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# The problems of eukaryotic and prokaryotic DNA packaging and in vivo conformation posed by superhelix density heterogeneity

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## ABSTRACT

Systems for gel electrophoresis in the presence of one of the intercalative unwinding ligands, ethidium or chloroquine, have been developed which permit the resolution of highly supercoiled closed circular DNA molecules differing by unit values of the topological winding number, $\alpha$. All native closed circular DNAs examined, including the viral and intracellular forms of SV40 and polyoma DNA, bacterial plasmid DNAs, and the double stranded closed circular DNA genome of the marine bacteriophage, PM2, are more heterogeneous with respect to the number of superhelical turns present than are the thermal distributions observed in the limit products of the action of nicking-closing (N-C) enzyme on the respective DNAs. In the cases of SV40 and polyoma, where it has been shown that the supercoiling is a combined consequence of the binding of the four nucleosomal histones, H2a, H2b, H3 and H4, and the action of N-C enzyme, the breadth of the distributions within the form I DNAs poses specific problems since the work of other laboratories indicates that the number of nucleosomes on the respective minichromosomes falls within a narrow distribution of 21. If it is assumed that all nucleosomes have identical structures, and that the DNA within a nucleosome is not free to rotate, the native DNA would be anticipated to be less heterogeneous than the thermal equilibrium mixtures present in N-C enzyme relaxed SV40 and polyoma DNAs.

The absolute number of superhelical turns (at 37°C in 0.2 M NaCl) in virion polyoma DNA has been determined to be 26 ± 1, which is the same value obtained for virion SV40 DNA. This is consistent with the observations that polyoma DNA has a higher molecular weight, a lower superhelix density, but the same number of nucleosomes as SV40 DNA. In addition, the distributions within the virion and intracellular form I DNAs of both SV40 and polyoma were found to be indistinguishable.

## INTRODUCTION

The phenomenon of supercoiling in closed circular DNA has been a subject of continuing interest since its original description by Vinograd et al. (31). Elucidation of the origins of supercoiling is expected to provide insight into mechanisms of DNA packaging. Quantitative studies of the supercoiled DNAs provide parameters which must be considered when defining models for the in vivo conformation of both eukaryotic and prokary-

otic chromosomes.

The significance of the above has been underlined for eukaryotic organisms by Germond et al. (9) who have demonstrated that most, if not all of the supercoiling present in the closed circular DNA genome of the papovavirus, SV40, can be accounted for by the binding of the four cellular histones H2a, H2b, H3 and H4. They also observed that native supercoiled virion SV40 DNA is heterogeneous with respect to the number of superhelical turns. The DNA of polyoma, a closely related papovavirus, is closed circular and supercoiled, and is found complexed both within the cell and after encapsidation, with the four nucleosomal histones (8, 19). The relationship of histones to the supercoiling of SV40 and polyoma DNAs makes these viruses useful probes for the analysis of eukaryotic chromatin structure.

Many other closed circular DNAs exist, however, where the origin of supercoiling has not yet been identified. Among these are bacterial plasmids, bacteriophage replicative intermediates, the encapsidated genome of the marine bacteriophage PM2, as well as chloroplast and mitochondrial DNAs. Furthermore, the chromosomes of several prokaryotes have been observed to be condensed in nucleoids, which in two cases, (E. coli and Mycoplasma hyorhinis) have been isolated and have been shown to be looped supercoiled structures (21, 28, 34). Although it is not known whether extrachromosomal DNAs exist in nucleoid-like configurations, recent electron microscopic observations of Griffith (12) have shown that after gentle lysis, both the E. coli chromosome and the DNA of superinfecting $\lambda$ appear condensed in beaded structures which are visibly similar to the nucleosomes of eukaryotic chromatin (20).

Because the dimensions of bacteria, cell nuclei and virus particles are invariably smaller than the lengths of their respective genomes, mechanisms for DNA condensation are universally required. Closed circular DNA is a particularly favorable system for the study of DNA packaging because one feature of its in vivo conformation, the topological winding number ($\alpha$), is preserved upon isolation. In the present work we examine the heterogeneity in $\alpha$ of several closed circular DNAs, isolated from a variety of sources.

The topological relationship for duplex closed circular DNA has been defined by the equation,

$$\alpha = \tau + \beta \qquad 1$$

where $\alpha$, the topological winding number, is the number of revolutions one

strand of the duplex makes about its complement if the molecule is constrained to lie in a plane. $\alpha$ must be integral and cannot be altered without a nicking-closing event. $\beta$, the mean duplex winding number, is the average number of turns that would be present in the nicked circular counterpart when at equilibrium with its environment. The value of $\beta$ need not be integral and is dependent upon the environmental conditions. $\tau$ is the number of superhelical turns in the molecule and is normally negative for naturally occurring closed circular DNAs; like $\beta$ it need not be integral, and will vary with $\beta$ in response to changing environmental conditions. The superhelix density, $\sigma \equiv \tau/\beta^\circ$ (where $\beta^\circ$ is 1/10 the number of base pairs in the molecule), is the quantity used when comparing supercoiled DNAs of different molecular weights.

The introduction of gel electrophoresis for the study of closed circular DNA (29) has permitted the resolution of molecules differing in the number of superhelical turns (17). Since $\alpha$ must be integral, and $\beta$ is the same for all molecules under a given set of conditions, resolved species must differ by an integral number of superhelical turns. In combination with the use of N-C enzymes, the foregoing has enabled the measurement of the absolute number of superhelical turns in a closed circular DNA molecule (18, 26).

In previous work we have shown that the limit product of the action of N-C enzyme on closed circular DNA is a distribution of species, heterogeneous in $\alpha$. The relative masses of the species within the limit product conform to a Boltzmann distribution defined by the free energy of supercoiling (22). Similar distributions have been demonstrated in the products of the action of ligase on nicked circular DNA (4, 5, 22).

As noted above, the work of Germond (9) indicated that virion SV40 DNA is also heterogeneous in $\alpha$; however, the distribution of species was not completely resolved by the electrophoresis conditions used. In general, gel electrophoretic techniques cannot resolve species, which under the electrophoresis conditions are either highly supercoiled or contain low numbers of superhelical turns. Since $\beta$, and therefore $\tau$, vary in response to environmental changes, it is frequently possible to optimize the resolution of the species present by adjusting the electrophoresis conditions. In the present work, the distributions of species in highly supercoiled native DNAs have been resolved by electrophoresis in the presence of small unwinding ligands. These cause a decrease in $\beta$ and therefore, decrease the number of negative superhelical turns in all closed species. The ligands

used are ethidium, which has been employed by others to titrate the superhelical turns present in closed circular DNAs (3, 7, 18) and chloroquine, an antimalarial drug which has been shown to bind to DNA in an intercalative manner (33). The naturally occurring DNAs examined in the present study are: SV40, polyoma, PM2, Minicol, ColE1 and pSM1. In each case the distribution of species is more heterogeneous than the thermal distribution resulting from N-C enzyme action on the corresponding DNA.

