, corresponds to an average difference of 5 ± 1 superhelical turns. Plasmid psvoa1p3d remains at this lower level of superhelicity for at least 72 hr. The distribution in linking numbers of the topoisomers of psvoa1p3d isolated from transfected COS cells is also more heterogeneous than that of SV40 DNA. These results suggest that the regulation of DNA supercoiling and chromatin assembly may be closely associated with specific DNA sequences. The approach presented here should have a wide application in the study of the regulation and functional role(s) of DNA supercoiling of plasmids in mammalian cells.

DNA supercoiling is an intrinsic property of the chromosomes of both procaryotic and eukaryotic cells (1–3). Supercoils are also present on the circular double-stranded DNA of plasmids and viruses (for review, see refs. 4, 5). In prokaryotes, supercoiling plays an essential role in a variety of genetic processes, including replication, recombination, and transcription (5). Such a role is not yet well-defined in eukaryotic systems.

In eukaryotic cells, most of the DNA supercoils are constrained within the nucleosomes (ref. 6; for review, see refs. 7, 8). Recent studies of simian virus 40 (SV40) minichromosomes and 5S RNA gene-containing plasmids injected into Xenopus oocytes have suggested that a significant portion of the minichromosomes in vivo exist in a torsionally strained state and that this strained subpopulation is transcriptionally active (9, 10). A more direct relationship between DNA supercoiling and enhancement of transcription in vivo was implied in a study of the herpesvirus thymidine kinase gene (11).

Although the degrees of supercoiling of many bacterial plasmids and animal virus DNAs have been studied extensively (refs. 12–16; for review, see ref. 5), recombinant plasmids isolated from transfected mammalian cell cultures have not been studied, possibly because of the low amount of replicated DNA that can be recovered for direct visualization on agarose gels. This information is essential for two reasons. First, most transfection experiments of recombinant plasmids have been carried out to study their transcription or recombination in vivo (for examples, see refs. 17, 18). These processes could be affected by the level of DNA supercoiling of the particular recombinant plasmid in the nuclei of transfected cells. Second, since the recombinant plasmids can easily be modified by molecular cloning, knowledge of their superhelicity before and after transfection allows one to study the possible regulation of DNA supercoiling in mammalian cells by specific DNA sequences present in cis (e.g., Z-DNA, enhancer sequences) and by factors acting in trans. For these purposes, we have applied a simple procedure that combines use of the Hirt method of isolating supercoiled DNA (19), chloroquine/agarose gel electrophoresis for separating different supercoiled species (15), and the Southern blot-hybridization technique (20) for examination of the superhelicity of plasmids. As an initial test, we have used this procedure to estimate the specific linking difference of a recombinant plasmid after its replication in a monkey cell line, COS-7. Surprisingly, we found that its superhelicity density (σ) is much lower than that of SV40 DNA.

MATERIALS AND METHODS

DNA Samples. Supercoiled SV40 DNA (gifts from Sophia Kondoleon, Leslie Hallick, and Robert Tjian) was isolated from virus-infected CV-1 cells 72 hr after infection. The plasmid vector psvod (21) was a gift from P. Mellon and T. Maniatis. Plasmid psvoa1p3d, constructed according to Mellon et al. (21), was isolated from bacterial strain RII grown at 37°C. Supercoiled plasmid DNAs were purified from bacteria by the alkaline extraction procedure and ethidium bromide/CsCl gradient centrifugation (22). Relaxed circular DNAs were prepared by treatment with HeLa DNA topoisomerase I (23).

DNA Transfection. Plasmid DNA or SV40 DNA was introduced into COS-7 cells (24) by the DEAE-dextran procedure (25). COS-7 cells were grown on 10-cm plates at 37°C (6% CO2) in Dulbecco’s modified Eagle’s medium/10% fetal calf serum/1% penicillin/streptomycin. At 60–80% confluence, the cells were washed and transfected with plasmid DNA or SV40 DNA (0.1 μg per 10-cm plate) according to Myers and Tjian (26). The only modification was that, after addition of the DNA/DEAE-dextran mixture, the plates were kept at 30°C (instead of 37°C) for 4 hr.

