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# Supercoiling in prokaryotic and eukaryotic DNA: changes in response to topological perturbation of plasmids in *E. coli* and SV40 *in vitro*, in nuclei and in CV-1 cells

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## ABSTRACT

Changes in DNA linking number have been observed in plasmid DNA purified from E. coli cells after the cells were treated with chloroquine. Chloroquine, a DNA intercalating drug, unwinds the DNA, decreasing the levels of negative supercoiling. Following this in vivo topological perturbation, within minutes DNA gyrase decreases DNA linking number producing more negatively supercoiled DNA topoisomers. Following the removal of the drug from cells, within minutes topoisomerase I or DNA gyrase increases the linking number restoring the original level of supercoiling. Analogous changes in DNA linking number after addition of chloroquine are observed in purified plasmid DNA, and in purified SV40 minichromosomes in the presence of exogenous topoisomerase. Changes in linking number are also observed in SV40 chromosomes in isolated nuclei and in SV40 DNA purified from CV-1 cells following topological perturbation with chloroquine. These results suggest that eukaryotic cells may have mechanisms to maintain a defined level of DNA supercoiling.

## INTRODUCTION

Most DNA isolated from natural sources is negatively supercoiled. In prokaryotic cells it has been demonstrated that much of the free energy inherent in supercoiled DNA is equilibrated as unrestrained torsional tension in the winding of the DNA double helix (1). However, not all the supercoils in DNA exist in an unrestrained form. It appears that part of the supercoils are restrained by the association of DNA with the nucleosome-like HU proteins of bacterial (2). It has been shown that HU in vitro can restrain about half the natural level of torsional tension that exists in DNA in vivo (3). Plasmid DNA in E. coli acts as though it has only half the number of supercoils measured for that plasmid when purified from cells as evidenced by the formation of cruciforms and Z-DNA (4-7). The level of DNA supercoiling in E. coli appears to be maintained by two enzymatic activities; DNA topoisomerase I and DNA gyrase (8-10). From analysis of differences in linking number of plasmids extracted from bacterial cells grown at different temperatures, Goldstein and Drlica (11) demonstrated that E. coli have homeostatic mechanisms which finely control the level of DNA supercoiling in vivo. It has been clearly demonstrated that DNA supercoiling and probably the precise level of supercoiling is important in DNA replication, recombination, and in the control of gene expression in prokaryotes (8,9).

The DNA of eukaryotic cells is organized somewhat differently than the DNA in prokaryotic cells. Although the DNA is wound with negative superhelical tension, most of the supercoils appear to be restrained by the organization of the DNA in nucleosomes. Thus, the DNA of eukaryotic cells, on average, acts as though it is relaxed (1). The supercoils are associated with the wrapping of the core DNA around the histone octomers and the DNA that comprises the linker is then presumably torsionally relaxed. As a result of nucleosomal organization only a small and perhaps variable fraction of DNA may be wound with unrestrained torsional tension, but there has been no direct evidence for the existence of unrestrained DNA supercoils in living eukaryotic cells. Temperature shift experiments, by Saavedra and Huberman, have shown that in yeast a linking number change is observed following a change in temperature (12) and that either topoisomerase I or topoisomerase II can reequilibrate the linking number in vivo. These experiments suggest that the DNA in non-nucleosomal associated yeast DNA in vivo is maintained in a relaxed state (12). Similar temperature shift experiments with SV40 have not shown a re-equilibration of linking number following temperature shifts (13). Consequently, it has not been possible to ask if topological perturbation of linking number in vivo results in re-equilibration of the linking number in higher eukaryotes.

In the present study we utilize a novel approach to perturb the level of supercoiling in vivo to ask if eukaryotic cells have mechanisms to regulate the level of DNA supercoiling. Our approach is based on the use of chloroquine, a DNA intercalating drug (14,15), to alter the topology of supercoiled DNA. Eukaryotic cells appear to tolerate certain concentrations of the drug for limited times (16), suggesting it may be useful for altering DNA topology in vivo. The topology of covalently closed circular DNA is described by the equation $L = T + W$, where L is the topological linking number or the number of times the two strands of the DNA are intertwined, T is the number of helical turns in DNA, and W is the writhing number of DNA (the intuitive concept of DNA supercoiling). In vitro the intercalative binding of chloroquine effectively decreases the number of helical turns, T, in DNA. This results in a relaxation of negative DNA supercoils, W. Since $L = T + W$, if cells recognize and maintain a precise level of supercoiling, or effectively W, following chloroquine addition a compensatory decrease in L should be observed. This should result in an increase in negative supercoiling after chloroquine is removed. Experimentally, it is possible to measure $\Delta L$, where $\Delta L = L - L_o$, and Lo is the linking number of relaxed DNA. Chloroquine binding effectively lowers Lo such that a compensating decrease in L must occur if cells maintain a constant level of supercoiling (or $L - Lo$). We observe changes in DNA supercoiling levels after perturbation of DNA topology by addition of chloroquine to plasmids in E. coli cells which have clearly demonstrated mechanisms to regulate levels of supercoiling (10,11). Similar changes in linking number are observed in purified SV40 minichromosomes in the

presence of the chloroquine after the addition of exogenous topoisomerase as well as in SV40 DNA extracted from nuclei or infected CV-1 cells treated with chloroquine.

## MATERIALS AND METHODS

Bacterial strains and plasmid purification: E. coli strains used were HB101; SD108, a topA$^+$ derivative of DM800, a strain containing gyrB225; and recA56 derivatives of JTT1 and RS2 (TopA10) (17). The latter three strains were obtained from Karl Drlica. Cells were grown in K medium (1) with 25 μg/ml tetracycline or 25 μg/ml ampicillin. 10 μg/ml antibiotic concentrations were used for RS2 (18). Plasmids used were pMB9, pCGTA-C (7) and pBR322. During exponential growth in K media at 2-6 x $10^8$ cells/ml various concentrations of chloroquine diphosphate (Sigma) were added as described in the Figure Legends. 10 ml samples were chilled in an ice-water bath, cells were immediately concentrated by centrifugation at 4°C, washed once with cold M9 buffer (1), suspended in 200 μl of a buffer containing 8% sucrose, 5% Triton X100, 50 mM EDTA, 50 mM Tris pH 8, and 10 μl of a 10 mg/ml solution of lysosome was added. After 1 minute at room temperature, the cell suspension was placed in a boiling water bath for 1 minute and DNA was purified as described (19). At least 0.5-1 μg of plasmid DNA was obtained from 10 ml culture.

