I. STUDIES OF CLOSED CIRCULAR DNA: PHYSICAL AND BIOLOGICAL IMPLICATIONS OF HETEROGENEITY IN THE TOPOLOGICAL WINDING NUMBER, α

II. THE STRUCTURE OF VIRION SV40 DNA IN SITU EXAMINED BY CHEMICAL MODIFICATION WITH DIMETHYLSULFATE

Thesis by

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In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1979

(Submitted October 13, 1978)

In Memory of Jerome Vinograd

ACKNOWLEDGMENTS

The first four chapters of this thesis contain work performed in the laboratory of Jerome Vinograd. I am grateful to him, as well as to both John Mayfield and Joseph Gall, in whose laboratories I worked while an undergraduate, for providing encouragement, enthusiasm, and atmospheres of true scientific curiosity, while at the same time, a large degree of independence.

During the past four years, my financial support was provided by an Institute Earle C. Anthony Fellowship and by an NSF Graduate Fellowship.

The preparation of this thesis was aided both by a grant from the Jean Weigle Memorial Fund and by the capability and patience of Bernita Larsh, to whom I would like to express sincere thanks.

Finally, I would like to thank Onyx for her cooperation in not shredding any of the pages of this manuscript.

ABSTRACT

Methods for gel electrophoresis have been developed which permit the resolution of closed circular DNA molecules differing by unit values in their topological winding number, α. Relaxed or nonsupercoiled closed circular DNAs, native supercoiled DNAs, as well as DNAs having intermediate numbers of superhelical turns, can be resolved into sets of bands by one or more of the different electrophoresis conditions. In addition, a method based on theory (presented in Chapter IV) has been developed for the photographic quantitation of fluorescent substances. DNA stained with ethidium in agarose gels is used as an example. In the course of developing this method, it has been demonstrated that the empirical methods employed by other authors can give rise to large systematic errors.

The methods for electrophoresis, used in conjunction with the method for photographic quantitation of fluorescence, have permitted a quantitative examination of closed circular DNA. The results of such studies (presented in Chapters I, II, and III) are summarized below.

The limit product of the action of nicking-closing (N-C) enzyme on closed circular DNA is not a homogeneous species but rather a distribution. Each distribution has a mean degree of supercoiling of approximately zero. The individual species within the distributions differ by $\Delta\tau = \pm 1, \pm 2$, etc., and the relative masses fit a Boltzmann distribution. It has also been demonstrated that "nonsupercoiled" closed circular duplex molecules serve as substrates for N-C enzyme and that a distribution of topological isomers is generated. Polynucleotide ligase, acting on nicked circular DNA, forms under the same conditions, the same set of closed DNAs. The latter enzyme freezes the population into sets of molecules otherwise in configurational equilibrium in solution.

By a method of overlapping the results obtained after agarose gel electrophoresis under two different sets of conditions, it has become possible to determine the number of superhelical turns in a given DNA by counting the bands present after partially relaxing the DNA with N-C enzyme. Because native supercoiled DNA is heterogeneous with respect to α, an average number of superhelical turns was determined. Virion SV40 DNA contains 26 ± 0.5 superhelical turns in 0.2 M NaCl and at 37°C, the conditions under which the enzymatic relaxations were performed. Under this same set of conditions, virion polyoma DNA contains 26 ± 1 superhelical turns, which is consistent with the observations that polyoma DNA has a higher molecular weight, a lower superhelix density, but the same number of nucleosomes as has SV40 DNA.

The average number of superhelical turns in SV40, 26, combined with the value, 21, for the average number of nucleosomes per SV40 genome, yields an average of 1.25 superhelical turns per 1/21 of the SV40 genome. If the regions of internucleosomal DNA are fully relaxed, 1.25 corresponds to the average number of superhelical turns within a nucleosome.

The superhelix densities determined by the band counting method have been compared with those determined by buoyant equilibrium in propidium diiodide (PDI)-CsCl gradients. A comparison of the values obtained by the two methods permits a calculation of an unwinding angle for ethidium. The mean value determined from experiments with SV40 DNA is $23 \pm 3°$.

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, α. All native closed circular

DNAs examined, including the viral and intracellular forms of SV40 and polyoma DNAs, 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 enzyme on the respective DNAs. In addition, the distributions within the virion and the intracellular form I DNAs of SV40 were found to be indistinguishable. This was also found to be the case for the virion and the intracellular form I DNAs of polyoma.

In the cases of both 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 around 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 DNAs would be anticipated to be less heterogeneous than the thermal equilibrium mixtures present in the N-C enzyme relaxed SV40 and polyoma DNAs.

Finally (see Chapter V), the structure of virion SV40 DNA in situ has been examined by chemical modification with dimethylsulfate (DMS). Virions are permeable to DMS and remain physically intact while the DNA (specifically, the bases adenine and guanine) within the virus particles becomes methylated. This approach permits the examination of specific protein-DNA interactions in the absence of artifacts of isolation and reconstitution.

A total length of approximately 3600 nucleotides (35% of the nucleotides in SV40 DNA) was scanned using this method. A total of 15 segments of the SV40 genome (obtained by kinasing 15 different 5' termini produced by treatment with different restriction endonucleases) was screened for decreases or increases in levels of methylation relative to naked SV40 DNA. The segments represented both strands of the DNA and were chosen to obtain samples representative of the entire genome, with a particular emphasis on fragments covering and surrounding the origin(s) of DNA replication and both late and early RNA transcription. No major or convincing differences were ever observed between purine patterns obtained after parallel treatment of corresponding samples derived from naked SV40 DNA which had been treated with DMS and from DNA within SV40 virions which had been treated with DMS.

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CHAPTER I

Action of Nicking-Closing Enzyme on Supercoiled and Nonsupercoiled Closed Circular DNA: Formation of a Boltzmann Distribution of Topological Isomers

Reprinted from
Proc. Nat. Acad. Sci. USA
Vol. 72, No. 11, pp. 4280–4284, November 1975
Biochemistry

Action of nicking-closing enzyme on supercoiled and nonsupercoiled closed circular DNA: Formation of a Boltzmann distribution of topological isomers

(polynucleotide ligase/gel electrophoresis)

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Contributed by Jerome Vinograd, August 25, 1975

ABSTRACT **Highly purified nicking-closing enzyme from mouse cells in 20-fold enzyme/substrate excess converts closed circular native PM2, ColE1, and Minicol DNA into limit product sets of DNAs. Each set has a mean degree of supercoiling of approximately zero. The individual species in the sets differ by $\Delta\tau = \pm 1, \pm 2$, etc., and the relative masses fit a Boltzmann distribution. It was also demonstrated that "nonsupercoiled" closed circular duplex molecules serve as substrates for the nicking-closing enzyme, and that a distribution of topological isomers is generated. Polynucleotide ligase, acting on nicked circular DNA, forms under the same conditions, the same set of closed DNAs. The latter enzyme freezes the population into sets of molecules otherwise in configurational equilibrium in solution.**

Nicking-closing (N-C) activities that alter the topological winding number (α) of closed circular DNA occur widely in nature (1–5). The topological winding number is the number of revolutions that one strand makes about the other if the molecule is constrained to lie in a plane. The activities have been demonstrated to be enzymatic with proteins from *Escherichia coli* (6), mouse (7), and human (8) cells in culture. The N-C enzyme from mouse LA9 cell nuclei, purified to homogeneity in good yield, is a major constituent of chromatin and accounts for about 1% of the total protein (H-P. Vosberg and J. Vinograd, unpublished work). It is similar to other eukaryotic N-C enzymes in its ability to relax both positive and negative superhelical turns. A probable *in vivo* role for the enzyme is to provide the transient swivels required for DNA replication. Such swivels may also be required in transcription, and in the condensation and decondensation of chromatin.