## EXPERIMENTAL PROCEDURES

### SV40 DNA

#### Viral DNA

TC-7 cells were grown on 9 cm Petri dishes to approximately 95% confluence. The cultures were then infected at a multiplicity of 0.01 pfu/cell with a stock of twice plaque purified SV40 (strain sp12). Virus was harvested when a full cytopathic effect was observed (10-12 days post-infection at 37°C). Virus was purified by the combination of methods previously described by Kasamatsu and Wu (16). Purified virions were lysed with SDS and the closed circular DNA was purified by buoyant banding in ethidium bromide (EtdBr)-CsCl gradients as described by Tai et al. (27).

#### Intracellular SV40 DNA

TC-7 cells were grown as described above and were infected at a multiplicity of 1 pfu/cell. The initial stages of the DNA purification were carried out at 37°C. At 70 hrs post-infection, the medium was removed and the cells were washed twice with 5 mls of TD buffer (0.14 M NaCl, 5 mM KCl, 0.7 mM $Na_2HPO_4$, 25 mM Tris HCl pH 7.4), prewarmed to 37°C. The cells were lysed by the addition of 1 ml of one of the following solutions (also prewarmed to 37°C) to each dish.

a) 0.6% SDS, 10 mM Tris HCl, 10 mM EDTA pH 7.8.

b) 1% sodium deoxycholate (DOC), 0.8 M NaCl, 10 mM Tris HCl, 10 mM EDTA pH 7.8.

c) 1% DOC, 1 M CsCl, 10 mM Tris HCl, 10 mM EDTA pH 7.8.

Solution b) must be prepared immediately before use, since gelation occurs upon standing. Solution c) is a modification of b) designed to minimize this problem; however this solution will also form a gel after prolonged standing.

Solutions b) and c) were designed to lyse the cells, but not virions, and to inhibit the action of N-C enzyme on the intracellular DNA. In all three cases lysis was instantaneous. The lysate was incubated for 10-15

min at 37°C. Further purification proceeded at room temperature, or at 4°C. The lysate obtained with solution a) was processed according to the method of Hirt (15) modified by the substitution of 1 M CsCl for 1 M NaCl for precipitation of the dodecyl sulfate. After removal of precipitated detergent and high molecular weight DNA by centrifugation, the closed circular SV40 DNA was purified by buoyant centrifugation in EtdBr-CsCl density gradients (24).

The lysates obtained with solutions b) and c) were centrifuged in a Beckman type 30 rotor at 25,000 rpm, 4°C for 3.5 hrs to pellet both high molecular weight DNA and virus particles. The supernatants, containing unencapsidated intracellular SV40 DNA, were treated with pancreatic RNase (20 μg/ml, 37°C, 1 hr) and then with pronase (50 μg/ml, 37°C, 1 hr). DNA was then precipitated with ethanol and the form I SV40 DNA was purified by banding in EtdBr-CsCl gradients.

Polyoma DNA

Purified intracellular polyoma DNA was a gift of Dr. M. Vogt. Samples of defective-free polyoma virus and viral DNA were provided by Drs. M. Vogt, J. Seehafer and W. Eckhart. DNA was extracted from the virions and was further purified as described above for SV40 DNA. Both the intracellular and viral DNA samples were from infected cells maintained at 37°C.

PM2 DNA

Bacteriophage PM2, purified by buoyant centrifugation in CsCl, was provided by R. M. Watson. The purified bacteriophage was lysed with 0.1% SDS and the lysate was deproteinized by repeated extractions (4-5 times) with an equal volume of a 3:1 (v/v) mixture of chloroform: n-butanol. The DNA was further purified by buoyant centrifugation in CsCl-EtdBr gradients.

Plasmid DNAs

A sample of purified pSM1 DNA, as well as the bacterial strain harboring the pSM1 plasmid were gifts of Dr. S. Mickel. The bacterial strain harboring the plasmid pVH51 (Minicol) was obtained from Dr. H. W. Boyer.

E. coli carrying the Minicol plasmid were grown with vigorous aeration at 37°C in 12 liters M9 casamino acids medium supplemented with 5 g/l glucose, 1 μg/ml thymidine, 1 μg/ml thiamine, 5 μg/ml tryptophan. After 12-24 hrs in stationary phase the cells were harvested by centrifugation, washed once with fresh M9 medium and resuspended in 75 mls of the above supplemented M9 medium. 15 ml aliquots were incubated at each of five temperatures (37°C, 30°C, 23°C, 13°C, 3°C). In different experi-

ments time points between 0.5 and 4.5 hrs were taken. The cells were lysed by the rapid addition of an equal volume of 2.5% lithium dodecyl sulfate, 0.2 M lithium EDTA pH 7.4 which had been pre-equilibrated at the appropriate temperature. Although lysis appeared to be instantaneous, the lysates were maintained at their respective temperatures for 1 hr, after which they were heated to 65°C for 5 min. The dodecyl sulfate was precipitated by the addition of CsCl to a final concentration of 1 M, and the precipitate, as well as the high molecular weight DNA, were removed by centrifugation at 30,000 rpm for 1 hr at 4°C in a Beckman Ti60 rotor. Removal of CsCl and concentration of the samples were performed by dialysis against 25% w/v polyethyleneglycol 6000, 10 mM Tris HCl pH 7.8, 1 mM EDTA at room temperature for approximately 7 hrs. After 1 hr of dialysis pancreatic RNase was added to a final concentration of 50 μg/ml. After an additional 2 hrs, pronase was added to an approximate final concentration of 200 μg/ml. The form I plasmid DNA was purified from each dialysate by equilibrium centrifugation in EtdBr-CsCl gradients.

The control experiment (see Results) to test for relaxation during lysis was performed by adding 20 μg/ml of purified plasmid DNA (pSM1) to the lysis buffer prior to its addition to the bacterial suspensions at the five temperatures. After incubation, the five pSM1 samples were compared with the original pSM1 DNA sample by gel electrophoresis in the presence of 85 μg/ml chloroquine phosphate. In all cases, the distributions were indistinguishable (results not shown).