Eukaryotic cells and SV40 DNA purification: African green monkey kidney cells (CV-1) were grown in Dulbecco's modified Eagles medium, supplemented with 50 U/ml Penicillin, 50 μg/ml Streptomycin, 10% fetal calf serum, and 2 mM glutamine, at 5% $CO_2$ and 37°C. At a density of 2-4 x $10^6$/100 mm plastic dish, cells were infected with 100 pfu/cell of SV40 (strain 776) for 1 hr. Forty eight hours after infection, chloroquine was added as described in the Figure Legends. After chloroquine treatment, cells were washed with PBS (1) and lysed as described by Chen and Hsu (20) with some modifications. Briefly, 1 ml of lysis buffer (1% SDS, 0.4 M NaCl, 10 mM Tris pH 7.6, 1 mM EDTA) was added to each dish; dishes was rocked at room temperature for 5 minutes and then 200 μl of 5 M NaCl were added. The lysate was harvested and samples were immediately frozen at -20°C for at least 1 hr and then thawed and centrifuged at 17,000 g for 15' at 4°C. Nucleic acids in the supernatants were precipitated by addition of 2 Vol EtOH, resuspended in 2 ml TEN (10 mM Tris pH 7.6, 1mM EDTA, 50 mM NaCl), extracted twice with phenol, three times with chloroform:isoamyl alcohol (24:1) and the DNA reprecipitated. In some experiments samples were extracted before ethanol precipitation. Supercoiled SV40 DNA was then purified from an ethidium bromide CsCl gradient. For a modified Hirt (21) purification procedure cells were incubated 5 mins at 20°C in 0.8 ml of 0.1 M NaCl, 10 mM Tris pH 7.5, 20 mM EDTA, 0.2% SDS. 50 μg/ml Proteinase K was then added for 30 min at 37°C, NaCl was adjusted to 1 M, and cells were scraped into tubes and stored at 4°C overnight. SV40 DNA was purified from the

supernatant as described above. To minimize any differences in superhelical densities of SV40 populations of different dishes, all samples consisted of a mixture of three culture dishes of cells.

SV40 minichromosomes were purified as described by Barsoum and Berg (22). A typical mixture for the relaxation assay of SV40 minichromosomes contained: 10 µg/ml supercoiled DNA, 1 to 5 units of topoisomerase extract/µg DNA in a buffer containing 10% sucrose, 0.18 M NaCl, 10 mM Hepes pH 7.8, 1 mM $Na_2EDTA$, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) in a final volume of 50µl. Topoisomerase extracts were prepared from HeLa or CV-1 cells as described by Germond et al. (23). Purified DNA was relaxed in the above Hepes buffer with 10% sucrose. Topoisomerase reactions were stopped by the addition of 0.5% SDS. SV40 DNA was purified from minichromosomes by proteinase K digestion (50 µg/ml) for 30 minutes at 37°, phenol and $CHCl_3$ extractions, and EtOH precipitation.

For purification of nuclei, CV-1 cells, 48 hrs after infection, were washed and collected as described by Barsoum and Berg (22). The pellet containing the nuclei was suspended in buffer containing 10 mM Tris pH 6.8, 0.1 M NaCl, 10 mM EDTA, 0.1 M PMSF and incubated at 37°C for 30 minutes and gently mixed occasionally without or with different chloroquine concentrations as described in Figure Legends. After incubation, nuclei were collected by centrifugation and resuspended in 10 mM Tris pH 7.6, 0.4 M NaCl, 5 mM EDTA. 1% SDS was added, and after 1M NaCl addition, samples were frozen and SV40 DNA was extracted as described above.

Agarose gel electrophoresis: 1% agarose and 1.5-3.0 µg/ml chloroquine were used for the SV40 DNA analysis. 1-1.5% agarose and 3-7 µg/ml chloroquine were used for plasmid DNAs analyses. Gel and running buffer were TAE (40 mM tris, 25 mM Na Acetate, 1 mM EDTA pH 8.3). Gels (30 x 15 x .3 cm) were run at 75V (2.5 V/cm) for 24 hours with recirculation of buffer. Gels were washed in distilled water, stained with 0.5 µg/ml of ethidium bromide and photographed. Densitometric analyses were done using an EC Densitometer equipped with a 3390 Hewlett Packard integrator.

## RESULTS

### Chloroquine Induced Linking Number Changes in a Prokaryotic System

Levels of DNA supercoiling in plasmids in E. coli change following chloroquine treatment: To test the applicability of topological perturbation by addition of chloroquine we examined the effect on plasmids in E. coli where mechanisms for maintaining a fixed or precise level of DNA supercoiling in E. coli have been clearly demonstrated (11). Intercalation of chloroquine unwinds the DNA helix and removes negative superhelical turns. Thus, we expect that following addition of chloroquine, a linking number decrease should occur, and an increase in negative supercoiling should be observed after purification of DNA from cells.

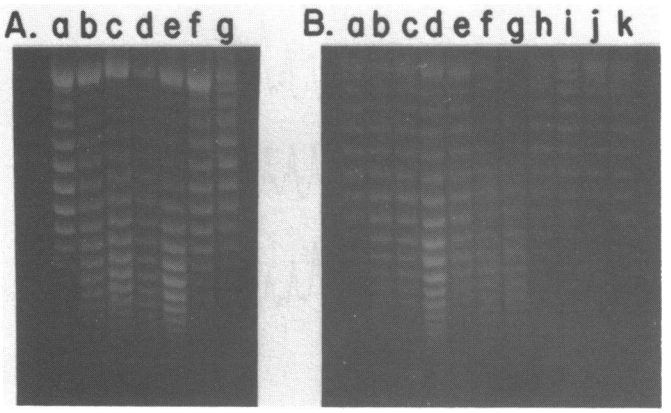


Figure 1: DNA linking number in pBR322 in E. coli changes following chloroquine treatment. Panel A: E. coli cells were grown in presence of chloroquine from 1 to 5 x $10^8$ cells/ml. pBR322 DNA was then purified and analyzed on an agarose-chloroquine gel. Lane a: control DNA (no chloroquine treatment). Lanes b-d are DNA purified from cells grown in media containing 500, 750, 1000 μg/ml chloroquine respectively. Lanes e-g are DNA samples purified from cells grown in presence of 1000 μg/ml chloroquine then washed with media to remove the drug, and incubated in media for 0, 1.5 and 3 hrs respectively. Panel B: Lanes a-f: E. coli cells were grown in a density of 4 x $10^8$ cells/ml. Lane a: Control DNA (no chloroquine treatment). Lanes b-e are DNA samples purified from cells treated with 1000 μg/ml chloroquine for 15 minutes, 30 minutes, 60 minutes and 120 minutes respectively. Lanes f-k: cells were grown 1000 μg/ml chloroquine containing medium for 2 1/2 generations and DNA purified from the cells (lane f) or DNA was purified from cells after being washed and resuspended in K medium for 0, 0.5, 1.0, 2.0 and 3 hrs respectively (lanes g-k).