In this study we have examined the limit product of the action of N-C enzyme on several closed circular DNAs by gel electrophoresis. Under appropriate analytical conditions, the limit product separates into a set of species differing in topological winding number. Individual species, isolated from the set, regenerate the original distribution upon incubation with the N-C enzyme. We view the foregoing as the consequence of four necessary events: nicking of the DNA, relaxation, random rotation about the swivel, and closure (Fig. 1). A set of species is also found when *E. coli* polynucleotide ligase is used to close a nicked circular DNA (9, 10). It is shown here that distribution of products obtained with ligase is indistinguishable from that obtained with N-C enzyme when the incubation conditions are the same for both reactions.

The relative masses of the species, when plotted against α, fall on a Gaussian curve. Such a curve is anticipated for a Boltzmann distribution, when the energy of supercoiling is proportional to the square (11) of the degree of supercoiling. The similarity of the products obtained with two different enzyme systems and the further similarities of the values of the free energy of supercoiling obtained here and by nonenzymatic procedures strongly indicate that the Boltzmann distributions are characteristic of the thermally induced torsional fluctuations of free DNA uninfluenced by the presence of an enzyme.

Abbreviations: N-C, nicking-closing; EtdBr, ethidium bromide.

MATERIALS AND METHODS

Enzymes and DNA. *E. coli* polynucleotide ligase was a gift of Dr. H. Boyer. N-C enzyme was prepared from mouse LA9 cell nuclei (H-P. Vosberg and J. Vinograd, unpublished work). DNase I was purchased from Worthington. PM2 DNA was prepared according to (12). ColE1 DNA was prepared from bacterial strain JC411 (ColE1) (13). Minicol DNA was prepared from bacterial strain PVH51 supplied by Dr. H. Boyer (14).

Relaxation of Closed Circular DNA. DNA (5–50 μg/ml) was incubated in 0.2 M NaCl, 0.01 M Tris-HCl, 0.1 mM EDTA at pH 7.4 with 5–20 units of N-C enzyme per μg of DNA for 24 hr at 37°. When relaxations were performed at other temperatures, a second addition of 10 units of enzyme per μg of DNA was made after 24 hr and the incubation was continued for a further 24 hr. Reactions were terminated by the addition of sodium dodecyl sulfate to a final concentration of 0.1%. For comparison of the enzymatically relaxed DNA with ligase closed DNA, both reactions were carried out in 0.2 M NaCl, 3 mM $MgCl_2$, 5 mM $(NH_4)_2SO_4$, 33 μM NAD, 0.2 mM EDTA, 0.1 mM spermidine, 20 μg/ml bovine

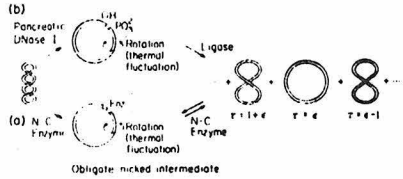

FIG. 1. The formation of a Boltzmann distribution of topological isomers of closed circular DNA. (*a*) The action of N-C enzyme on a closed circular DNA substrate. The diagram is not intended to specify the mechanism for N-C action. The obligate elementary steps include nicking, releasing preexisting supercoils, random rotation at the swivel, and closure. The quantity ϵ is the difference between the duplex winding number (β) of a nicked DNA and the duplex winding number of a similar hypothetical molecule with $\tau = 0$. The fractional turn ϵ is not illustrated in Fig. 1. (*b*) The closure of nicked circular DNA by polynucleotide ligase.

serum albumin, 30 mM Tris·HCl at pH 7.8 for 8 hr at 37°. Five micrograms of DNA and 140 units of N-C enzyme and/or 2×10^{-3} units of ligase were used in the reactions.

Electrophoresis. A vertical slab gel electrophoresis apparatus (Aquebogue) was used. Gels contained 1% agarose (Sea Kem), 40 mM Tris-acetate at pH 7.8, 0.5 mM EDTA. Magnesium acetate (5 mM) was added in most instances. Samples (5–100 μl) containing 200–400 ng of DNA were layered into the sample wells in a solution containing approximately 10% Ficoll 70 (Pharmacia) and 2 mM EDTA at pH 8. Up to 250 μg of DNA in 1 ml of the same solution was layered into the large sample well of a preparative gel. Two volts per cm were applied to the 8 mm preparative gels and 3 V/cm to the 4 mm analytical gels. Electrophoresis times were varied according to voltage gradient, molecular weight of the DNA, and temperature.

Determination of Relative Masses of DNA Species by Fluorescence Photography. Gels were stained in the dark overnight in 10 mM Tris·HCl, 2 mM EDTA, 2 μg/ml of ethidium bromide (EtdBr). The gels were illuminated from below with short wavelength ultraviolet light from a Transilluminator (Ultra Violet Products Inc.) and photographed on Kodak Plus X film. The films were traced on a Joyce-Loebl microdensitometer. Traces were evaluated with a Hewlett Packard 9864A Digitizer Platen and 9820A calculator. Relative fluorescence intensities (J_i) were evaluated and integrated over the band to obtain the relative mass with the equation

$$\sum_h J_i \Delta x_i = \sum_h (10^{D_i/\gamma} - 1)\Delta x_i \qquad [1]$$

where D_i is the optical density above background and γ is the slope of the characteristic curve of the film (γ was evaluated for each film). Where appropriate, the areas under each peak in the optical density traces were also evaluated. The areas give a good approximation to the relative concentrations of species providing $D/\gamma \ll 1$. Procedures for calculating ϵ and B (defined below) for the distributions were incorporated into the integration program. A full description of the experimental method will be presented elsewhere (D. E. Pulleyblank and J. Vinograd, unpublished work).

Extraction of DNA from Gels. Stained gels were placed on a mask with 3 mm slots parallel to the direction of the electrophoresis, and portions of the DNA bands were visualized by illumination from below. The gel was sliced so as to separate bands, and the sections that had received direct UV illumination were discarded. The remaining gel fragments were frozen and thawed three times and then centrifuged at 40,000 × *g* for 1 hr. The supernatant, containing approximately 30–50% of the DNA in the gel slice, was freed of ethidium by extraction with 1-butanol. The DNA was precipitated from the aqueous phase by the addition of 2 volumes of cold ethanol, followed by centrifugation in an SW 50.1 rotor at 35,000 rpm at 4° for 1 hr.

RESULTS

The Existence of Multiple Species of Closed Circular DNA in the Limit Product of N-C Enzyme Action. Keller and Wendel (4) were able to resolve a series of species in partially relaxed closed circular simian virus 40 DNA using agarose gel electrophoresis. Their assumption that adjacent, resolved species differ by a single superhelical turn has been used throughout the present study of DNAs with low numbers of superhelical turns. The validity of the assumption is considered in the *Discussion* section.

1 2 3 4 5 6 7 8 9 10

FIG. 2. Demonstration that non-supercoiled DNA is a substrate for the action of N-C enzyme and that N-C enzyme reacts with a homogeneously supercoiled species of closed circular DNA ($\tau \approx 0$) to form a thermal distribution of species. (*1*) Minicol I and II; (*2*, *5*, and *8*) limit products of the initial reaction of Minicol I with N-C enzyme; (*3*, *6*, and *9*) purified species with $\tau \approx +1, 0, -1$, respectively; (*4*, *7*, and *10*) limit products regenerated after reaction of purified species with N-C enzyme. Bands migrating slower than Minicol II in channels 4, 7, and 10 are sets of relaxed ColE1 DNA. Native ColE1 DNA was added to the reaction mixtures to monitor the activity of the enzyme.