Electrophoresis

A vertical slab gel electrophoresis apparatus (Aquebogue) was used for gels 15 cm in length. Long gels (30 cm) were run in an electrophoresis apparatus modified for this purpose by R. M. Watson. Gels (4 mm thick) consisted of 1% or 1.2% agarose in either of the following electrophoresis buffers:

1) 40 mM Tris acetate pH 7.8 (4.84 g Tris base, 1.53 g acetic acid/l), 5 mM sodium acetate, 1 mM EDTA and a concentration of ethidium bromide between 10 and 30 ng/ml depending upon the DNA species being resolved.

or 2) 50 mM Tris phosphate pH 7.2 (6.06 g Tris base, 2.85 g 85% $H_3PO_4$/l), 1 mM EDTA, with a concentration of chloroquine phosphate (K & K Laboratories, Inc.) between 7.5 and 1000 μg/ml, depending upon the DNA species being resolved.

Electrophoresis was at room temperature (23°C) at 2-3 v/cm, and

electrophoresis times (17-38 hrs) were varied according to agarose concentration and molecular weight of the DNA. The buffer was recirculated in all cases. Gels containing ethidium were run in the dark. Gels were stained for 4 hrs in 10 mM Tris HCl pH 7.8, 2 mM EDTA, 1 $\mu$g/ml EtdBr. Because of the strong effect of superhelix density on the binding of ethidium, the closed circular DNA was nicked photolytically by exposure to high intensity ultraviolet light prior to restaining overnight in fresh staining solution. Extensive staining is required because of competition by chloroquine with ethidium for DNA binding sites.

Photography and Quantitation

Gels were photographed on either Kodak Plus X or Ilford FP4 4" x 5" sheet film and the photographs were quantitated as described previously (22, 23).

## RESULTS

The multiple species present within virion and intracellular SV40 form I DNA have been resolved by electrophoresis on agarose gels in the presence of ethidium and are presented in Figure 1, (A) and (B), respectively. Under the conditions used, the species present migrate as negatively supercoiled molecules, with the most supercoiled species having the greatest mobility. Native Minicol, ColE1, and polyoma DNAs have also been resolved into multiple species by the use of appropriate ethidium concentrations (results not shown); however, the quality of resolution obtained was found to be excessively sensitive to minor variations in the ambient conditions as well as to the amount of DNA applied per channel. These factors, among others, have made desirable the use of an unwinding ligand with a lower affinity for DNA. A second buffer system has therefore been developed in which

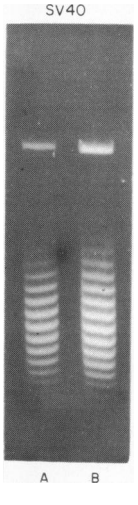


FIGURE 1. The Resolved Species of SV40 Form I DNA after Electrophoresis in the Presence of Ethidium.

The uppermost band in both channels is SV40 form II DNA. In (A) and (B) are virion and intracellular SV40 form I DNAs respectively. The intracellular DNA was prepared by Hirt lysis of infected cells at 23°C. Electrophoresis was in the presence of 25 ng/ml ethidium bromide, in a 1.2% agarose gel at 3 v/cm for 22 hrs.

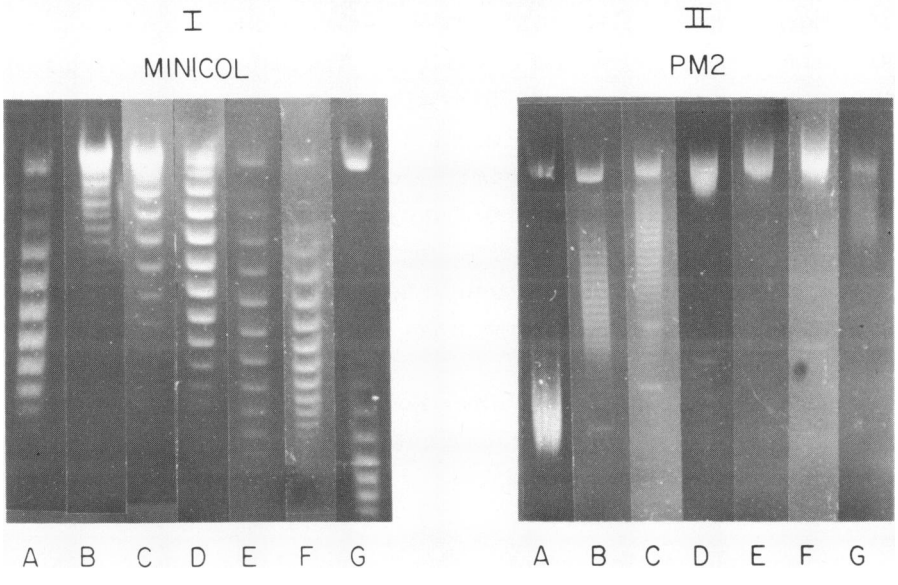


FIGURE 2. Development of the Chloroquine Phosphate Gel Electrophoresis System.

Panel I: Minicol DNA and Panel II: PM2 DNA after electrophoresis in the presence of: (A) 7.5 μg/ml, (B) 15 μg/ml, (C) 25 μg/ml, (D) 50 μg/ml, (E) 75 μg/ml, (F) 100 μg/ml, and (G) 200 μg/ml, chloroquine phosphate. Electrophoresis was for 17 hrs at 5 v/cm in 1% agarose slab gels.

the antimalarial drug, chloroquine, has been used as the unwinding ligand.

The panels in Figure 2 show the results obtained after electrophoresis of native Minicol DNA (I) and PM2 DNA (II) in the presence of increasing levels of chloroquine phosphate. Minima are observed in the mobilities of form I DNAs relative to those of the form II DNAs. This behavior is similar to that observed for SV40 DNA when electrophoresed in the presence of increasing levels of ethidium (3, 7, 18). Experiments with very high concentrations of chloroquine phosphate (500 μg/ml and 1000 μg/ml) have confirmed that PM2 form I DNA can be resolved as a set of positively supercoiled species (results not shown).

The initial decrease in the mobilities of these DNAs in the presence of increasing levels of the unwinding ligand is due to the titration of the negative superhelical turns initially present in the molecules. The minimum relative mobilities of the form I DNAs are reached when the concentration of chloroquine in the electrophoresis buffer is sufficient to remove all of these superhelical turns. Beyond this point any further unwinding of

the primary helix (by increasing levels of chloroquine) leads to the generation of superhelical turns of the opposite (positive) sense. In this range of chloroquine concentrations the most positively supercoiled species (i.e. that which initially was the least negatively supercoiled) has the greatest mobility. As anticipated above, there are two regions in the titration curves, one on each side of the minimum, where the multiple species present within the form I DNAs are resolved.