Figure 1 shows the DNA topoisomer distribution of pBR322 extracted from E. coli cells treated with different chloroquine concentrations: lanes a-d of panel A show an experiment in which E. coli cells have been grown in the presence of 0, 500, 750, and 1000 μg/ml chloroquine for 2 1/2 generations. The negative superhelical densities of DNA from the chloroquine treated cells are higher than those of DNA from control untreated cells. A chloroquine concentration of 100 μg/ml had little effect on the topoisomer distribution (data not shown); at drug concentrations between 500 and 2000 μg/ml this increase in negative supercoiling appears to be "saturated" in that there was little difference in the linking number change between these concentrations.

A time course following chloroquine addition at 1 mg/ml is shown in lanes a-e of Figure 1 (panel B). Densitometric analysis of the gel showed that the change in linking number was essentially complete by 15 minutes. Results obtained at earlier times following drug treatment are shown in a densitometric scan of a gel in Figure 2 where changes were measured between 0-5 minutes. By 2.5 minutes a linking number change

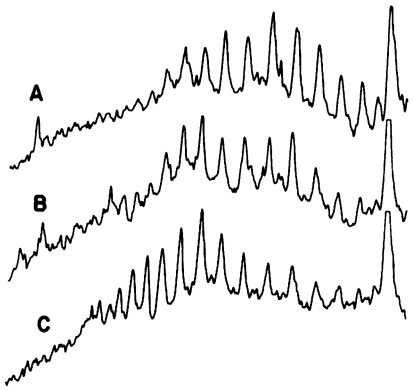


Figure 2: Linking number changes in pBR322 DNA in E. coli following chloroquine treatment. pBR322 DNA was purified from E coli cells treated with 1000 μg/ml chloroquine for 0, 2.5 and 5 minutes. Shown are densitometric tracings of the gel photographs for the 0, 2.5 and 5 min samples, scans A-C, respectively. The lanes scanned from top (relaxed) to bottom (supercoiled) are shown from right to left. The large peak at the far right is nicked DNA.

was observed. We have observed changes in DNA linking number only 30 sec after chloroquine addition (data not shown). There is a shift in the Gaussian center of the topoisomer distribution of $\Delta L = -3.5 \pm 1$ between the control and the 5 minutes drug treated sample. This shift results from a reduction in the peak heights of the slow migrating (less supercoiled) bands with a concominant increase in the faster (more supercoiled) bands. In addition, there is the appearance of more negatively supercoiled topological isomers than can be accounted for in the shift of the Gaussian peak.

If cellular enzymatic activities are able to re-establish normal DNA supercoiling levels after chloroquine perturbation, analogous mechanisms of compensation may act after the removal of the drug to restore the original DNA linking number. To test this, chloroquine was washed from cells after a 30 minutes pulse and the cells were allowed to recover up to 3 hrs. The results shown in Figure 1A, lanes e to g and Figure 1B, lanes g to k, indicate that the cells re-equilibrate the linking number of DNA back to original levels. These reverse mechanisms are also very rapid, acting significantly within 0.5-1 minutes after the removal of chloroquine (data not shown).

Effect of topA10 and gyrB225 mutations on chloroquine induced linking number changes: The observed changes of the DNA linking number are presumably the result of a balance between topoisomerase I and DNA gyrase. Analogous experiments were performed using E. coli strains carrying mutations in topoisomerase I (topA10) or DNA gyrase (gyrB225). Consistent with the results of Pruss et al. (18,24) the negative

**TABLE 1**

**Linking Number Changes in E. coli Mutants Following Chloroquine Treatment**

| E.coli Strain/Plasmid | Addition of 1 mg/ml chloroquine[a] | | Removal of 1 mg/ml chloroquine[b] | |
|---|---|---|---|---|
| | Time after addition min | ΔL[c] | Time after removal min | ΔL |
| HB101/pBR322 wild type | 2.5 | -3 ± 1.5 | | |
| | 5 | -3.5 ± 1 | | |
| | 30 | -3.5 ± 1 | 30 | +3.5 ± 1 |
| JTT1/pCGTA-C wild type | 30 | -1.5 ± 0.5 | | |
| RS2/pCGTA-C topoA 10 | 30 | -4.5 ± 2 | 5 | +4.5 ± 2 |
| SD108/pCGTA-C gyrB225 | 30 | 0 | | |
| | 60 | 0 | | |

a,b E. coli cells were grown and DNA purified as described in Materials and Methods.
c Linking number changes. The quantitation was from densitographic analyses of the gels. ΔL represents the shift of the Gaussian center between the control and the chloroquine treated cells or between chloroquine treated and chloroquine washed cells. All experiments were repeated at leat three times. There was more variation in the linking number change in RS2 perhaps due to the more heterogenous topoisomer distribution (24).

superhelical density of pCGTA-C (2715 bp) was higher by ΔL = -2 (or 14%) in the topA10 strain (RS2) compared to the isogenic wild-type strain (JTT1). As shown in Table 1, DNA in the topA10 mutant (RS2) showed a larger linking number change (ΔL = -4.5) following chloroquine treatment than DNA in the wild-type strain. The magnitude of this change was more variable than that in wild type cells. It is not known if this is due to increased permeability to chloroquine or a function of the increased heterogeneity of linking number or other consequence of the topA10 mutation (24). DNA from the gyrB225 strain (SD108) (which was less negatively supercoiled than DNA from wild-type cells (18)) showed no change in linking number in reported experiments even after longer times of treatment. This suggests that the decrease in linking number to produce higher levels of negative supercoiling after chloroquine perturbation may be dependent on DNA gyrase activity. The increase in linking number to produce lower levels of negative supercoiling following removal of chloroquine occurred within 5 minutes in the topA10 mutant (ΔL = + 4.5) suggesting that E. coli DNA gyrase or the residual topoisomerase I activity was sufficient to re-establish natural levels of DNA supercoiling. This result that gyrase can relax supercoils in vivo in absence of topA10 is consistent with recent observation of Pruss et al. (25).