In a study of the effects of electrophoresis conditions on the resolution of closed circular DNAs with differing values of α, it was found that the limit product of N-C enzyme action on closed circular DNA could be resolved into multiple species. Optimum resolution was achieved at 4° in the presence of 5 mM Mg^{++}. Under these electrophoresis conditions, the mean duplex winding number (β) is greater than during the relaxation reaction (15). The increase is compensated by the generation of $\Delta\tau$ negative superhelical turns in all members of the set according to the equation $\Delta\beta = -\Delta\tau$. Comigration of pairs of species that initially had equal numbers of positive and negative superhelical turns is eliminated, and the magnitudes of τ are such that the gel resolves species that differ by unit values of τ.

The Generation of a Set of Species from an Isolated Homogeneous Species. The thermal nature of the distribution observed in the limit products of the N-C enzyme action on closed circular DNA has been established with experiments such as shown in Fig. 2. The three dominant species of Minicol DNA present in the N-C enzyme limit products (channels 2, 5, and 8) were isolated separately from a preparative agarose gel and purified. These materials (channels 3, 6, and 9) were treated with the N-C enzyme under the conditions used to generate the original set of limit products. In each case a new set of products was generated (channels 4, 7, and 10) with the same distribution as the original set.

The above results lead to the following conclusions. (*i*) The species found in the original reaction mixture are the limit products of the reaction and are not generated by incomplete relaxation of the supercoiled substrate. Therefore, under the conditions of reaction, the materials in channels 3, 6, and 9 contained approximately +1, 0, and −1 superhelical turns, respectively. (*ii*) Species are generated with higher and lower superhelical winding numbers than the substrates for the rereaction. Rotation about the swivel must, therefore, be driven by thermal fluctuations. The creation of a multi-

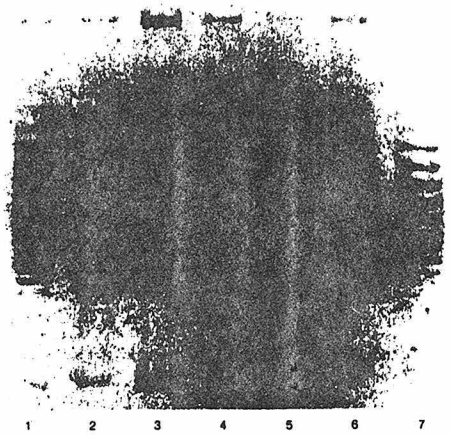

FIG. 3. The limit product of the N-C enzyme is indistinguishable from the ligase-closed product. (*1*) PM2 II + Ligase; (2) PM2 I + N-C Enzyme; (*3*) I, II; (*4*) mixture of products in (*1*) and (2); (*5*) PM2 I, II + N-C Enzyme+Ligase; (*6*) PM2 II + N-C Enzyme+Ligase; (*7*) ligase closed PM2 + N-C Enzyme. Reactions were carried out in 0.2 M NaCl, 3 mM $MgCl_2$, 5 mM $(NH_4)_2SO_4$, 33 μM NAD, 0.2 mM EDTA, 0.1 mM spermidine, 20 μg/ml of bovine serum albumin, 30 mM Tris·HCl at pH 7.8 for 8 hr at 37°; 5 μg of DNA, 140 units of N-C Enzyme, 2×10^{-3} units of ligase were used in the reactions. Nicked and linear DNA were removed by EtdBr–CsCl buoyant centrifugation. PM2 I (10 ng) was added to each channel.

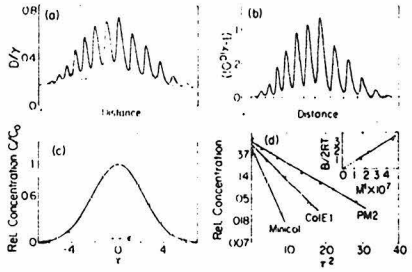

FIG. 4. Processing of data for the relative masses in each set. The Boltzmann distribution. (*a*) Absorbance trace of a photograph of an ethidium stained gel of relaxed PM2 DNA with background traces from both sides of the sample channel. Sample background (- - -) was calculated by averaging the background traces. The ordinate is plotted in units of D/γ. (*b*) Plot of fluorescence intensity against distance, generated from the optical density trace shown in 4a. The curve was calculated by the equation $J = 10^{D/\gamma} - 1$, where D is the optical density above background. The peaks were integrated between the indicated limits, to determine the relative masses of the species present. (*c*) The concentration of species present in solution are normalized by C_0, the concentration of a theoretical species lacking supercoils. The curve through the points is the calculated Gaussian curve with the best least squares parameters, 0.105 and 0.33 for $B/2RT$ and ϵ, respectively. The point lying above the curve at $\tau = 0.67$ was not used in the least squares fit because of contamination of this species by linear PM2 DNA. (*d*) The natural logarithm of the relative masses of species in the limit products of three closed DNAs treated with N-C enzyme, plotted against the square of the superhelical winding number. The intercepts of the traces have been displaced for clarity of presentation.

ple set from a single species represents an increase in the entropy of the system. (*iii*) Supercoiling of the closed circular substrate is *not* a requirement for the action of the mouse N-C enzyme. Linear and nicked circular DNAs can therefore, with confidence, be considered substrates for the enzyme.

Sets of Species Formed with N-C Enzyme and Closed Circular DNA Are Indistinguishable from Those Formed with Ligase and Nicked Circular DNA. Ligase closure of nicked circular DNA also leads to a set of species with different topological winding numbers (10). The distribution of species was compared with the distribution of N-C enzyme products (Fig. 3). Since environmental conditions affect the duplex winding number, and hence the position of the center of the distribution (see below), it was necessary to perform the reactions under conditions that were as similar as possible. The observed distributions were indistinguishable (channels 1 and 2). The addition of a second enzyme and/or substrate to a reaction mixture caused no detectable change in the distributions (channels 4, 5, 6, and 7), and ruled out the possibility that the correspondence between the distributions in channels 1 and 2 was due to adventitious effects of protein binding. The N-C enzyme did not close the ligase substrate PM2 II (data not shown).

We conclude that the forms of the distributions generated by each of the two systems are the same, with respect to both the center of the distribution and, with less accuracy, to the relative concentrations of the species present. The comparison of products generated by the two enzymatic systems corroborate conclusion (*i*) of the previous section.

The Boltzmann Distribution. A set of molecules at thermal equilibrium contains a distribution of states with respect to all degrees of freedom available to the molecules. The number of molecules in a given state (N_i) within the set (N_t) is related to the energy of the state (E_i) by the Boltzmann equation

$$N_i/N_t = A \exp(-E_i/RT) \qquad [2]$$

where A is a normalization factor; R and T have their usual meanings. A closed circular DNA with a given value of α cannot come to thermal equilibrium with respect to α, because of the requirement for breakage and reformation of a covalent bond in the phosphodiester backbone. The N-C enzyme catalyzes these reactions, and allows the system to come to equilibrium.

The molar free energy associated with supercoiling of closed circular DNA (G_τ) has been determined for superhelical simian virus 40 DNA (11) and PM2 DNA (15) and is related to the number of superhelical turns by the equation

$$G_\tau = (B\tau^2/2) \qquad [3]$$

where B is a constant. The superhelix density, $\sigma = 20\,\tau/N$ where N is the number of nucleotides in the DNA is substituted into [3]

$$G_\tau = B(N\sigma/20)^2/2 \qquad [4]$$

We observe, providing G_τ is proportional to N when σ is constant, that B must be inversely proportional to N.