For the Minicol DNA preparation used here the optimal resolution is observed at a concentration of 75 μg/ml of chloroquine phosphate. At this concentration the species present in the DNA migrate as positively supercoiled molecules. In the case of PM2 DNA optimal resolution of the species within the form I DNA is obtained around 15 μg/ml of chloroquine phosphate, where the species are still negatively supercoiled. In several attempts to resolve PM2 DNA into its constituent species through the use of ethidium as the unwinding ligand, only slight indication of heterogeneity was observed. The general improvement in resolution obtained by electrophoresis in the presence of chloroquine is illustrated by the relative ease with which the PM2 species were resolved.

The panels in Figure 3 present the resolution of the form I DNAs of the papovaviruses, polyoma and SV40, after electrophoresis in the presence of chloroquine. The uppermost band in each of the channels is the nicked circular form (II) of the DNA. A comparison of the relative mobilities of the form II DNAs in panel I shows that polyoma DNA (D) is slightly larger than SV40 DNA (A, B, and C), in agreement with the results of Helling, Goodman and Boyer (14). Despite the slightly larger genome size of polyoma, the set of species within polyoma form I DNA migrates ahead of those present within SV40 I DNA. Because, under these electrophoresis conditions, the resolved species are positively supercoiled, we conclude that in the absence of chloroquine, polyoma DNA has a lower (i.e. less negative) superhelix density than does SV40 DNA. This is in agreement with previous results obtained by sedimentation velocity-dye titrations and by buoyant equilibrium centrifugation (10). By the use of the band counting method we have determined that there are 26 ± 1 superhelical turns in virion polyoma DNA at 37°C in 0.2 M NaCl (results not shown), which is the same value as that previously determined for SV40 DNA (26). The constancy of the number of superhelical turns is especially interesting in light of the difference in the molecular weights of these two DNAs.

As shown in Figure 3, panel III, the multiple species within native

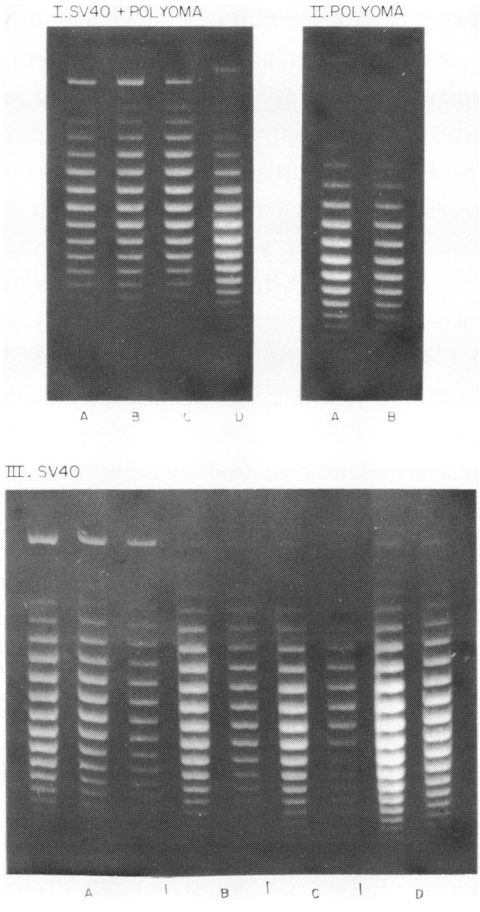


FIGURE 3. Comparison of the Intracellular and Virion Form I DNAs of Polyoma and SV40. The uppermost band in each channel is the nicked circular form (II) of the DNA. Panel I: (A) and (B) contain intracellular SV40 form I DNA prepared by Hirt lysis of infected cells at 23°C and 37°C respectively. (C) contains virion SV40 form I DNA and (D) contains virion polyoma DNA. Electrophoresis was at 3 v/cm for 24 hrs in a 1.2% agarose gel in the presence of 50 μg/ml chloroquine phosphate. Panel II: (A) and (B) contain samples of virion and intracellular form I polyoma DNA respectively. Electrophoresis conditions were the same as those in panel I. Panel III: (A) contains virion SV40 form I DNA. (B), (C), and (D) contain intracellular SV40 form I DNA prepared by lysis of infected cells at 37°C with the following 3 solutions respectively: (B) 1% DOC, 1 M CsCl, 10 mM Tris HCl pH 7.8, 10 mM EDTA. (C) 1% DOC, 0.8 M NaCl, 10 mM Tris HCl pH 7.8, 10 mM EDTA. (D) 0.6% SDS, 10 mM Tris HCl pH 7.8, 10 mM EDTA. The first two solutions were designed to inhibit N-C enzyme, while at the same time leaving virus particles intact. Electrophoresis was for 24 hrs at 3 v/cm in the presence of 75 μg/ml chloroquine phosphate in a 1.2% agarose gel.

form I SV40 DNA were optimally resolved when electrophoresed on agarose gels in the presence of 75 μg/ml chloroquine phosphate. In panel III (A) samples of non-defective virion SV40 form I DNA are resolved into species with adjacent bands differing by a single turn. Intracellular SV40 DNA prepared by a Hirt lysis (at 37°C) of infected cells is shown in panel III (D). Preparations of intracellular SV40 DNA that are free of packaged viral DNA were made (also at 37°C) as an extra precaution against the possibility that viral DNA constituted a major fraction of the closed circular DNA in the Hirt lysates. Samples of these are shown in panel III (B) and (C). The distributions of species in the various virion and intracellular samples have been shown to be indistinguishable by a quantitative treatment of the fluorescence photographs of ethidium-stained gels (23). It is significant that DNA prepared by Hirt lysis of the cells at 23°C is also indistinguishable from the virion DNA, as shown in panel I, (A) and (C), respectively.

The slightly lower superhelix density of polyoma DNA resulted in optimal resolution being obtained after electrophoresis in the presence of 50 μg/ml chloroquine phosphate. Samples of non-defective form I polyoma DNA isolated from virions and from infected cells are shown in panel II, (A) and (B), respectively. A quantitative comparison has shown that the distributions present in virion and intracellular polyoma DNAs are also indistinguishable.

The distributions of species within the form I DNAs of both polyoma and SV40 are much broader than the purely thermal distributions generated by the action of N-C enzyme on these DNAs. This is illustrated for SV40 in Figure 4, where densitometric traces of fluorescence photographs of native SV40 form I DNA (A) and the limit product of the action of N-C enzyme on SV40 DNA (B) are shown. The limit product shown in (B) was prepared under standard conditions (at 37°C in 0.2 M NaCl).