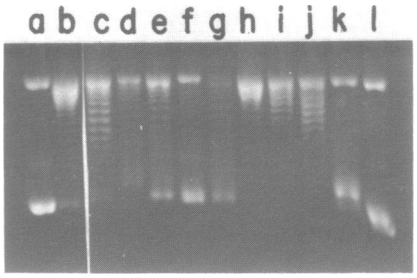


Figure 3: Chloroquine induced linking number changes in pMB9 DNA in vitro. Lane a: supercoiled pMB9 (control). Lanes b-g: pMB9 DNA samples were first relaxed at 37°C for 30 minutes by DNA topoisomerase. Following relaxation, 0, 10, 50, 100, 1000, and 10,000 μg/ml chloroquine were added and incubation was continued for 30 minutes, lanes b-g, respectively. Lanes h-l: are aliquots of samples c-g respectively, incubated again at 37°C for 30 minutes after a 1:10 dilution in topoisomerase buffer and addition of additional topoisomerase activity.

Chloroquine Induced Linking Number Changes in a Eukaryotic System: SV40

Chloroquine induced linking number changes in purified DNA: Before examining the effect of chloroquine on linking number changes in SV40 we wished to test any possible interference by this drug on the relaxing activity of DNA topoisomerase I (Figure 3). The addition of chloroquine concentrations from 10 to 10,000 μg/ml to purified relaxed DNA results in the introduction of positive supercoils. The addition of either HeLa or CV-1 eukaryotic topoisomerase I activity relaxed these positive supercoils and on removal of the chloroquine led to the production of more negatively supercoiled DNA molecules (lanes c-g). These results indicate that eukaryotic topoisomerase I was still active in the presence of at least 1 mg/ml chloroquine. At 10 mg/ml topoisomerase activity was inhibited. Following the addition of chloroquine and topoisomerase, the subsequent addition of buffer to dilute the concentrations of chloroquine led to the generation of negatively supercoiled molecules. The topoisomerase re-equilibrated these molecules, producing a decrease in supercoiling levels (lanes h - l) as expected. These results show that topoisomerase I activity can relax positively or negatively supercoiled DNA molecules in the presence of chloroquine.

Experiments similar to those just described above were performed to carefully quantitate the linking number changes of purified pMB9 in the presence of concentrations of chloroquine below 100 μg/ml. From these, shown in Table 2, data it is possible to calculate the unwinding expected for SV40 minichromosomes, as described below.

Re-equilibration of linking number of purified SV40 minichromosomes in presence of chloroquine: To examine the effect of topological perturbation by chloroquine, SV40 minichromosomes, purified in 0.36 M NaCl to contain no endogenous topoisomerase

**TABLE 2**

**Quantitation of Linking Number Change in Purified pMB9 and SV40 Minichromosome**

| Chloroquine μg/ml | ΔL pMB9[a] | ΔL SV40 calc. calculated[b] | Δ L SV40 Minichromosomes[c] | Δ L SV40 in nuclei[d] | Δ L SV40 Minichromosomes, expected[e] |
|---|---|---|---|---|---|
| 0 | 0 | | 0 | | |
| 10 | -3 | -2.95 | -1.5 ± 1 | | -0.9 |
| 50 | -9.75 | -9.57 | -2.5 ± 1 | | -2.9 |
| 100 | -16.5 | -16.20 | -5 ± 1 | -5 ± 1 | -5.3 ± 0.5 |

a) Linking number changes, ΔL, were measured by densitometric analysis on a series of chloroquine gels that allowed resolution of the topoisomers in 0, 10, 50, and 100 μg/ml chloroquine treated samples.

b) ΔL SV40 was calculated assuming 5340 bp for pMB9 and 5243 bp for SV40.

c) ΔL SV40 minichromosomes were determined from the gel shown in Figure 4, smearing in the gel made precise quantitation difficult, thus the high error estimates.

d) ΔL SV40 in nuclei was determined from the 100 μg/ml chloroquine treated sample shown in Figure 5.

e) ΔL SV40 minichromosome, expected was calculated assuming 25 nucleosomes per SV40 DNA molecule, each restraining 145 bp DNA, which would result in 3625 bp being unavailable for chloroquine binding. The ΔL change is that resulting from chloroquine binding to the 1618 bp of available linker DNA.

activity (22), were treated with increasing amounts of chloroquine in the presence of exogenous topoisomerase I from either CV-1 or HeLa cells (with identical results). Treatment of purified SV40 minichromosomes at 37° for 30 minutes in the presence of topoisomerase but in the absence of chloroquine resulted in the reproducible introduction of slightly more negative supercoils in SV40 DNA (Figure 4, lanes a,b). This is opposite from the result of Barsoum and Berg (22), but does appear to be very similar to results shown by Rodriguez-Campos et al. (26) (their Figure 4, lanes 1 and 2). This may reflect interaction between proteins in the topoisomerase extract and the SV40 minichromosomes or differences in the reaction conditions. The re-equilibration of linking number following chloroquine addition is shown in Figure 4, lanes c-h. The entire population of topoisomers shifts to a more negatively supercoiled position after increasing concentrations of chloroquine up to 1 mg/ml. Following a 1:10 dilution of the chloroquine concentration by addition of buffer to the samples, the level of supercoiling of these samples (lanes i-l), returns to a topological isomer distribution comparable to that of the final expected chloroquine concentration. As shown above, 10 mg/ml chloroquine begins to inhibit topoisomerase activity (lane h). These results suggest that the topology of SV40 minichromosomes is altered by chloroquine addition and that topoisomerases can re-equilibrate positive or negative supercoils back to, presumably, a relaxed state.