Isolation of Extrachromosomal DNA from Transfected COS-7 Cells. Extrachromosomal DNA was isolated from the transfected COS-7 cells according to Hirt (19) with some modifications. At various times after transfection, the plates were washed with phosphate-buffered saline (15 mM sodium phosphate/150 mM NaCl, pH 7.5; PBS), and the cells were

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Abbreviations: SV40, simian virus 40; kb, kilobase(s).
scraped from the plates into PBS. The cells were collected by spinning at room temperature in a clinical centrifuge (Dynac II, Clay Adams) at 2000 rpm for 5 min. The cell pellet from each plate was then suspended in 750 μl of 10 mM Tris-HCl, pH 7.5–8.0/10 mM EDTA in a 1.5-ml Eppendorf tube. Fifty microfilters of 10% NaODsO₄ and 200 μl of 5 M NaCl were added successively to each tube, and the tubes were kept at 4°C overnight. The tubes were spun in an Eppendorf centrifuge at 4°C for 30 min. Proteinase K (Sigma) was added to the supernatants to 150 μg/ml and the mixtures were incubated at 37°C for 3 hr. The samples were then extracted sequentially with phenol, phenol/Sevag [chloroform/isoamyl alcohol, 20:1 (vol/vol)], and ether. The DNA molecules from each tube were recovered by precipitation with ethanol and then suspended in 200 μl of 10 mM Tris-HCl, pH 8.0/1 mM EDTA.

**Chloroquine/Agarose Gel Electrophoresis and Blot Hybridization.** Electrophoresis of DNA in 0.8% agarose gels containing various concentrations of chloroquine was carried out under the conditions of Shure et al. (15) at room temperature in a 40 × 20 cm horizontal apparatus at 50 V, 70 mA for 40–60 hr. The buffer contained appropriate concentrations of chloroquine. Usually 1/20th of the extrachromosomal DNA isolated from a plate was loaded into a slot, and 1–2 μg of plasmid DNA and/or SV40 DNA was loaded into a separate slot as marker. After electrophoresis, the gels were stained for 30 min with ethidium bromide (0.5 μg/ml of 40 mM Tris-HCl, pH 8.0/8 mM NaOAc/1 mM EDTA). According to the positions of forms I and II of the DNA markers, the appropriate parts of the gels were cut out and irradiated with short-wavelength UV light for 5 min to induce nicks in the supercoiled DNA molecules.

After irradiation, the DNAs in the gel(s) were denatured, neutralized, and blotted onto nitrocellulose filters according to Southern (20). They were then hybridized with plasmid DNA or SV40 DNA labeled with ³²P by nick-translation (27) and autoradiographed on Kodak x-ray films.

To quantitate the relative amounts of different supercoiled DNA species (see Fig. 4), the x-ray films were scanned in a microdensitometer (Quick Scan Jr., Helena Laboratory). Because errors in the values of ρ are approximately ±1, the measured values of ρ were rounded to the nearest integer.

**RESULTS**

**Replication and Supercoiling of Transfected SV40 DNA.** Supercoiled DNAs (form I) isolated from SV40 virions or from SV40-infected cells late in the infection cycle have been analyzed by gel electrophoresis (12, 15). The various species having different linking numbers (topoisomers) were separated on either ethidium bromide/agarose or chloroquine/agarose gels and visualized by ethidium bromide staining, and their distribution was found to be Gaussian. From those experiments, it was concluded that both virion and intracellular SV40 DNA (form I) have an average number of negative supercoiling densities (ρ) of −25 ± 1.

As a control experiment, we have studied the change in supercoiling of SV40 DNA after transfection into COS cells. Supercoiled SV40 DNA (ρ = −25) or relaxed SV40 DNA (prepared by topoisomerase I treatment in vitro) was introduced into COS cells by the DEAE-dextran method. As shown in Fig. 1, relaxed SV40 DNA starts to replicate efficiently 20–28 hr after transfection (lanes 2–7) and attains a topoisomeron distribution similar to that of supercoiled SV40 DNA isolated from virus-infected cells (compare lanes 4–7 with lane 1). Transfection of supercoiled SV40 DNA gave results identical to those for the relaxed form (data not shown).

**Replication of a Recombinant Plasmid, psvo1p3d, in COS Cells.** Having established a procedure to analyze the superhelical distribution of replicated SV40 DNA in mammalian cells, we set out to test whether plasmids having different sequences would attain superhelical densities similar to or different from that of SV40 DNA after replication in COS cells.