Upon incorporating Eq. [3], the Boltzmann Eq. [2] becomes

$$N_i/N_t = A \exp(-B\tau^2/2RT) \qquad [5]$$

The equation is Gaussian, and a plot of ln N_i against τ^2 should be linear. We have defined ϵ ($-0.5 < \epsilon \leq 0.5$) as the difference between the duplex winding number β of a

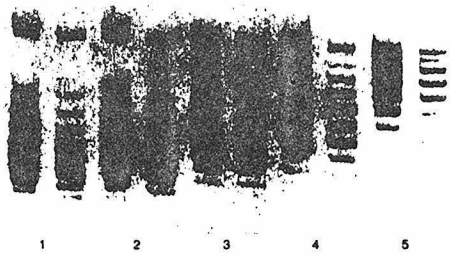

FIG. 5. The effect of the incubation temperature on the position of limit products in a slab-gel electrophoresis experiment. The paired samples contained 100 and 200 ng of DNA. The incubation temperatures were 41.5°, 37.2°, 29.6°, 22.5°, and 13.4° in (*1*) to (*5*).

nicked DNA and the duplex winding number of a similar hypothetical closed molecule with $\tau = 0$ in the same environment. The number of superhelical turns in a member of the distribution is defined by the equation $\tau = I + \epsilon$ where I is an integer.

The relative concentrations of the species present in the limit products of N-C enzyme action on Minicol, ColE1, and PM2 DNAs were determined from traces of photographs of ethidium stained gels (Fig. 4a and b). The relative concentrations were fitted by a least squares procedure to a Gaussian equation to determine the best value for ϵ and $B/2RT$ (Fig. 4c). The natural logarithm of the relative concentrations are plotted against τ^2 (Fig. 4d). The slopes of the lines ($B/2RT$) are inversely proportional to the molecular weight of the DNA (Fig. 4d, insert); this is in agreement with the prediction made above. The values of $B/2RT$ measured at 37° for PM2, ColE1, and Minicol DNA were 0.10, 0.17, and 0.32, respectively. The reproducibility was ±10% for measurement of 16 channels in two gels for ColE1 DNA, and measurements of 12 channels in two gels for PM2 and Minicol DNAs. Note that the number of species observed is approximately proportional to the square root of the molecular weight of the DNA (Fig. 4d).

The Thermal Unwinding of DNA. The value of the equilibrium duplex winding number (β) is sensitive to small changes in the environment of the DNA. Analysis of the distributions obtained after N-C enzyme action on closed circular DNA give accurate values for ϵ. Changes in β can be measured by counting the number of turns including partial turns (ϵ) between the center of a sample distribution and the center of a reference distribution. In general ϵ can be estimated to within ±0.1 duplex turn; this corresponds to a limit of accuracy for PM2 DNA of about ±0.2 turn in the 10^3 duplex turns.

Temperature and protein binding are examples of factors that affect β and $\tilde{\alpha}$ (see *Discussion* for the definition of $\tilde{\alpha}$). Here closed circular PM2, ColE1, and Minicol DNA samples were relaxed at different temperatures (Fig. 5). Each distribution was analyzed by the least squares procedure to obtain the value of ϵ, and hence the position of the center of the distribution. One of the species in the distributions was used as an arbitrary marker, and the number of helical turns to the center of each distribution was calculated and plotted against the temperature of reaction (Fig. 6). The temperature dependence of the rotation angle was $-1.4 \pm 0.1 \times 10^{-2}$ °/C°, base pair.

Quantitation of the mass distributions in Fig. 5 showed that the values of $B/2RT$ were constant to within ±10%,

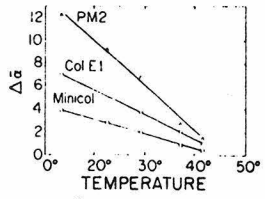

FIG. 6. The temperature dependence of the center of the Boltzmann distribution. Each distribution was analyzed by the least squares procedure to obtain the value of ϵ, and hence the position of the center of the distribution. A corresponding band was used in each set as a reference; the number of turns including partial turns (ϵ) to the center of each distribution was measured. The center of the distribution corresponds to $\tilde{\alpha}$ as defined in the *Discussion*.

which indicates that the entropy of supercoiling is the major component of the superhelix free energy.

DISCUSSION

The assumption that each resolved species differs from its neighbors by a single turn in its topological winding number is supported by two arguments. It is the simplest explanation for the observation of discrete bands. Other proposals, for example, that adjacent species differ by two turns in the topological winding number, appear artificial and especially unlikely in view of the similarity of the results obtained here with N-C enzyme and polynucleotide ligase. If a shift in the center of the distribution by a single band is equivalent to a 360° change in the winding of the duplex, we calculate the thermal unwinding angle of duplex DNA to be $-1.4 \pm 0.1 \times 10^{-2}$ °/C°, base pair. This value corresponds well with $-1 \pm 0.2 \times 10^{-2}$ °/C°, base pair calculated from the results of Wang (16) by using 26° instead of 12° for the unwinding angle (ϕ) of ethidium when bound to DNA (17, 18).

The Free Energy of Supercoiling. The free energy of supercoiling has been determined in the past by studying the relative binding affinities of a closed circular DNA and its nicked circular counterpart for the unwinding ligand, ethidium (11, 15). To compare the more direct results presented here with those obtained previously, it is necessary to know ϕ. Provided that the DNA is negatively supercoiled, a term ν_c is defined as the molar ratio of bound ethidium to DNA phosphate when all superhelical turns have been removed. If ϕ is expressed in degrees $\tau = N\,\phi\nu_c/360$, where N is the number of nucleotides in the DNA. Eq. [3] can then be written

$$G_\tau = (B/2)(N\phi\nu_c/360)^2 \qquad [6]$$

As noted previously, B is inversely proportional to the molecular weight of the DNA. We define a molecular weight independent term $b \equiv BN/2$ to compare the present results with those obtained previously by others. Eq. [3] assumes the form $G_\tau = b\tau^2/N$ where G is in cal mol^{-1} when RT is in cal mol^{-1}. The value of b/RT calculated for the three DNAs in the present study is $2.06 \pm 0.14 \times 10^3$. We have reevaluated the coefficient of Eq. [24] of Bauer and Vinograd (11), with the more recent value of 26° for ϕ, and obtained 1.78×10^3. With the value of a_1 as defined and determined by Wang (ref. 15, cf. Eq. [14e] and Eq. [6] above), we obtain a value for b/RT of 1.05×10^3. The correspondence between these values is satisfying in view of the widely differing experimental methods and the present uncertainties about the cor-

rect value of ϕ (18). It should be pointed out that the correspondences may be the result of compensating errors, in particular since the salt concentration was 0.2 M instead of 3.0 and 5 M in the previous studies.

Superhelix Density Heterogeneity and the Relationships among the Winding Numbers. We define a time dependent variable β_t as the number of duplex turns in a nicked circular DNA molecule. The equilibrium duplex winding number β is defined as the time-averaged or ensemble-averaged value of β_t. Since the system of closed circular molecules generated by the closure of a nicked circle is not homogeneous with respect to α, we define a new term $\tilde{\alpha}$ as the median of the Gaussian curve that fits the Boltzmann distribution in α (Fig. 4c). The term $\tilde{\alpha}$ is equal to the value of β at the time of ring closure, and approximates the average value of α. In addition, the term $\tilde{\tau}$ is defined by $\tilde{\tau} = \tilde{\alpha} - \beta$. Under the conditions of ring closure, $\tilde{\tau} = 0$, whereas individual molecules have values of $\tau = I + \epsilon$, where I is integral. The equation $\tilde{\tau} = \tilde{\alpha} - \beta$ replaces, in the case of a Gaussian distribution, the previous equation, $\tau = \alpha - \beta$, derived for a single species.

Mechanism of Action of N-C Enzyme. The results presented here do not allow us to distinguish between alternatives for one aspect of the N-C enzyme action on "nonsupercoiled" DNA: the single turn mechanism in which rotation at the swivel is limited to one turn during a nicking-closing event, and the multiturn mechanism in which several rotations can occur during the nicking-closing event. The direction of rotation in both cases would be biased by the free energy of supercoiling. Comparable alternative explanations have been considered for the appearance of intermediates during the relaxation of highly supercoiled DNA (4, 7).