There are two arguments that can be made suggesting that these concentrations of

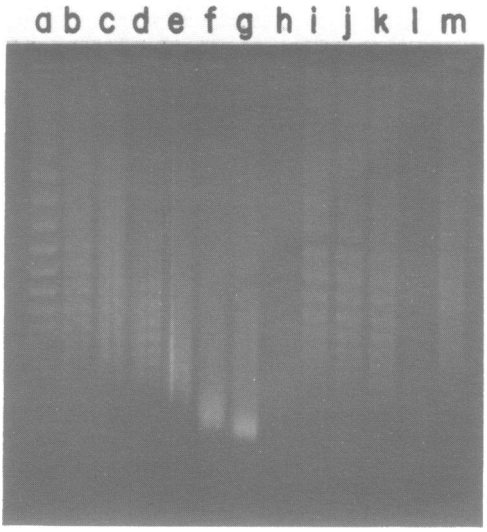


Figure 4: Chloroquine induced linking number changes in purified SV40 minichromosomes. Lane a: DNA from purified minichromosomes. Lane b: purified minichromosomes relaxed by the addition of exogenous topoisomerase at 37°C for 30 minutes. Lanes c-h: As lane b but with the addition of 10,50, 100, 500, 1000 and 10,000 µg/ml chloroquine. Lanes i-l: are aliquots of samples e to h respectively which were diluted 1:10 in topoisomerase dilution buffer and incubated an additional 30 minutes at 37°C following readdition of topoisomerase. Lane m: Is an aliquot of sample b which was treated as lanes i-l.

chloroquine below 100 µg/ml are not displacing nucleosomes from the SV40 minichromosomes. First, the expected $\Delta L$ for SV40 minichromosomes containing 25 nucleosomes can be calculated assuming that the 3625 bp in the nucleosome cores are not available for topological perturbation of chloroquine and only the 1618 bp comprising the linker DNA can bind chloroquine. It is expected that at equal chloroquine concentrations, free DNA or linker DNA in a minichromosome should have a similar occupation of binding sites in terms of chloroquine molecules per kbp DNA. This should result in only about 30% of the $\Delta L$ change observed for naked DNA. The linking number change measured for purified SV40 minichromosomes treated with 10, 50, and 100 µg/ml chloroquine are shown in Table 2. It is evident that the observed $\Delta L$ values are identical, within experimental error, to the theoretical $\Delta L$ values calculated for SV40 minichromosomes, as described above, and are not consistent with the $\Delta L$ calculated for naked SV40 DNA (Table 2). Second, we have compared the sedimentation of $^{3}H$-labeled SV40 purified minichromosomes incubated 30 minutes at 37°C in the presence of 0, 1, or 10 mg/ml chloroquine and then sedimented on neutral sucrose gradients. This allows testing of higher concentrations of chloroquine than is easily measured on gels. Migration positions of SV40

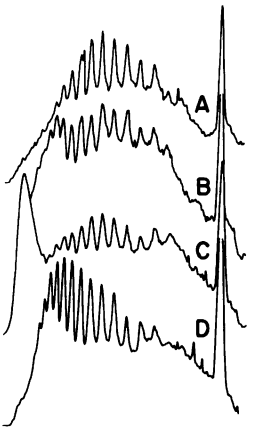


Figure 5: Effect of chloroquine concentration on SV40 DNA in purified nuclei. SV40 nuclei were purified as described under Methods, and incubated at 37°C for 30 minutes with 0, 100, and 1000 μg/ml chloroquine (scans a-c, respectively). During incubation, samples were occasionally mixed gently. After 30 min, an aliquot of the sample treated with 1000 μg/ml chloroquine was diluted 10-fold by the addition of buffer and reincubated for an additional 30 minutes at 37°C (scan D). Lanes scanned from top to bottom are shown from right to left. The large peak at the far right is nicked DNA.

minichromosomes treated with 0 and 1 mg/ml chloroquine were indistinguishable suggesting that no significant structural rearrangments or loss of nucleosomes had occurred (data not shown). SV40 minichromosomes treated with 10 mg/ml chloroquine showed a reduced sedimentation rate, although a greater rate than that for minichromosomes treated with proteinase K and SDS (data not shown). Although this analysis is not as sensitive as measuring linking number changes it also suggests that chloroquine at concentrations less than 1 mg/ml is not drastically disrupting or altering nucleosomal organization.

Linking number of SV40 DNA in purified nuclei changes following chloroquine treatment: We asked if SV40 in nuclei purified 48 hr after infection would change DNA linking number in response to chloroquine addition in a fashion similar to that for purified minichromosomes. The effect of chloroquine treatment on SV40 nuclei is shown in Figure 5. Purified nuclei were incubated 30 minutes at 37°C with 0, 100 and 1000 μg/ml chloroquine (scans a-c, respectively). The effect on the distribution of topological isomers observed following chloroquine treatment was essentially identical to that for purified minichromosomes. The linking number change was reversible as shown in scan d where an aliquot of the sample treated with 1000 μg/ml chloroquine for 30 minutes was diluted 10-fold and incubated an additional 30 minutes at 37°C. Following the 10-fold dilution the level of supercoiling was comparable to that of the 100 μg/ml chloroquine treated sample (lane b) showing that re-equilibration occurred. Interestingly, about 35%

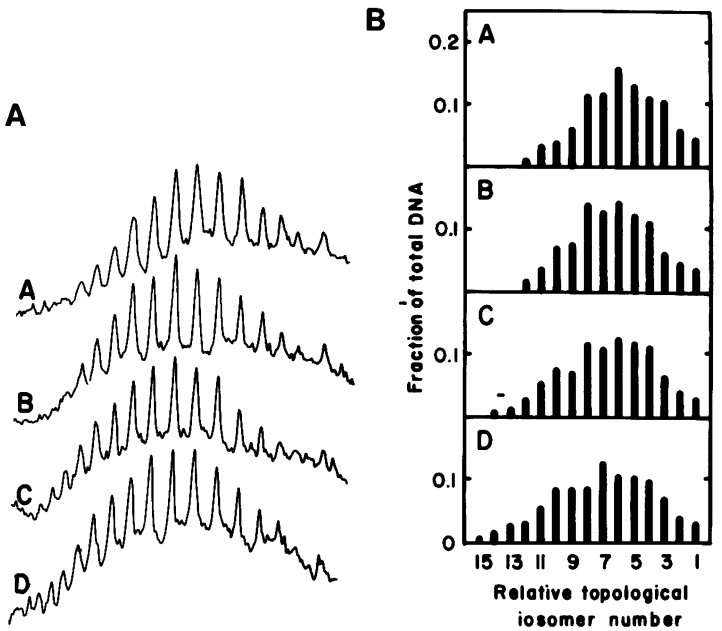


Figure 6: DNA linking number of SV40 minichromosomes in CV-1 cells changes following chloroquine treatment. Panel A: CV-1 cells were treated with chloroquine 48 hrs after SV40 infection. SV40 DNA was then purified as described under Methods and analyzed on an agarose-chloroquine gel. Scan A: Control DNA (no chloroquine treatment). Scans B-D: SV40 DNA purified from CV-1 cells treated with 1000 μg/ml chloroquine for 5, 10, and 15 minutes respectively. The scans from top to bottom are shown from right (top) to left (bottom). Panel B: Quantitative analysis corresponding to densitometric traces of panel A. On the x axis is relative isomer number and on the y axis is the fraction of total DNA which exists as a particular topological isomer.