The relative masses of the species, $m_\alpha$, were determined by a quantitative treatment of the fluorescence photographs. Because each DNA sample is homogeneous with respect to molecular weight, the relative mass of a species, $m_\alpha$, is proportional to the number, $N_\alpha$, of molecules having a given value of $\alpha$. Plots of $N_\alpha$ vs. $\Delta\alpha$, shown in Figure 5, indicate that the species present within both form I polyoma DNA and form I SV40 DNA conform to Gaussian distributions of the form:

$$N_\alpha / N_t = Ae^{-C(\alpha - \overline{\alpha})^2} \qquad 2$$

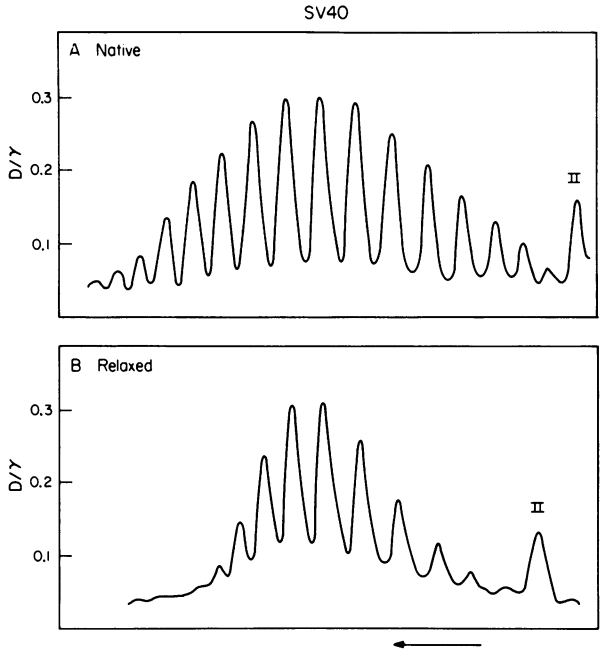


FIGURE 4. Comparison of the Distribution Within Native SV40 Form I DNA and the Thermal Distribution Within the Limit Product of N-C Enzyme Action on SV40 Form I DNA.

Densitometric traces of photographs of ethidium stained gels. (A) is a trace of virion SV40 form I DNA (Figure 3, panel III (A) ). (B) is the limit product of N-C enzyme action on SV40 form I DNA prepared under the standard conditions described in Shure and Vinograd, 1976 (26). The species are resolved as negatively supercoiled molecules by electrophoresis at 4°C in the presence of 5 mM magnesium acetate (22). The ordinate of these traces is in units of the logarithm of the intensity of fluorescence ($D/\gamma$). The arrow on the abscissa indicates the direction of electrophoresis.

where $N_t$ is the total number of molecules in the distribution, C is a constant determining the shape of the distribution, and $\bar{\alpha}$ is the median of the Gaussian curve. The term $(\alpha - \bar{\alpha})$ is equivalent to $\tau$ when $\bar{\alpha} = \beta$. Therefore, the difference in the topological winding number between two species, $\Delta\alpha$, is equivalent to the difference in the respective number of superhelical turns, $\Delta\tau$.

The values of C obtained for virion and intracellular SV40 DNA are $0.051 \pm 0.004$ and $0.050 \pm 0.004$, respectively, and for virion and intracellular polyoma DNA, $0.053 \pm 0.005$ and $0.050 \pm 0.006$, respectively. Since the value of $\bar{\alpha}$ need not be integral, the center of a distribution will

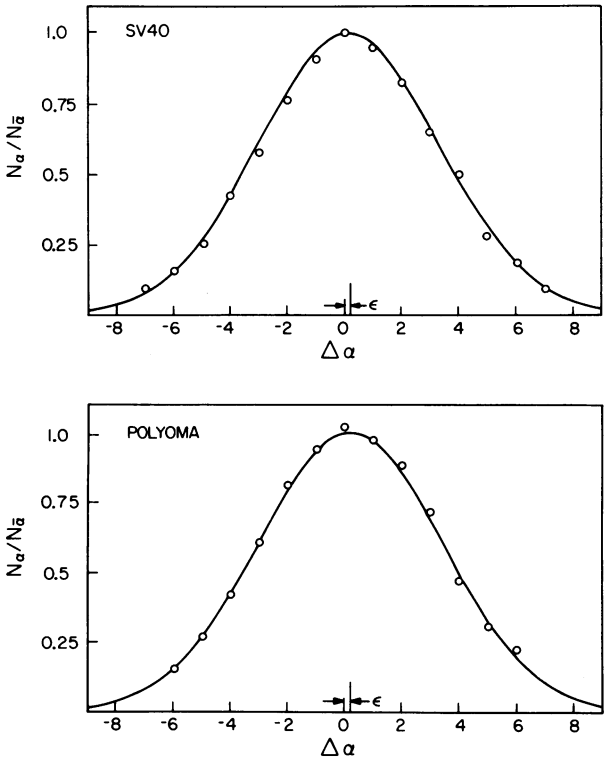


FIGURE 5. The Distributions Present Within SV40 and Polyoma DNAs are Gaussian.

The number ($N_\alpha$) of molecules having a given value of $\alpha$ were normalized by $N_{\bar{\alpha}}$, the number of molecules of a theoretical species having a value of $\alpha = \bar{\alpha}$, where $\bar{\alpha}$ is the median of the distribution. $N_\alpha/N_{\bar{\alpha}}$ is plotted against $\Delta\alpha$. The species closest to the median has arbitrarily been given a value of $\Delta\alpha = 0$. The fractional turn between this species and the median of the distributions is designated by $\epsilon$. The curves through the points are the best least squares Gaussian curves calculated for single channels of SV40 and polyoma DNAs. Species on the left hand side of the curves have the lowest values of $\alpha$ and are the most negatively supercoiled.

not necessarily coincide with a species. We therefore define a quantity, $\epsilon$ ($-0.5 < \epsilon \leq 0.5$) as the non-integral part of $\bar{\alpha}$. $\epsilon$ values determined for the virion and intracellular SV40 form I distributions are $-0.187 \pm 0.030$ and $-0.187 \pm 0.015$, respectively, and for the virion and intracellular polyoma form I distributions, $-0.211 \pm 0.040$ and $-0.187 \pm 0.005$, respectively, as indicated in Figure 5.

Plasmid DNAs

In earlier work from this laboratory the form I DNA of the E. coli Minicol plasmid was shown to be heterogeneous in $\alpha$ (26). The use of the ethidium and chloroquine gel systems has permitted detailed examination of the heterogeneity of this and other bacterial plasmid DNAs. Various lysis procedures have been examined with the intention of optimizing the yield of form I plasmid DNA from the host strains of E. coli. We have noted, however, that the isolation procedure has a great effect on the supercoiling of the form I plasmid DNA. In particular methods which involve the initial formation of spheroplasts result in preparations of partially relaxed DNA.