We wish to thank H. W. Boyer, University of California, San Francisco, for generously providing the polynucleotide-ligase and the bacterial strains harboring plasmids and R. B. Watson for advice and assistance with the gel electrophoresis experiments. This work was supported in part by USPHS National Institutes of Health grants GM15327 and CA08014. D.E.P. is a recipient of a Medical Research Council of Canada fellowship. This is Contribution no. 5156 from the Division of Chemistry and Chemical Engineering.

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CHAPTER II

The Number of Superhelical Turns in Native Virion SV40 DNA and Minicol DNA Determined by the Band Counting Method

Cell, Vol. 8, 215–226, June 1976, Copyright © 1976 by MIT

The Number of Superhelical Turns in Native Virion SV40 DNA and Minicol DNA Determined by the Band Counting Method

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Summary

By a method of overlapping the results obtained after agarose gel electrophoresis under two different sets of conditions, it has become possible to determine the number of superhelical turns in a given DNA by counting the bands present after partially relaxing the DNA (Keller and Wendel, 1974) with highly purified nicking-closing (N-C) enzyme from LA9 mouse cell nuclei. Because native supercoiled DNA is heterogeneous with respect to superhelix density, an average number of superhelical turns was determined. Virion SV40 DNA contains 26 ± 0.5 superhelical turns, and native Minicol DNA contains 19 ± 0.5 superhelical turns. The above are values at 0.2 M NaCl and at 37°C, the condition under which the enzymatic relaxations were performed.

The superhelix densities determined by the band counting method have been compared with superhelix densities determined by buoyant equilibrium in PDI-CsCl gradients. The Gray, Upholt, and Vinograd (1971) calculation procedure has been used for evaluating the superhelix densities by the latter method with the new statement, however, that relaxed DNA has zero superhelical turns. Comparison of the superhelix densities obtained by both methods permits a calculation of an unwinding angle for ethidium. The mean value from experiments with SV40 DNA is 23 ± 3°.

The average number of superhelical turns in SV40, 26, combined with the value, 21, obtained by both Griffith (1975) and Germond et al. (1975) for the average number of nucleosomes per SV40 genome, yields an average of 1.25 superhelical turns per 1/21 of the SV40 genome. If the regions of internucleosomal DNA are fully relaxed, 1.25 corresponds to the average number of superhelical turns within a nucleosome.

When analyzed under identical conditions, the limit product generated by ligating a nicked circular substrate in the presence of 0.001 M Mg^{2+} at 37°C (ligation conditions) is slightly more positively supercoiled than the limit product obtained when the N-C reaction is performed in 0.2 M NaCl at 37°C. The difference in superhelix density as measured in gels between the two sets of limit products for both Minicol and SV40 DNAs is 0.0059 ± 0.0005. This result indicates that the DNA duplex is overwound in the ligation solvent relative to its state in 0.2 M NaCl.

Introduction

DNA, as extracted from many sources, appears as double-stranded, closed circular, supercoiled molecules. The sources are varied and include bacteriophages, bacteriophage replicating forms, bacterial plasmids, both intracellular and viral forms of eucaryotic DNA viruses, mitochondrial DNA, and chloroplast and kinetoplast DNAs. Both the number of supercoils in a given DNA, as well as their origin, have been questions of interest for some time.

Previously, five methods were used to determine the superhelix density (σ) of a DNA, that is, the number of superhelical turns (τ) per 10 base pairs, β°. These methods include four based on the binding of ethidium bromide (EtdBr): titrations of the supercoiled DNA monitored by sedimentation velocity, by buoyant density, and by viscometry, and the buoyant separation method in high concentrations of EtdBr or propidium diiodide (PDI). The fifth method is alkaline buoyant density titration. Upholt, Gray, and Vinograd (1971) titrated SV40 DNA and determined a "standard" superhelix density in 3 M CsCl (σ_o) of −0.039. Since then SV40 DNA has been used as a standard against which the superhelix densities of other DNAs have been measured. Native virion SV40 DNA is, in fact, the only DNA which may be used as a standard in the buoyant separation method of Gray et al. (1971), because in the equation relating the difference in superhelix density ($\Delta\sigma_o$) to the separation between bands, the coefficient was derived using SV40 I DNA as a standard.

The EtdBr methods require knowledge of the EtdBr unwinding angle (ϕ_{EB}), which was taken to be 12°. Recent work by Wang (1974) and by Pulleyblank and Morgan (1975) indicates that the unwinding angle is two to three times larger than the 12° value.

More recently, Keller and Wendel (1974) estimated the number of supercoils in SV40 DNA by counting the resolved bands present after agarose gel electrophoresis of SV40 DNA which had been partially relaxed by the nicking-closing (N-C) enzyme isolated from KB cells. They counted 21 bands between the fully supercoiled virion DNA and the DNA which had been completely relaxed by the KB cell N-C enzyme at 0°C. Under their electrophoresis conditions, both the native virion DNA and the relaxed DNA migrate as single species. Although the above method does not involve the ethidium unwinding angle, it contains problems which were not apparent at the time the method was originated.

Recent work from this laboratory (Pulleyblank et al., 1975) has shown that the limit product of the action of the LA9 cell N-C enzyme on closed circular DNA is not a homogeneous species, but rather a Boltzmann distribution of closed circular DNAs having values of τ centered around zero superhelical turns. Germond et al. (1975) showed that native virion SV40 DNA is also heterogeneous with respect to τ. These results indicated that certain species near the fully relaxed DNA and near the center of mass of the virion DNA must have been missed in the original work. In the present study, two gel systems were used which alternatively resolve the species present within native DNA and within the limit product of N-C enzyme action.

The value of τ, that is, the number of bands in gels in which all species are resolved, is also dependent upon the conditions under which the enzymatic incubation occurred. The foregoing statement is illustrated here for the effect of temperature during the incubation. We note first the topological relation for duplex closed circular DNA,

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

where α is the topological winding number which is normally invariant, but can be altered by the action of the N-C enzyme. β is the mean duplex winding number, a value which varies with temperature and other environmental conditions. Such variations affect the value of τ which is normally negative in naturally occurring closed circular DNA. Consider the effect of conducting the relaxation with the N-C enzyme at two temperatures, T_1 and T_2 where $T_2 > T_1$, in the hypothetical case in which both the starting material and the completely relaxed material are homogeneous with respect to τ. The reactions can be considered in the following individual steps. Aliquots of the same DNA are placed at the two temperatures with the indicated effects on the winding numbers:

$$(\alpha, \beta, \tau)_T \rightarrow (\alpha, \beta_1, \tau_1)_{T_1};\ (\alpha, \beta, \tau)_T \rightarrow (\alpha, \beta_2, \tau_2)_{T_2} \qquad (2)$$

At these temperatures, the N-C reactions occur.

$$(\alpha, \beta_1, \tau_1)_{T_1} \rightarrow (\alpha_1, \beta_1, \tau = 0)_{T_1};\ (\alpha, \beta_2, \tau_2)_{T_2} \rightarrow (\alpha_2, \beta_2, \tau = 0)_{T_2} \qquad (3)$$

The products are then brought to the original temperature, T, for analysis.

$$(\alpha_1, \beta_1, \tau = 0)_{T_1} \rightarrow (\alpha_1, \beta, \tau_1')_T;\ (\alpha_2, \beta_2, \tau = 0)_{T_2} \rightarrow (\alpha_2, \beta, \tau_2')_T \qquad (4)$$

Because in equation (2) $\beta_1 > \beta_2$ (Wang, 1969), $\alpha_1 > \alpha_2$ in equation (3). Therefore in equation (4), in which β is the same in the two samples, $\tau_1' > \tau_2'$ and $|\tau_1'| < |\tau_2'|$. In a band counting experiment, the number of species present will increase as the temperature of the enzymatic incubation decreases. Since SV40 DNA is normally formed at 37°C, it is appropriate to conduct the N-C reaction at 37°C, or to correct for the effect of changes of temperature on β (Pulleyblank et al., 1975; Depew and Wang, 1975). In SV40 DNA, this corresponds to a reduction of seven superhelical turns for a ΔT of +37°C.