of the DNA topoisomers are not shifted to more negatively supercoiled positions after chloroquine treatment, as evident by the presence of the bands remaining at the position of control untreated DNA samples (lanes b and c). This suggests the presence of different SV40 subpopulations, some which may not be associated with topoisomerase activity, or it may reflect the packaging of SV40 DNA in virions, where it may be inaccessible to chloroquine or otherwise topologically non-perturable.

DNA linking number of SV40 in CV-1 cells changes following chloroquine treatment: SV40 infected CV-1 cells were treated with chloroquine 48 after viral infection to determine if a change in DNA linking number could be observed in eukaryotic cells. Reproducible changes in the distribution of topoisomers were observed although higher concentrations of chloroquine were required than for purified SV40 or SV40 in purified nuclei. This may be due to a decrease in permeability of chloroquine for cells compared

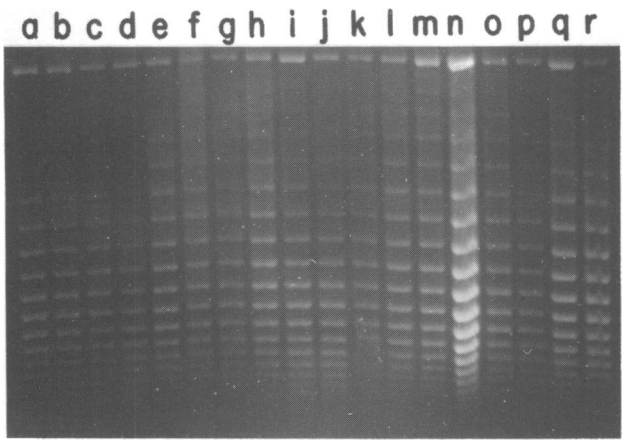


Figure 7: Effect of purification protocol, chloroquine concentration, temperature, and novobiocin on linking number changes of SV40 minichromosomes in CV-1 cells. Lanes a and b: SV40 DNA purified by the Hirt procedure. Lane a: control infected CV-1 cells (untreated). Lane b: cells treated with 1000 μg/ml chloroquine for 15 minutes. Lane c-r: DNAs purified as described in Experimental Procedures and as shown in Figure 6. Lane c: control DNA. Lane d: Chloroquine treated cells, 1000 μg/ml for 15 minutes. Lane e: SV40 DNA purified from cells treated with chloroquine (as in lane d) but following washing and a 15 minute incubation in medium without chloroquine. Lanes f: As lane e, but DNA isolated 30 minutes after washing. Lane g: Control DNA (as in lane c). Lane h: SV40 DNA purified from cells incubated at 7°C in media for 3 hrs. Lanes i and j: DNA samples purified from CV-1 cells treated with 5 and 10 mg/ml chloroquine for 15 minutes. Lane k: Control DNA (as in lane c). Lanes l and m: DNA samples purified from cells treated with 125 μg/ml chloroquine for 15 and 60 minutes respectively. Lanes n and o: DNA from cells treated with 250 μg/ml for 15 and 60 minutes, respectively with chloroquine. Lane p: Control DNA (as in lane c). Lanes q and r: SV40 DNA purified from cells treated with 100 and 1000 μg/ml novobiocin, respectively, for 30 minutes.

to nuclei. After the addition of chloroquine to the culture medium, a small but significant change in the distribution of topological isomers of SV40 DNA was observed. Figures 6A and B show the densitometric profile and the quantitative analysis of SV40 DNA samples purified from cells treated with 1000 μg/ml chloroquine. Soon after chloroquine addition there is a decrease in the intensity of the slower migrating DNA topoisomers and a concominant increase in the more negatively supercoiled (faster migrating) topoisomers. Significantly, the appearance of new more negatively supercoiled topoisomers is also observed (see the fastest migrating bands in Figure 6A and isomers numbers 13, 14, and 15 in Figure 6B). From comparison of the control and 1000 μg/ml chloroquine sample treated for 15 minutes (Figure 6B panels A and D), it was determined that there was an 18% increase in fraction of total topoisomers migrating as relative topoisomers 9-15 (with a concominant 18% decrease in the topoisomers

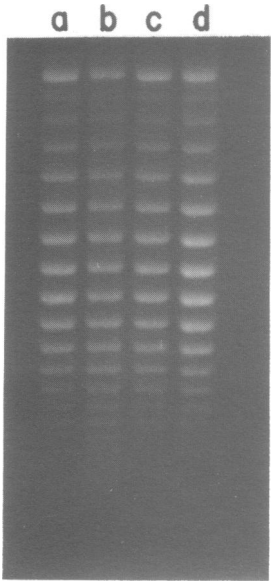


Figure 8: Effect of ammonium chloride on linking number changes of SV40 minichromosomes in CV-1 cells. SV40 DNA purified from CV-1 cells at 48 hr after infection following no treatment (lane a), 15 minute treatment with 10 mg/ml chloroquine (lane b), 15 minute treatment with 20 mM $NH_4Cl$ (lane c), or 15 minute treatment with 100 mM $NH_4Cl$ (lane d).

migrating as isomer numbers 1-8). Control experiments showed that a 15 to 30 minute treatment with 1 mg/ml chloroquine, as used in these experiments, is not toxic to uninfected cells. Upon removal of the drug the cells behave normally and their growth rate is the same as that of untreated cells. SV40 infected CV-1 cells were also treated with 5 and 10 mg/ml chloroquine for 15 minutes. Longer incubation in the presence of 10 mg/ml chloroquine lead to severe vacuolization of the cytoplasm and to detachment of CV-1 cells from culture dishes. As shown in Figure 7 (lanes i and j), treatment with 5 and 10 mg/ml chloroquine also produced the characteristic increase in the more negatively supercoiled topoisomers and the change was more pronounced than that observed following treatment with 1 mg/ml. This change is especially pronounced in experiment shown in Figure 8, in which at least six new more negatively supercoiled topological isomers are observed in the 10 mg/ml treated sample compared to the control sample. Densitometric analysis of this experiment showed a 16% increase in topoisomers migrating at positions indicative of a decrease in linking number. Only slight changes in topological isomer distributions were observed following chloroquine treatment with 125 or 250 μg/ml as shown in Figure 7, lanes l-o. The experiments demonstrating linking number changes in SV40 DNA from infected cells were extremely reproducible and essentially identical results have been repeated 6 times for 1 mg/ml chloroquine and 4 times with 10 mg/ml chloroquine.