There are two ways to measure the number of superhelical turns in a circular DNA molecule. The “band-counting” method devised by Keller (12) is a direct approach. This approach requires extensive gel analysis of preparations of plasmid samples having various numbers of superhelical turns (ρ) centered around different mean values (ρ̄). We have used, instead, as an initial attempt, an indirect method. Whenever possible, SV40 origin-containing plasmid DNAs having molecular weights similar to that of SV40 DNA are used to transfected COS-7 cells. Replicated supercoiled DNAs are then purified, and their topoisomeron distributions are compared side-by-side with that of SV40 DNA by agarose gel electrophoresis and blot hybridization. When appropriate concentrations of chloroquine are used, it should be possible to determine the difference in ρ values between a plasmid and SV40 DNA directly and thus estimate the superhelical density of the plasmid.

The recombinant plasmid, psvo1p3d (Fig. 2A), was constructed by inserting a 1.5-kilobase (kb) Pst I fragment containing the human α₂-globin gene into the Pst I site of the plasmid vector psVod (21). psVod contains SV40 replication origin region (base 5107 to base 97) and lacks the pBR322 sequence inhibitory to replication in mammalian cells (28). To assay psvo1p3d replication in mammalian cells, both the highly supercoiled form (prepared from bacteria strain RR1) and its relaxed form were transfected into COS-7 cells. As shown in Fig. 2B, both forms replicate efficiently. Only a small amount of the transfected DNA remain unreplicated, as demonstrated by Dyn1 and MboI digestion of the Hirt DNA preparation (Fig. 2C).

**DNA Supercoiling of psvo1p3d in COS Cells: Broadening of the Topoisomeron Distribution and Low Superhelical Density.** Close examination of the autoradiographs shown in Fig. 2B and C reveals a number of weak bands in the replicated psvo1p3d population. These bands most likely correspond
to DNA species having absolute numbers of supercoil turns ranging from 1 to 12. Relaxation of these species by *Drosophila* topoisomerase I in vitro (C.-K.J.S., unpublished results) supports this hypothesis. As shown in Fig. 3, these bands are not observed in samples recovered from SV40 DNA-transfected COS-7 cells. Thus, psvoa1p3d has a broader distribution of topoisomer than SV40 DNA after replication in monkey cells. Similar to the results for SV40 DNA, transfection with the supercoiled or relaxed forms of psvoa1p3d yields DNA having the same topoisomer distribution after replication in COS cells (see below).

The different supercoiled species of psvoa1p3d can be resolved on agarose gels containing different concentrations of chloroquine. When the superhelical distributions of SV40 DNA and psvoa1p3d replicated in COS cells were analyzed on the same chloroquine-containing gels, it was found that the topoisomers of replicated SV40 DNA migrated much faster than those of psvoa1p3d. In other words, SV40 DNA is more negatively supercoiled. A comparison of the two DNAs in a gel containing chloroquine at 5 μg/ml is shown in Fig. 4A. From the migration patterns of the same samples on gels containing chloroquine at 2.5 and 7.5 μg/ml (data not shown), respectively, we have concluded that the supercoiled species in lanes 1, 2, 4–6, and 8 of Fig. 4A migrate as negative supercoils. Densitometric tracings of the migration patterns of lanes 4 and 8 of Fig. 4A are shown in Fig. 4B and C. Since the molecular weights of SV40 DNA (5.2 kb) and of psvoa1p3d (5.3 kb) are close, it can be estimated from the positions of the maximum-intensity bands that the average number of supercoil turns of psvoa1p3d in COS cells is \((-20 \pm 1,\) which is equivalent to an approximate superhelical density of \(-0.038\). Comparison of lanes 4 and 5 also suggests that plasmid psvoa1p3d isolated from bacteria RRI has approximately \(-27 \pm 1\) supercoil turns. A cotransfection experiment (Fig. 5) has shown that the observed differences in \(r\) values of SV40 DNA and psvoa1p3d does not result from differences in the transfection and DNA isolation conditions.

**DISCUSSION**

The high-sensitivity procedure presented above has allowed us to study DNA supercoiling of minute amounts of extrachromosomal DNA in mammalian cells. A similar procedure has been used previously to study the population of topoisomeres of plasmids of *Escherichia coli* under different physiological conditions (29). Although nanogram amounts of DNA were used in this study, amounts as low as 1 pg can easily be detected after blot hybridization and autoradiography (data not shown). With further refinement of the procedure, it should be possible to detect differences of 1 or 2 in linking numbers of DNA samples. If necessary, the band-counting method (12) can be used in combination with our procedure for estimating the number of supercoil turns of episomes isolated from mammalian cells directly.