The problems encountered in preparing plasmid DNAs under controlled conditions, while at the same time preventing nicking-closing activity, have led us to develop a lysis procedure that can be used under a variety of conditions. Minicol DNA has been prepared by this procedure. A control experiment in which purified pSM1 DNA was mixed with the lysis buffer before its addition to the bacterial suspension showed no indication of nicking-closing activity as evidenced by preservation of the input distribution of pSM1 species (results not shown). Furthermore, species of very low superhelix density are absent from the Minicol DNA prepared by this method, as shown in Figure 6.

Figure 6 shows the results of an experiment in which aliquots of a stationary phase culture of the E. coli strain harboring the Minicol plasmid were incubated for 2 hrs at different temperatures prior to lysis at those temperatures. As is the case with the papovaviruses, the distributions of species within the plasmid DNAs are much broader than the thermal distributions generated by the action of N-C enzyme on the respective form I DNAs. Plots of $N_\alpha$ vs. $\Delta\alpha$ have shown that the species within Minicol DNA, such as the sample shown in (F), sometimes conform to a Gaussian distribution of the form given in Equation 2. More frequently, the distributions are skewed as illustrated by the samples shown in (A-E) which were prepared at 3°C, 12°C, 23°C, 30°C, and 37°C, respectively, as described in the above experiment. Physiological factors other than temperature may also have important roles in determining the form of the distribution, since the DNA samples shown in (E) and (F) were both prepared at 37°C by the same method, but from separate stationary phase cultures.

The center of mass of the distribution responds reproducibly in a

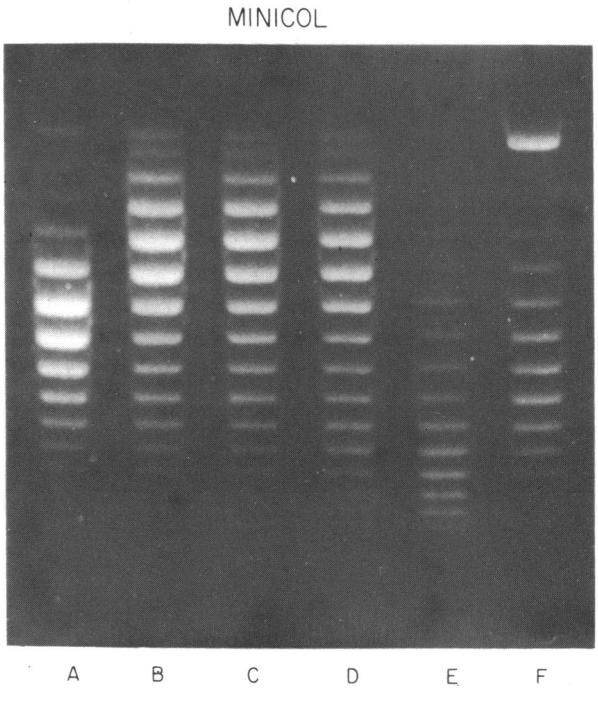


FIGURE 6. The Effect of Incubation Temperature on the Distribution of Species Within Native Form I Minicol DNA.

(A) through (F) contain samples of Minicol DNA prepared by lysis of the host cells in the presence of 1.25% lithium dodecyl sulfate, 0.1 M lithium EDTA pH 7.4 (see Experimental Procedures). Samples in (A) through (E) were prepared from aliquots of a culture which had been incubated for 2 hrs at 3°C, 12°C, 23°C, 30°C, and 37°C respectively. The sample in (F) was prepared from another culture of the host strain, incubated at 37°C prior to lysis. Electrophoresis was at 3 v/cm for 17 hrs in the presence of 125 μg/ml chloroquine phosphate in a 1.2% agarose gel.

non-linear fashion to changes in temperature. An initial decrease in temperature from 37°C is always accompanied by an increase in the average superhelix density of the plasmid DNA. A further decrease in temperature to 0-4°C consistently results in a reversal of this effect. These temperature effects are quite different from those observed for DNA in vitro, where a decrease in temperature causes a monotonic increase in the duplex winding angle.

PM2

PM2 DNA, having a molecular weight of $6.4 \times 10^6$ daltons, is the largest of the DNAs yet examined. It is also exceptional in having a superhelix density considerably higher than that known for any other natu-

rally occurring closed circle (10). As shown in Figure 2 the species present in virion PM2 DNA can be resolved by the use of chloroquine as an unwinding ligand. Because of the large number of species it was necessary to electrophorese this DNA on 30 cm gels in order to obtain sufficiently good resolution for quantitation of the individual species.

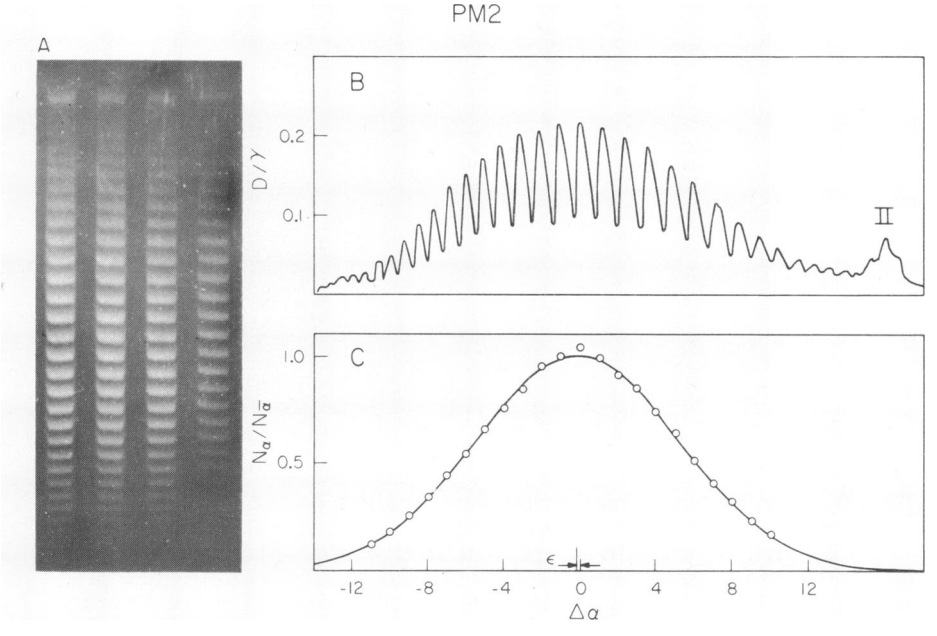


FIGURE 7. The Distribution of Species Within PM2 Form I DNA.
Panel A shows the species present in PM2 form I DNA resolved after electrophoresis at 3 v/cm, for 38 hrs in the presence of 20 $\mu$g/ml of chloroquine phosphate in a 1% agarose gel. Panel B is a densitometric trace of one of the channels shown in A. The large peak on the right hand side is PM2 form II DNA. A plot of the number of molecules, $N_\alpha / N_{\bar{\alpha}}$, against $\Delta\alpha$ is shown in panel C. The normalization of $N_\alpha$ with respect to $N_{\bar{\alpha}}$ as well as the sign of $\Delta\alpha$ and the determination of $\epsilon$ are explained in the legend to Figure 5.