Experiments were performed to determine if the changes in linking number following the addition of chloroquine were reversible upon removal of the drug. SV40

infected CV-1 cells were treated with 1000 μg/ml chloroquine for 15 minutes, 48 hrs after viral infection. The chloroquine-containing medium was then removed, cells were rapidly washed and incubated in fresh medium at 37°C for 15 and 30 minutes. As shown in Figure 7, (and in Figure 9) the topoisomer distribution of these samples (in Figure 7, lanes e and f) appears similar to that of the untreated control sample (Figure 7, lane c). The rate of this cellular response to re-equilibrate the level of supercoiling to control levels is very fast. Five minutes after washing, significant reversal was observed (data not shown). These reversible changes were also observed following a 15 minutes pulse with 10 mg/ml chloroquine (data not shown).

There may to be several explanations for the observation that only about 15-20% of the topoisomers are shifting (in a measurable fashion) to a more negatively supercoiled position in the analyses of the chloroquine treatment in vivo as mentioned in the Discussion. To try to enrich for free SV40 minichromosomes in our in vivo analysis we analyzed the SV40 minichromosome peak from sucrose gradients in a purification procedure based on that of Blasquez et al. (27). The peak fractions of slowly sedementing $^{3}$H-labelled SV40 minichromosomes were analyzed on chloroquine gels as shown in Figure 9. The SV40 minichromosome and virion peaks were not as distinct as those shown by Blasquez although we made a number of modifications in the purification procedure to minimize the potential action of topoisomerases. The increase in salt concentration used presumably resulted in the disruption of some virions as reported by Ambrose et al (28). The results presented in Figure 9A show that 1 mg/ml chloroquine treatment resulted in the characteristic appearance of several new topological isomers. This purification procedure does result in an amplification of topoisomer distribution towards a decreased linking number. In this experiment, analyzed densitometrically in Figure 9B, there was a 30% increase in more negatively supercoiled topoisomers. There is still no major shift of the Gaussian center of the peak. The 15 minute incubation after washing cells to remove chloroquine resulted in a re-equilibration of linking number although not completely back to original levels.

Figure 7, lanes a-b shows DNA from untreated and chloroquine treated samples purified using the modified Hirt method described under Methods, which involves a 30 minute incubation at 37°C prior to addition of 1 M NaCl. It is evident that the difference in linking number is not as pronounced in these samples as with the more rapid purification protocol used in the rest of this study (lanes c and d). This may be consistent with the occurrence of some degree of a re-equilibration of linking number during the Hirt purification protocol.

Alteration of levels of DNA supercoiling by temperature shifts in bacterial and yeast cells have been shown to induce compensating changes in DNA linking number (11,12). Control experiments have been performed to study if analogous mechanisms of

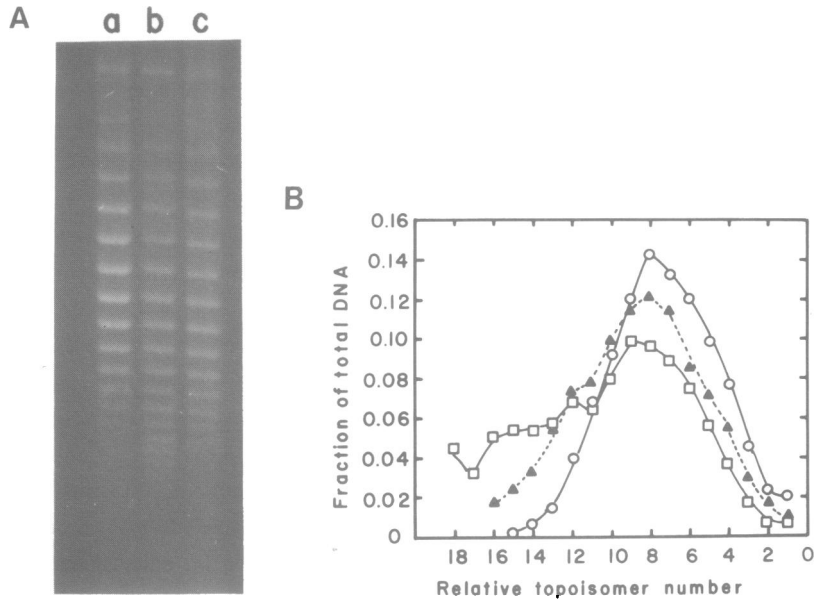


Figure 9: Enrichment for free SV40 minichromosomes in the in vivo analysis of chloroquine treatment. 48 hr after infection $^{3}$H-labelled SV40 minichromosomes were purified from control, untreated cells; cells treated with 1 mg/ml chloroquine for 15 minutes; and cells treated with chloroquine for 15 minutes followed by washing and a 15 minute incubation in medium without chloroquine. In the purification procedure we tried to separate free minichromosomes from those packaged in virons. Cells on plates were washed three times with ice cold Hanks Salts (28), scraped into tubes, centrifuged and resuspended at 0°C in 1 ml of 10 mM Tris pH 7.6, 0.4 M NaCl, 5 mM EDTA, and 0.1 mM PMSF. Cells were dounce homogenized and shaken gently for 30 minutes at 4°C. After 30 minutes 0.5% NP40 was added and the samples were shaken an additional 60-90 minutes. Following a 10 minute centrifugation at 5000 g the supernatant was layered onto a 5-30% sucrose gradient containing 10 mM Hepes pH 7.8, 1 mM EDTA, 0.36 M NaCl, and 0.1 mM PMSF and centrifuged for 60 minutes at 4°C at 36,000 RPM in a Beckman SW41 rotor. The peak fractions migrating at about 75 S were purified by Proteinase K treatment, extraction with phenol and chloroform, and ethanol precipitation. These samples were not banded in ethidium bromide CsCl gradients which probably explains the light smearing throughout the gel. Panel A lanes a-c are control, chloroquine-treated, and chloroquine treated and washed samples, respectively run on a 1% agarose gel containing 3 μg/ml chloroquine. Panel B shows densitometric anslysis of the gel shown in panel A. Plotted are the fraction of total SV40 DNA topoisomers migrating at a particular topological isomer number (isomer 0 is the nicked, open circular peak). This analysis is comparable to that in Figure 6B although lines have been drawn between the points in place of a bar graph presentation. Lane a (o), lane b (□), lane c (▲).