Unlike prokaryotes, in which DNA supercoiling comes mainly from the balance between gyrase action and topoisomerase I action (for review, see ref. 5), the working model for DNA supercoiling in eukaryotic cells has two pathways that may function independently or collaboratively. First, much of the supercoiling may be accounted for by the binding of DNA duplexes to the histone octamers to form

![Fig. 2. Gel analysis of plasmid psvoa1p3d replication in transfected COS-7 cells. (A) Map of psvoa1p3d, which contains a 1.5-kb *Pst* I restriction fragment of human DNA cloned into the *Pst* I site of pBR322 and a 233-base-pair sequence containing the SV40 replication origin. For details of the construction, see ref. 21. (B) Electrophoresis pattern of psvoa1p3d DNA isolated from transfected COS cells. A 0.1-μg sample of relaxed (lanes 1–6) or supercoiled (lanes 7–12) psvoa1p3d DNA was transfected into COS-7 cells. Low molecular weight DNAs were isolated by the Hirt method at various times after transfection (lanes 1 and 7, 0 hr; lanes 2 and 8, 20 hr; lanes 3 and 9, 28 hr; lanes 4 and 10, 36 hr; lanes 5 and 11, 48 hr; lanes 6 and 12, 72 hr), electrophoresed in an 8% agarose gel, blotted, and hybridized with nick-translated psvoa1p3d. The blot was exposed to x-ray film for 24 hr without an intensifying screen. Note the presence of supercoiled species in between the form III DNA and the major band of supercoils. (C) Electrophoresis pattern of DNAs from lanes 1–6 of B after digestion with *Dpn* I (lanes 1–6) or *Mbo* I (lanes 7–12). The blot was autoradiographed for 24 hr without an intensifying screen.

![Fig. 3. Comparison of electrophoresis patterns of low molecular weight DNAs isolated from COS-7 cells transfected with relaxed form SV40 DNA (lanes 2–7) or psvoa1p3d (lanes 8–13). DNAs were electrophoresed in an 8% agarose gel at various times after transfection: lanes 2 and 8, 0 hr; lanes 3 and 9, 20 hr; lanes 4 and 10, 28 hr; lanes 5 and 11, 36 hr; lanes 6 and 12, 48 hr; lanes 7 and 13, 72 hr. Lane 1: 3 ng of relaxed form SV40 DNA (control sample). The hybridization probes were nick-translated SV40 DNA and psvoa1p3d. Exposure was for 24 hr without an intensifying screen.](image-url)
The molecular weight of the DNA and its relaxed form attain a mean value of \(-25 \pm 1\) after transfection and replication in monkey cells (ref. 12; Figs. 1 and 4). The sensitivity of our assay does not allow us to study the change in superhelical density of unreplicated plasmids (for example, see Fig. 2C). However, Ryoji and Worcel (10) were able to follow the time course of supercoiling and chromatin assembly of unreplicated plasmid after its injection into Xenopus oocytes.

The broadness in topoisomer distribution of the recombinant plasmid psvoalp3d and its low superhelical density may be associated with the chromatin assembly process in the monkey cells. Analysis of the transfected and replicated plasmids on chloroquine-free gels reveals a broader and more skewed topoisomer distribution of psvoalp3d than that of SV40 DNA (Fig. 3). Previous studies have shown that the topoisomer distribution of intracellular SV40 DNA is itself broader than the equilibrium thermal distribution (13-15). As discussed by previous workers, this heterogeneity is likely to be a reflection of the structural aspects of the chromatin. Taking the analogy, we argue that the heterogeneity in the topoisomer distribution of the replicated recombinant plasmid is the result of an "imperfection" of the chromatin assembly in the monkey cells. Certain sequences present on the pBR322 vector or the human DNA fragment may inhibit nucleosome formation. Alternatively, some specific sequences of the SV40 genome may be required for an efficient chromatin assembly process. For example, phasing of the nucleosomes on the Xenopus SS RNA gene seems to be modulated by a specific DNA sequence and a trans-acting factor (33, 34).