It is important to note that since the native DNA is heterogeneous with respect to superhelix density, some average is required to characterize $\bar{\tau}$ in a closed circular DNA. We thought it logical to count between the center of mass of the species within the native DNA and the center of the Boltzmann distribution of species formed by the action of N-C enzyme, that is, the zero-th band.

The band counting method, in common with all previous methods for determining the number of superhelical turns, measures the titratable or potential number of superhelical turns, rather than the actual or physical number of superhelical turns. The latter quantity will be the smaller one in a negatively supercoiled DNA, if the rotation angle of the duplex is reduced by the stress associated with the superhelical turns.

The unwinding angle (ϕ_{EB}) of EtdBr has been calculated from a comparison of the number of bands counted for SV40 DNA and the results of buoyant separations conducted in propidium diiodide–CsCl density gradients previously calibrated with standards having values of σ_o determined with EtdBr. The result, 23 ± 3°, supports the more recent higher values of ϕ_{EB} (Wang, 1974; Pulleyblank and Morgan, 1975).

Results

In an earlier study (Pulleyblank et al., 1975) of the limit products formed by the action of N-C enzyme, it was found that the multiple species were resolved when electrophoresed in the presence of Mg^{2+} (1–5 mM) at 4°C (Gel B system). Under such electrophoresis conditions, β (the mean duplex winding number) is greater than under the reaction conditions. The increase in β, $\Delta\beta$, is compensated by the generation of $\Delta\tau$ negative superhelical turns in each of the closed species, thereby shifting the average superhelix density of the set of limit products away from zero and into a range in which the gel offers optimum resolution between species differing by a single turn (Figure 1, D). The arrow indicates the center of the distribution of species within the limit product. Under the standard reaction conditions (0.2 M NaCl, 37°C), this species contained approximately zero superhelical turns. Native supercoiled DNA migrates as a single species, because it is too negatively supercoiled to be resolved by the gel (Figure 1, E).

The Gel A system (no Mg^{2+}, 23°C, 2% agarose) was used to resolve the multiple species within na-

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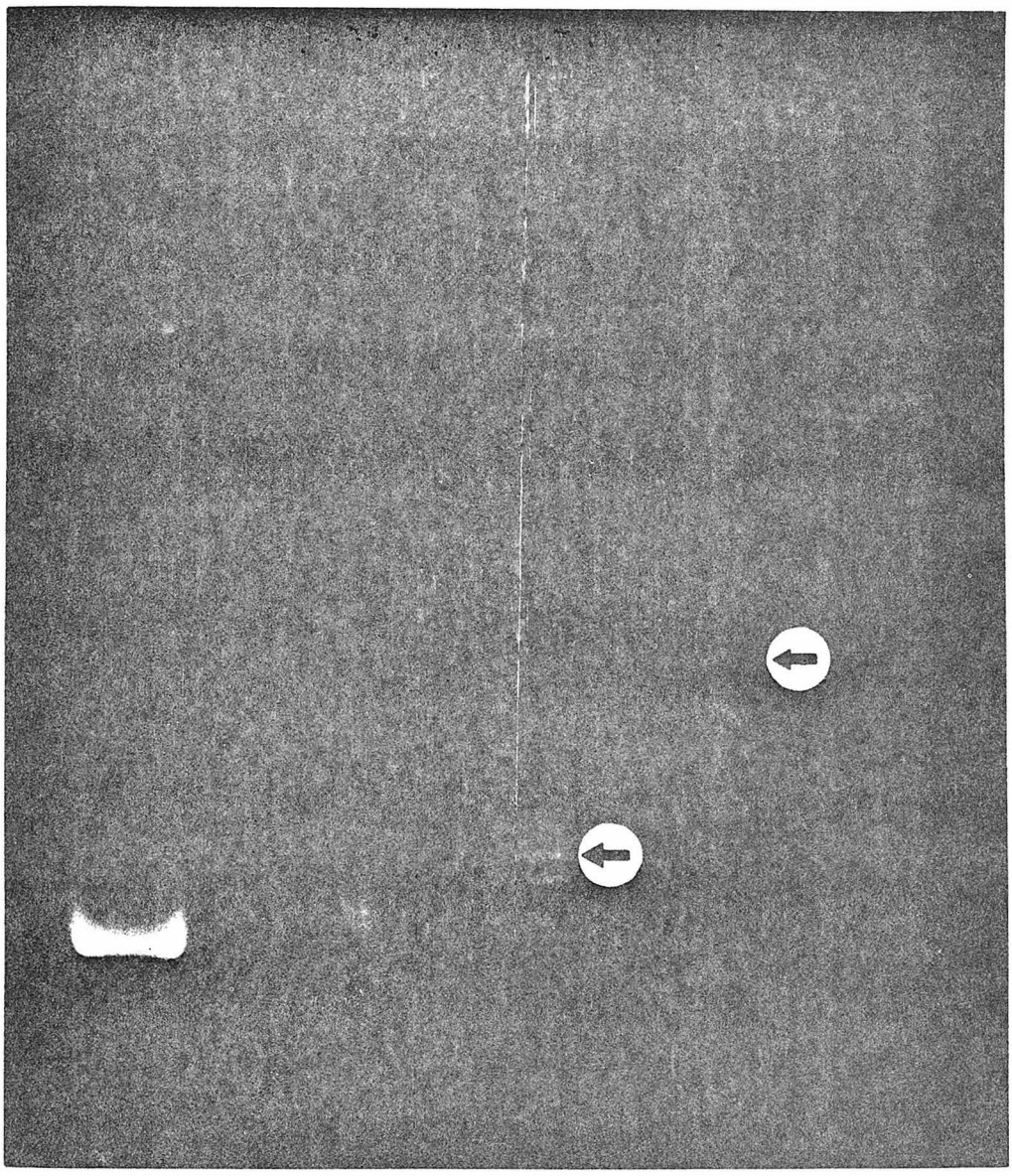
SV40



A B C D E

Figure 1. The Effect of the Presence of Varying Amounts of EtdBr during the Nicking-Closing Reaction on the Position of the Limit Products in the Gel B System

The top band in (A–D) corresponds to SV40 DNA II formed during the reaction. The upper band in (E) is SV40 DNA III added as a marker. The lowest band in (E) is SV40 DNA I. (D) presents the limit product generated in the absence of EtdBr; and (C), (B), and (A) present the limit products formed in the presence of 0.420, 0.588, and 0.756 μg/ml EtdBr, respectively, in incubations containing approximately 35 μg/ml SV40 DNA. The arrows designate the zero-th band under the reaction conditions.