Figure 7 (A) is an example of a gel in which the multiple species are resolved as negatively supercoiled molecules after electrophoresis in the presence of 20 $\mu$g/ml chloroquine phosphate. As shown in Figure 7 (C) the relative masses ($m_\alpha \propto N_\alpha/N_{\bar{\alpha}}$) of the species, when plotted against $\Delta\alpha$, fit a Gaussian curve, having a C value of 0.017 ± 0.001 and an $\epsilon$ value of 0.04 ± 0.09. PM2 is similar to the other DNAs so far examined

in that the distribution of species is much broader than a purely thermal distribution.

TABLE 1. Parameters of the Distributions of Species Within Native DNAs and Within the Corresponding Limit Products of N-C Enzyme Action

| | C | $\epsilon$ | Species | B/2RT | Species |
|---|---|---|---|---|---|
| SV40 Virion | 0.051±0.004 | -0.187±0.030 | 17-18 | 0.200 | 8-9 |
| SV40 Intracellular | 0.050±0.004 | -0.187±0.015 | 17-18 | | |
| Polyoma Virion | 0.053±0.005 | -0.211±0.040 | 17-18 | 0.192 | 8-9 |
| Polyoma Intracellular | 0.050±0.006 | -0.187±0.005 | 17-18 | | |
| PM2 | 0.017±0.001 | 0.04±0.09 | 26-30 | 0.107 | 13 |
| Minicol | 0.092±0.014 | 0.18±0.03 | 13-14 | 0.310 | 7 |

The values of C and of $\epsilon$ for each of the distributions are averages of a minimum of 10 determinations except for Minicol where the values are an average of 3 determinations. The values for B/2RT were calculated from Equation 4, where b/RT has previously been determined to be $2.06 \times 10^3$ (22) and the molecular weights of the DNAs were taken to be: SV40, $3.4 \times 10^6$ daltons; polyoma, $3.55 \times 10^6$ daltons; PM2, $6.4 \times 10^6$ daltons; and Minicol, $2.2 \times 10^6$ daltons.

Table 1 is a summary of the results obtained above for the various DNAs. In the first two columns are the values of C and $\epsilon$ for the native DNAs. In the third column are the approximate number of supercoiled species visible in the native form I DNAs. The fourth column contains the values of B/2RT, the molecular weight dependent free energy coefficient previously defined (22). B/2RT, a quantity analogous to C, describes the Gaussian curve which is defined by the relative masses present in a thermal distribution of species, such as that generated by the action of N-C enzyme on closed circular DNA. In the fifth column are the number of supercoiled species visible in the limit product of N-C enzyme action on the corresponding form I DNA.

## DISCUSSION

### The Papovaviruses

A brief report on the quantitation of the species present within intracellular SV40 form I has been published (4). The value of the C term for the distribution estimated by these authors (0.046) is similar to that determined in the present study (0.051). In disagreement with points made by

the above authors, it should be noted that the C value obtained for distributions in native DNAs, although formally equivalent to the B/2RT values of thermal distributions, cannot be considered to be a superhelix free energy coefficient for naked DNA, since the in vivo environments are not well characterized. As outlined in the Introduction, any two species within a resolved distribution must differ by an integral number of superhelical turns. This point disagrees with a statement of the above authors which attributes the breadth of the native SV40 distribution to the existence of two interleaved distributions where adjacent bands differ by approximately one-half of one superhelical turn.

The resolution obtained through the use of chloroquine has permitted accurate quantitation of the relative amounts of the species within the various native form I DNAs. In certain respects the heterogeneity of superhelix density within the different DNAs appears to be similar. In all cases the distributions are much broader than the thermal distributions generated by the action of N-C enzyme on the corresponding DNAs, but in each case where the origin of supercoiling is different, the breadth of the distribution presents a separate problem. Because the origin of the supercoils present within native polyoma and SV40 DNAs is known, problems posed by heterogeneity in $\alpha$ can be more clearly formulated if these two DNAs are first considered. The DNA of both polyoma and SV40 is covered to a large extent with nucleosomes and the reported distributions of nucleosomes on both DNAs fall within a narrow range of 20-21 with a deviation on each determination of only 1 to 1.5 (1, 9, 11). On the basis of these reports, heterogeneity in the number of nucleosomes could not account for the observed breadth of the distributions in $\alpha$.

In considering heterogeneity in $\alpha$, we first note the effect of molecular weight of the DNA on the breadth of thermal distributions. As has been shown previously (22), the species within the limit product of N-C enzyme action on closed circular DNA conform to a Boltzmann distribution defined by the free energy of supercoiling.

$$N_i/N_t = Ae^{-B(\tau - \bar{\tau})^2/2RT} \qquad 3$$

The equation is Gaussian and is formally equivalent to Equation 2. $N_i$ is the number of molecules having a value of $\tau = i$, $N_t$ is the total number of molecules in the distribution, R and T have their usual meanings, and $A = N_{\bar{\tau}}/N_t$ where $N_{\bar{\tau}}$ is the number of molecules of a theoretical species lacking supercoils ($\bar{\tau} = 0$) at the time of ring closure. B, the molar free

energy coefficient, is inversely proportional to the molecular weight (M) of the DNA. A molecular weight independent free energy coefficient, b, has previously been defined as:

$$b \equiv BM/662 \qquad 4$$

where 662 is taken to be the molecular weight of a base pair in sodium DNA. After appropriate substitutions, the following relation is obtained:

$$N_i/N_{\bar{\tau}} = e^{-331\ (b\tau^2/MRT)} \qquad 5$$

At a constant detectability limit for DNA, which can be defined by taking $N_i/N_{\bar{\tau}}$ as constant, it follows that $\tau_i^2$ is proportional to M. It can therefore be seen that at this limit, the number of species visible within a thermal distribution (bracketed by the values, $-\tau_i$ and $\tau_i$) is proportional to $\sqrt{M}$. From the molecular weights of SV40 and polyoma DNAs (taken to be $3.4 \times 10^6$ daltons and $3.55 \times 10^6$ daltons, respectively) and the previously published value for b/RT of $2.06 \times 10^3$, the values of B/2RT for thermal distributions of SV40 and polyoma DNAs are calculated to be 0.20 and 0.19, respectively. The number of visible species in the distributions is approximately 9.