supercoiling regulation were present in the mammalian system in response to changes of the growth temperature of the cells. In four separate experiments, we did not observe any change in DNA linking number of DNA samples purified from cells shifted from 37°C to 7°C for 3 hrs (for example see Figure 7, lane h). These data confirm previous

observations performed on the same SV40 system which showed no appreciable change in linking number (13). Treatment of SV40 infected CV-1 cells for 30 minutes in the presence of 100 and 1000 μg/ml novobiocin had no effect on linking number, as shown in Figure 7, lanes q and r.

Chloroquine is known to introduce a pH change in eukaryotic cells (29,30). To rule out the possibility that the observed change in linking number was due to an intracellular pH change, CV-1 cells were treated with ammonium chloride at two concentrations that should introduce pH changes similar to or greater than that for chloroquine (30). As shown in Figure 8 the *in vivo* linking number change in SV40 was observed following treatment with 10 mg/ml chloroquine but not with 20 or 100 mM ammonium chloride.

## DISCUSSION

### Regulation of Supercoiling in Prokaryotes

The results shown here demonstrate that *E. coli* cells re-equilibrate the level of supercoiling in plasmid DNA *in vivo* following topological perturbation by chloroquine treatment. In *E. coli* it is expected that chloroquine intercalatively binds to DNA and partially relaxes unrestrained torsional tension. Presumably, *E. coli* DNA gyrase then re-equilibrates the level of DNA supercoiling *in vivo* by decreasing the linking number. Following purification from cells and removal of chloroquine the DNA is more negatively supercoiled than from untreated cells. The change in linking number observed following chloroquine addition was completed within 5 minutes. This decrease in linking number was rapidly reversible when chloroquine was removed, with the change being completed within 5 minutes. This result confirms and extends the results by Goldstein and Drlica (11) showing that *E. coli* has mechanisms for regulating or maintaining a precise level of supercoiling *in vivo*. The re-equilibration observed of Goldstein and Drlica (11) following topological perturbation by temperature shifts required 30 to 60 minutes for completion. Our results suggest that the linking number re-equilibration can be very rapid and significant changes were observed after 30 seconds. This suggests that existing proteins within the cell are responsible for the linking number changes, and that new proteins need not be synthesized.

Analysis of linking number changes in the *gyrB225* strain is consistent with the idea that DNA gyrase is responsible for the linking number decrease since no increase in supercoiling was observed in this strain following chloroquine addition. Following removal of the drug in the *topA10* strain, relaxation of superhelical turns occurred suggesting involvement of DNA gyrase or residual topoisomerase I activity in the relaxation. The level of supercoiling appeared to be regulated in the topoisomerase I mutant even though the linking number of DNA in the *topA10* strain is lower than that in wild-type. This suggests that gyrase and topoisomerase I activity, although imbalanced

in the topA 10 strain, still act to maintain DNA at a reduced linking number. The possibility that gyrase may relax supercoils in the topA10 strain is consistent with a recent analysis of supercoiling in nucleoids from coumermycin treated topA10 cells by Pruss et al. (25).

Regulation of Supercoiling in Eukaryotes

The results presented here demonstrate that the linking number of SV40 minichromosomes in CV-1 cells changes rapidly in response to chloroquine addition or removal as expected if SV40 DNA is maintained at a precise level of supercoiling in vivo. This is the first demonstration that the level of DNA supercoiling can be "regulated" or maintained at a defined level in mammalian cells. The linker region of the SV40 minichromosomes is presumably torsionally relaxed although our results cannot actually distinguish whether this DNA is maintained in a relaxed or torsionally strained state in CV-1 cells. The linking number changes observed in vivo were small; about 20% of the population of total SV40 topoisomers changed linking number. However, this may represent the expected value if the SV40 DNA in virions is inert to topological perturbation or DNA topoisomerase and 20% represents the fraction of SV40 DNA as free minichromosomes (for instance see Blasquez, et al. (27), Figure 2A). An attempt to increase the fraction of free SV40 minichromosomes in the in vivo analysis (as described in Figure 9) resulted in a linking number change in at least 30% of the topoisomers isolated from CV-1 cells. Significantly, the linking number changes were rapid, reversible, and very reproducible. The fact that large and very similar reversible changes in linking number were observed for both SV40 in purified nuclei and for purified SV40 minichromosomes suggests that the smaller linking number changes observed in vivo may reflect a low intracellular or intranuclear chloroquine concentration coupled with a large population of SV40 inert to chloroquine. Assuming that the magnitude of the linking number change observed in vivo accurately reflects the intranuclear chloroquine concentration, it is clear from the control experiments described above that these chloroquine concentrations are probably not disrupting nucleosomes.

It is no doubt significant and paradoxical that the reequilibration of linking number in response to a temperature change occurs in E. coli (11) and in yeast (12) but has not been observed for SV40 (13). In numerous experiments we too failed to observe a change in linking number following up to 3 hr incubations at various temperatures between 7 and 37°C. Morse and Cantor (31,32) have recently shown that in reconstituted minichromosomes the thermal twisting of DNA in nucleosomal linker regions is prevented or constrained. Our results would suggest that twisting in linker regions generated by binding an intercalating drug is not prevented in vivo. This result is consistent with the observation that DNA cross-linking by intercalating psoralen derivatives is restricted to nucleosomal linker regions (33,34). Presumably, the interaction of linker DNA with the

nucleosome is stable to thermal perturbation but not to untwisting from intercalation. A 30°C temperature change would result in a change in twist of 20° in a 55bp linker region whereas binding of a single intercalating drug would unwind DNA by about 28°. Perhaps thermal twisting induced by a reduction in temperature is not sufficient to displace a possible "microdomain" defined by DNA linker-nucleosomal protein contacts. It is also possible that in vivo a topological change from thermally induced twisting of the DNA helix is compensated by a change in DNA protein interactions which may effectively change the negative writhe to compensate for the change in twist.

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