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tive DNA (Figure 2, H and I), as well as species present after partially relaxing the DNA (Figure 2, E, F, and G). In this gel system, the limit product of the N-C enzyme reaction prepared under standard conditions does not resolve, but migrates as an unresolved mass along with the nicked circular form (II) of the same DNA (Figure 2, D). Counting the resolved peaks present in densitometric traces of photographs of the bands in Figure 2, E, F, and G leads to a value of 26 for τ. This, fortuitously, is the correct number, but includes extra bands in the native material having values of $|\tau| > |\bar{\tau}|$ and excludes bands having low values of $|\tau|$. The arrow in Figure 2, H and I indicates the center of mass

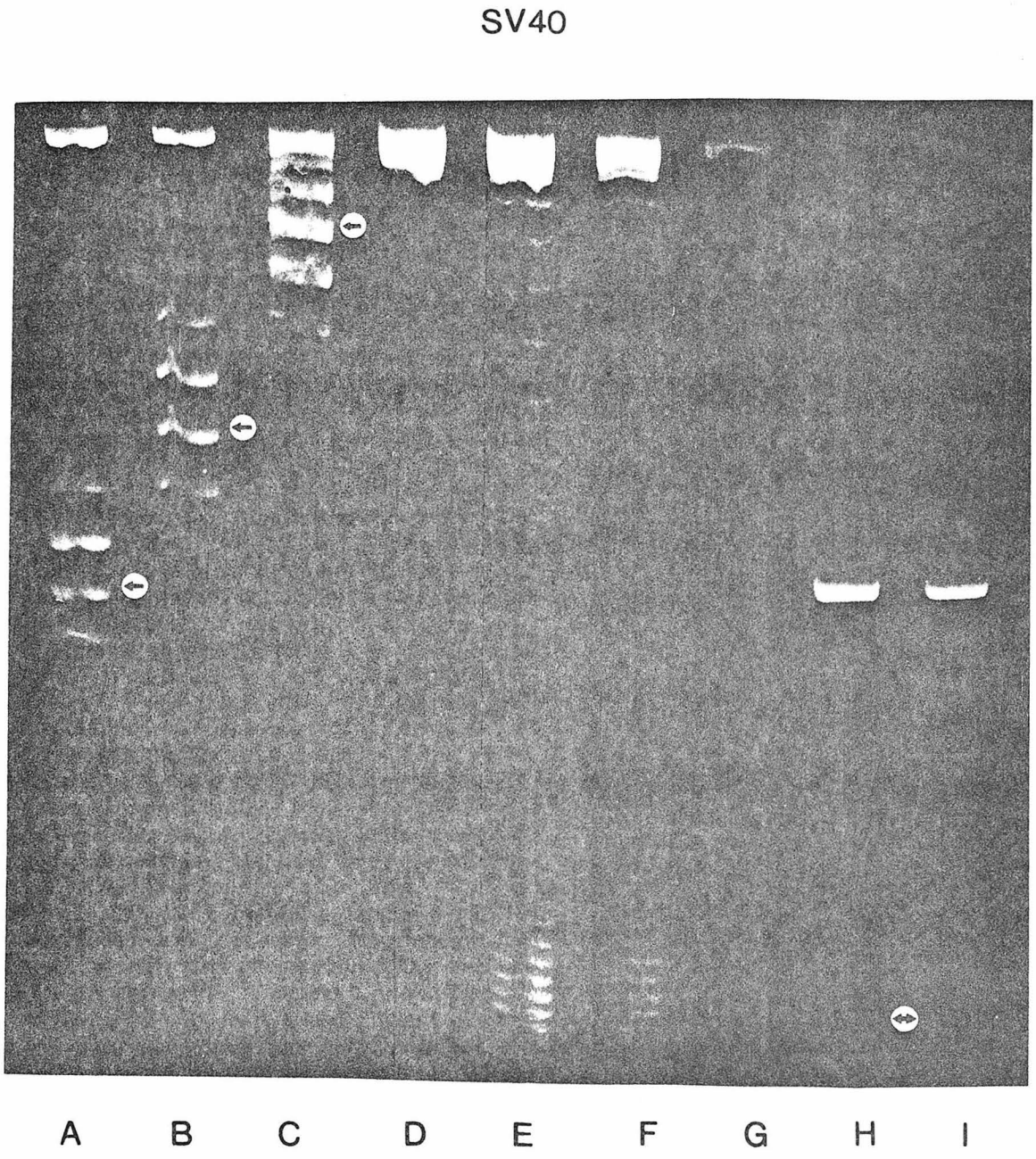

Figure 2. Multiple Band Patterns in the Gel A System

(A–D) contain the same materials as in Figure 1, A–D, respectively. (E), (F), and (G) contain different amounts of a sample of SV40 DNA partially relaxed as described in Experimental Procedures. (H) and (I) contain equal amounts of SV40 DNA III as a marker which migrated approximately halfway between form II and the native DNA. The arrow indicates the center of mass of the distribution of species present in virion DNA.

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of the distribution of species within the native virion SV40 DNA. The center of mass is also the number average value of τ.

To correlate bands within the two gel systems and thus identify species having the same values of τ in the reaction mixture, limit products which completely resolve in both gels were prepared by relaxation in the presence of EtdBr. Ethidium unwinds the primary helix, so that after complete relaxation and subsequent removal of the ethidium, the limit products are negatively supercoiled. The average superhelix densities of limit products prepared in this way become increasingly negative as increasing ethidium concentrations are used in the reaction mixture. The limit products prepared in the presence of increasing amounts of ethidium are resolved in the Gel A system (Figure 2, C, B, and A, respectively). Only the limit products prepared in the presence of the lowest amount of ethidium (Figure 1, C) resolve in the Gel B system. The two other sets of marker molecules (Figure 1, A and B) are too negatively supercoiled to be resolved. By slightly modifying the Gel B system, however, it was possible to resolve all three sets of limit products (Figure 3, C, B, and A, respectively). Under the slightly modified Gel B conditions, the limit products prepared under standard conditions can be resolved past the center of the distribution (Figure 3, D); however, part of the distribution co-migrates with the form II DNA.

The positions of the arrows in Figures 1, 2, and 3, indicating the centers of the distributions in the limit products and in the native DNA, were determined by a quantitative treatment of photographs of the ethidium-stained agarose gels, as described in Experimental Procedures. In earlier work from this laboratory (Pulleyblank et al., 1975), it was shown that the center of a distribution of closed circular DNAs need not fall on a band. In the work presented here, the band which lay closest to the determined center of mass for any given distribution has been used as a reference. This limits the accuracy of the band count to ± 0.5 turns.

The number of superhelical turns in SV40 DNA was evaluated from the densitometric traces (Figure 4) of the fluorescent photographs of the ethidium-stained agarose gels in Figures 1 and 2. Traces 1 and 2 are from Figure 1, D and C (Gel B system). Traces 3, 4, and 5 are from Figure 2, C, F, and H (Gel A system). Traces 2 and 3 represent the same limit products prepared in the presence of ethidium and are resolved in both gel systems. Here it is possible to align the maximum band of the EtdBr relaxed DNA in the two gel systems. The samples contained mobility markers of form II and form III (linear) SV40 DNA, so that the traces of the channels in any one slab gel could be aligned.

Trace 2 of the limit products prepared in the presence of the lowest EtdBr concentration is aligned, using II and III, with trace 1 of the limit products prepared under standard conditions. It is seen that