The following situation is now considered for SV40 and polyoma minichromosomes. Assuming i) that the number of nucleosomes per viral genome is constant at 21, ii) that all nucleosomes are identical in terms of the manner in which they affect the winding of the DNA, iii) that the minichromosomes are completely relaxed (i.e. that there can be a thermal fluctuation on the histone-DNA complex), and iv) that approximately 180 base pairs of DNA are held rigidly within a nucleosome, it follows that the effective free molecular weight of DNA in a minichromosome becomes $9 \times 10^5$ daltons for SV40 (i.e. 26% of the SV40 genome) and $1.05 \times 10^6$ daltons for polyoma (i.e. 29% of the polyoma genome). The values of B/2RT calculated on the basis of the preceding assumptions, for thermal distributions on the SV40 and polyoma minichromosomes, respectively are 0.76 and 0.65 which correspond to distributions in which 4-5 bands are visible. As noted above B/2RT is formally equivalent to C in that both quantities determine the shape of a Gaussian distribution. The values of C, 0.051 and 0.052, determined for the distributions of species within native SV40 and polyoma DNAs, are an order of magnitude smaller than the above calculated values for B/2RT. In addition, the number of species visible within these native DNAs, 18, is much greater than the predicted number of 4-5. The argument that only a small fraction of the DNA within the minichromosome is

free to rotate is supported by the lack of any significant difference in the distributions of intracellular SV40 DNA isolated at 23°C and at 37°C as shown in Figure 3 panel I (A) and (B). Previous work has shown that there is a substantial effect of temperature on the equilibrium winding of the duplex in free DNA (5, 22, 32). Since the cell contains a large excess of N-C enzyme it is probable that the minichromosomes are at thermal equilibrium within the cell. This would lead to the prediction that if all of the DNA within the minichromosome were free to rotate, a decrease in temperature of 14°C would result in the loss of 3 negative superhelical turns by all of the molecules. From the foregoing discussion it is clear that the heterogeneity in $\alpha$ within papovavirus DNAs presents a paradox. As shown here (see Table 1) the distributions of supercoiled species in the intracellular DNAs are indistinguishable from those in the corresponding viral DNAs. It can therefore be concluded that if encapsidation in any way alters the winding of the DNA helix, the associated stress is not relieved by a nicking-closing event. Conversely, if nicking-closing events occurred during the process of encapsidation, it could be concluded i) that packaging does not affect the winding of the DNA duplex and ii) that the heterogeneity in $\alpha$ in polyoma and SV40 DNAs is a phenomenon associated solely with nucleosomes. In either case, it is probable that the heterogeneity in $\alpha$ is a reflection of structural aspects of chromatin. The conservation of the number of nucleosomes on the polyoma and SV40 minichromosomes is reflected in the conservation of the number of superhelical turns in the DNAs ($26 \pm 1$). The constancy of these numbers, as well as the similarity in the shapes of the distributions (values of C) present in the form I DNAs suggest that the mechanisms of DNA packaging are similar for both viruses. However, since the value of $\epsilon$ is sensitive to small differences in the molecular weight of the DNA (5), the similarity between the SV40 and polyoma $\epsilon$ values is probably adventitious. Constancy in the number of nucleosomes on DNAs of different molecular weights implies that the physical distribution of nucleosomes on DNA can vary and may be controlled by factors other than purely stochastic processes.

PM2

The DNA of the marine bacteriophage, PM2 is at present the only known example of a packaged closed circular DNA of bacterial origin (6). The superhelix density of PM2 DNA is approximately 1.7 times that of native SV40 DNA (10). The high superhelix density of this DNA is paradoxical in view of the high ionic strength of the marine-like environment in which

the bacteriophage is grown (0.5 M NaCl, 50 mM $MgSO_4$, 10 mM $CaCl_2$) as well as the relatively low growth temperature (25°C), since both decreases in temperature and increases in ionic strength result in overwinding of the DNA duplex (32) and since in this respect magnesium ions have an anomalously large effect (26). It would be predicted that thermal equilibration of the DNA at 25°C under the above ionic conditions would result in a decrease in negative superhelix density rather than the observed increase relative to DNAs extracted from sources in which the intracellular environment is approximated by 0.2 M NaCl at 37°C. It is possible that folding of the DNA accompanying virus assembly may be responsible for both the high superhelix density and the heterogeneity in $\alpha$ of PM2 DNA.

Bacterial Plasmids

The mechanism of supercoiling of bacterial DNAs is at present unknown. There have been several reports of basic, histone-like proteins from E. coli and other prokaryotes (13, 25) which could possibly function in a simlar way to the histones. It has also been postulated (12) that the condensation of bacterial DNAs might be the result of interactions between the DNA and small molecules such as polyamines, high concentrations of which are present in bacterial cells.

A new line of evidence with regard to the origin of the supercoiling of bacterial plasmid DNAs comes from the experiments reported here in which the Minicol plasmid has been isolated after lysis of the cells at different temperatures. The results show that the average superhelix density of this plasmid is a temperature dependent quantity. This result argues against the hypothesis that the supercoiling of bacterial DNAs is due to the tight binding of an intracellular ligand. If the DNA were complexed with a tightly bound ligand, the degree of supercoiling would be expected to be related in a simple, continuous manner to the ambient temperature, where the magnitude of this effect would depend upon the amount of DNA free to rotate. Intracellular SV40 DNA provides us with an analogous case where, in the present work, the supercoiling has been shown to be unaffected by the temperature of isolation.

In view of the problems that have been experienced in obtaining samples of plasmid DNAs that do not show signs of partial relaxation, we feel that caution should be exercised in the interpretation of changes in superhelix density of plasmid DNAs (2, 30).

No attempt has been made to assess superhelix densities by measuring changes in mobilities of closed circular DNAs as a function of either

chloroquine or ethidium concentration. The potential accuracy of such titration procedures (3, 7) is severely limited by the uncertainties in determining both the binding constant and the free ligand concentration under the electrophoresis conditions. The band counting procedures previously reported (18, 26) are based on an absolute scale and avoid the inaccuracies associated with the above methods.

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