Side-by-side gel analysis (Figs. 4 and 5) has shown that psvoalp3d has, on the average, 5 fewer negative supercoils than SV40 DNA. This is unlikely to be a result of preferential binding of chloroquine to, and hence a greater unwinding of, psvoalp3d DNA in gels. The relaxed forms of SV40 DNA and psvoalp3d have similar mobilities in gels containing chloroquine at 0-2 \(\mu g/ml\) (unpublished results). The difference of 5 supercoils corresponds to a difference of 20% in superhelical density and a free energy difference of approximately 25 kcal/mol (1 kcal = 4.18 J) of free energy (35). Similar to the heterogeneity in the topoisomer distribution, there are several possible factors that may modulate the superhelical density of the recombinant plasmid. First, DNA supercoiling may be affected by the virion assembly process in SV40 DNA-transfected but not in psvoalp3d-transfected cells. However, this is unlikely because the topoisomer distribution of SV40 DNA remains the same between 28 and 72 hr after transfection (Fig. 1). Furthermore, Shure et al. (15) have shown that intracellular and virion SV40 DNAs have the same superhelical density (15). Second, the difference in superhelical density may be related to the transcriptional activities of the two plasmids. Throughout the infection cycle, only a minor fraction (less than 5%) of the SV40 DNA molecules are being transcribed (36). On psvoalp3d, the \(\alpha\)-globin gene is transcribed in COS-7 cells (21). If a major

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**Fig. 4.** Comparison of superhelical distributions of SV40 DNA and psvoalp3d replicated in transfected COS-7 cells. COS-7 cells were transfected with 0.1 \(\mu g\) of supercoiled SV40 DNA (lanes 1 and 2), relaxed form SV40 DNA (lanes 3 and 4), supercoiled psvoalp3d (lanes 5 and 6), or relaxed form psvoalp3d (lanes 7 and 8). Low molecular weight DNAs were isolated 0 hr (lanes 1, 3, 5, and 7) and 72 hr (2, 4, 6, and 8) after transfection and analyzed on an 0.8% agarose gel containing chloroquine at 5 \(\mu g/ml\). (A) Autoradiograph of the blot after hybridization with nick-translated SV40 DNA and psvoalp3d. (B and C) Microdensitometric tracings of lanes 4 and 8 of A. The arrows indicate the bands of maximum intensity.

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**Fig. 5.** Cotransfection experiment. psvoalp3d and SV40 DNA (0.1 \(\mu g\) of each) were mixed and cotransfected into COS-7 cells. Seventy-two hours after transfection, low molecular weight DNAs were isolated by Hirt procedure. Triplicate samples were electrophoresed on an 0.8% agarose gel containing chloroquine at 5 \(\mu g/ml\). The samples were then blotted onto nitrocellulose, the paper was cut, and three pieces of the blots were hybridized with nick-translated SV40 DNA (lane 1), pBR322 (lane 2), and (SV40 DNA/pBR322) (lane 3) and autoradiographed.
fraction of the psvoa1p3d molecules are transcriptionally active, then the observed linking number difference may reflect a direct or an indirect effect of the transcription process on supercoiling. It is worth note, however, that studies of minichromosomes in monkey cells (9) and in *Xenopus* oocytes (10, 37) have shown that the total number of supercoil turns constrained in transcriptionally active or inactive minichromosomes is the same. A third possibility is that psvoa1p3d lacks a specific sequence(s) that is required for either the phasing of nucleosomes, as described above, or the recognition by a gyrase-like activity in the monkey cells.

One interesting candidate for this missing sequence is the SV40 enhancer region, which has recently been suggested to be a preferential binding site of DNA topoisomerase II in vivo (38). Topoisomerase II is the eukaryotic equivalent of the bacterial gyrase (for discussion, see ref. 5), although its ability to introduce negative supercoil turns into covalently closed, duplex DNA has not been demonstrated explicitly either in vitro or in vivo. Finally, according to the first pathway of chromatin assembly mentioned above, there may be certain regions (e.g., the pBR322 sequence or the α1-globin gene) of psvoa1p3d that do not form a nucleosome structure.

Although many previous studies have shown that most, if not all, of the minichromosomes of recombinant plasmids do assemble in vivo into periodic nucleosome-like structures as judged by the generation of a nucleosomal ladder of DNA fragments after micrococcal nuclease digestion (ref. 37 and references therein), the assay is not sensitive enough to conclude whether most of the plasmids form nucleosomes throughout their entire length. Further transfection studies of different recombinant constructs using the approaches presented here should provide information on the nature of the DNA element(s) that is involved in the regulation of DNA supercoiling and chromatin assembly in mammalian cells.

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