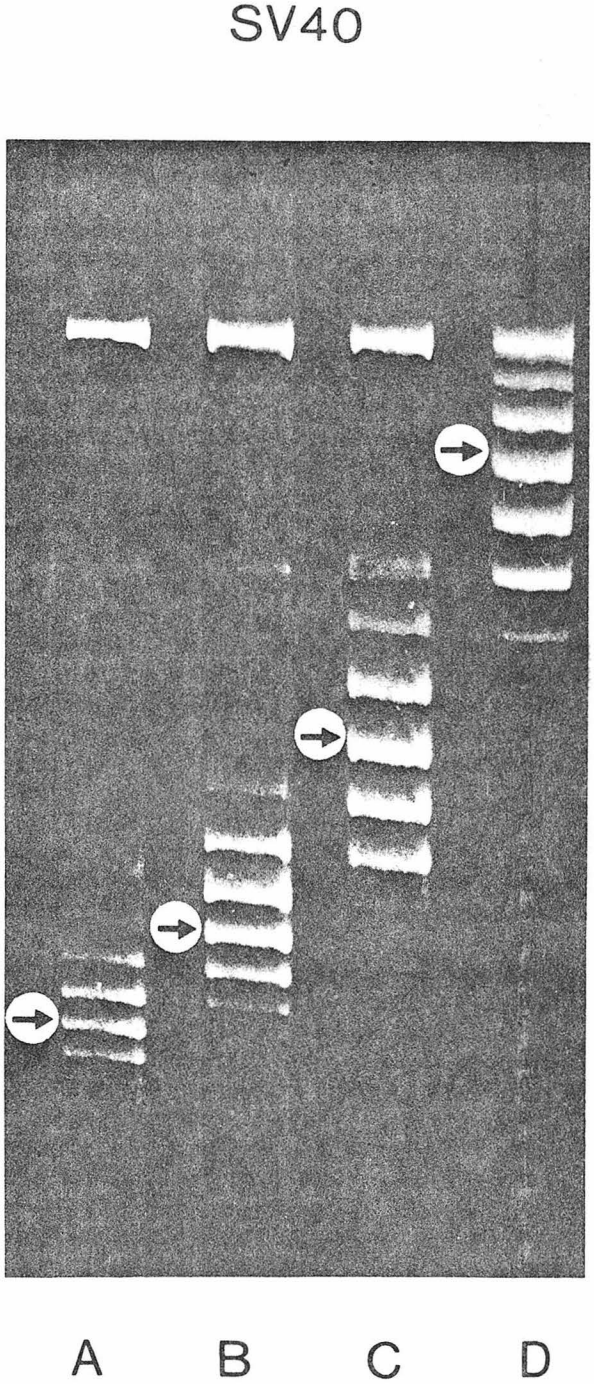

Figure 3. The Effect of the Presence of Varying Amounts of EtdBr during the Nicking-Closing Reaction on the Position of the Limit Products in the Modified Gel B System

(A-D) contain the same materials present in Figure 1, A-D, respectively.

the center of the distribution of limit products prepared in the presence of the lowest amount of ethidium corresponds to five superhelical turns relative to the center of the distribution in trace 1, which corresponds to approximately zero turns in the reaction medium. The maximum band in trace 3 corresponds to the maximum band in trace 2, and therefore had a value of $|\tau| = 5$ under the reaction conditions. We align trace 3 with the partially relaxed material in trace 4 using II, and with the native virion SV40 DNA in trace 5 using III. We count, on trace 4, from the reference band with $|\tau| = 5$ to the 26th band which corresponds to the center of mass of species within the native DNA, as determined in trace 5. We therefore conclude that there are 26 ± 0.5 titratable superhelical turns in native virion SV40 DNA at 37°C in 0.2 M NaCl.

The same value of 26 ± 0.5 was obtained when the entire procedure was repeated with the two other sets of limit products prepared in the presence of EtdBr. The centers of the distributions within these two sets correspond to $|\tau| = 9$ and $|\tau| = 12$. The above procedures have also been used to determine the number of superhelical turns in Minicol DNA. The value obtained was 19 ± 0.5.

A determination of the number of superhelical turns obtained by band counting, together with the molecular weight of the DNA, provide a value of the superhelix density, σ, where $\sigma \equiv \tau/\beta^{\circ}$, at 37°C in 0.2 M NaCl. These values are $(-5.05 \pm 0.1) \times 10^{-2}$ and $(-5.76 \pm 0.15) \times 10^{-2}$ for SV40 DNA and Minicol DNA, respectively. In the calculation, the molecular weights of SV40 and Minicol DNAs were taken to be 3.4×10^6 (Tooze, 1973) and 2.2×10^6 daltons, respectively (Hershfield et al., 1974; D. Tang, personal communication), and the mean molecular weight of a base pair in sodium DNA was taken to be 662 daltons.

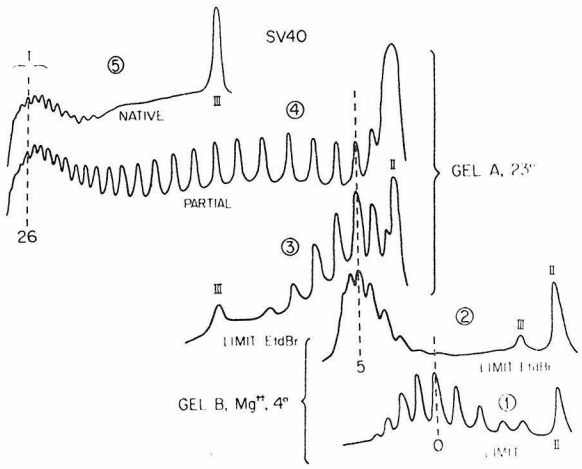

Figure 4. Densitometric Traces of Gel Patterns Aligned to Permit the Counting of Species in the Partially Relaxed SV40 DNA between the Zero-th Band in the Limit Product and the Center of Mass of the Native Virion SV40 DNA

The alignment procedures are described in the text.

The Effect of Mg^{2+} on the Winding of the DNA Duplex Relative to Its State in 0.2 M NaCl

Mg^{2+} (1–5 mM) is present in the electrophoresis buffer used to resolve the multiple species present within the limit product of N-C enzyme action generated in 0.2 M NaCl at 37°C. As shown above, when electrophoresed in the absence of Mg^{2+} at room temperature, the N-C enzyme limit products prepared under standard conditions do not resolve into multiple species, but rather co-migrate with the form II of the same DNA. Under similar electrophoresis conditions, however, the limit products of polynucleotide ligase, prepared in 0.001 M Mg^{2+} at 37°C, resolve as a series of positively supercoiled molecules (Depew and Wang, 1975). Because of these observations, it was of interest to determine the quantitative effect of Mg^{2+} on the DNA duplex relative to its state in 0.2 M NaCl. It has been shown (Pulleyblank et al., 1975) that if the ambient conditions for both reactions are identical, the sets of multiple species are indistinguishable. This indicates that the distributions generated are independent of the enzyme used.

To measure directly the winding effect of 0.001 M Mg^{2+} on the DNA duplex relative to its state in 0.2 M NaCl, the following experiments were performed using both Minicol and SV40 DNAs. A singly nicked form of the DNA was prepared, dialyzed against ligation buffer (containing 0.001 M Mg^{2+}), and treated with polynucleotide ligase at 37°C. Correspondingly, the native supercoiled form of the same DNA was treated with N-C enzyme under standard conditions (0.2 M NaCl, 37°C). The DNAs were placed on adjacent slots of an agarose gel and electrophoresed using the Gel B system (except in this case 10 mM Mg^{2+} was used in the electrophoresis buffer, because Mg^{2+} had been present during the ligations). The results of these experiments are presented in Figures 5a and 5b. Channels A in Figures 5a and 5b contain, respectively, the limit products of the action of polynucleotide ligase on singly nicked Minicol and SV40 DNAs. Channels B in Figure 5a and 5b contain, respectively, the limit products of the action of N-C enzyme on native supercoiled Minicol and SV40 DNAs. The arrows in this figure indicate the DNA species closest to the center of the distribution in question. Because the center of a distribution need not fall on a band (Pulleyblank et al., 1975), the difference between the centers of the distributions of limit products generated in 0.001 M Mg^{2+} and in 0.2 M NaCl need not be an integral number. The difference between the centers of the two distributions for each DNA

has been determined as described previously (Pulleyblank et al., 1975). For Minicol DNA (Figure 5a, A and B), the difference is $\Delta\tau = 2.0 \pm 0.2$ turns. For SV40 DNA (Figure 5b, A and B), the difference is $\Delta\tau = 3.0 \pm 0.2$ turns.

As can be seen, the limit products generated in 0.2 M NaCl at 37°C are more negatively supercoiled than are those generated in 0.001 M Mg^{2+} at 37°C. It can therefore be concluded that Mg^{2+} overwinds the DNA duplex relative to its state in 0.2 M NaCl. A determination of the shift in the centers of the distributions, together with the molecular weight of the DNA, provide a value for the change in superhelix density which results from transferring a closed

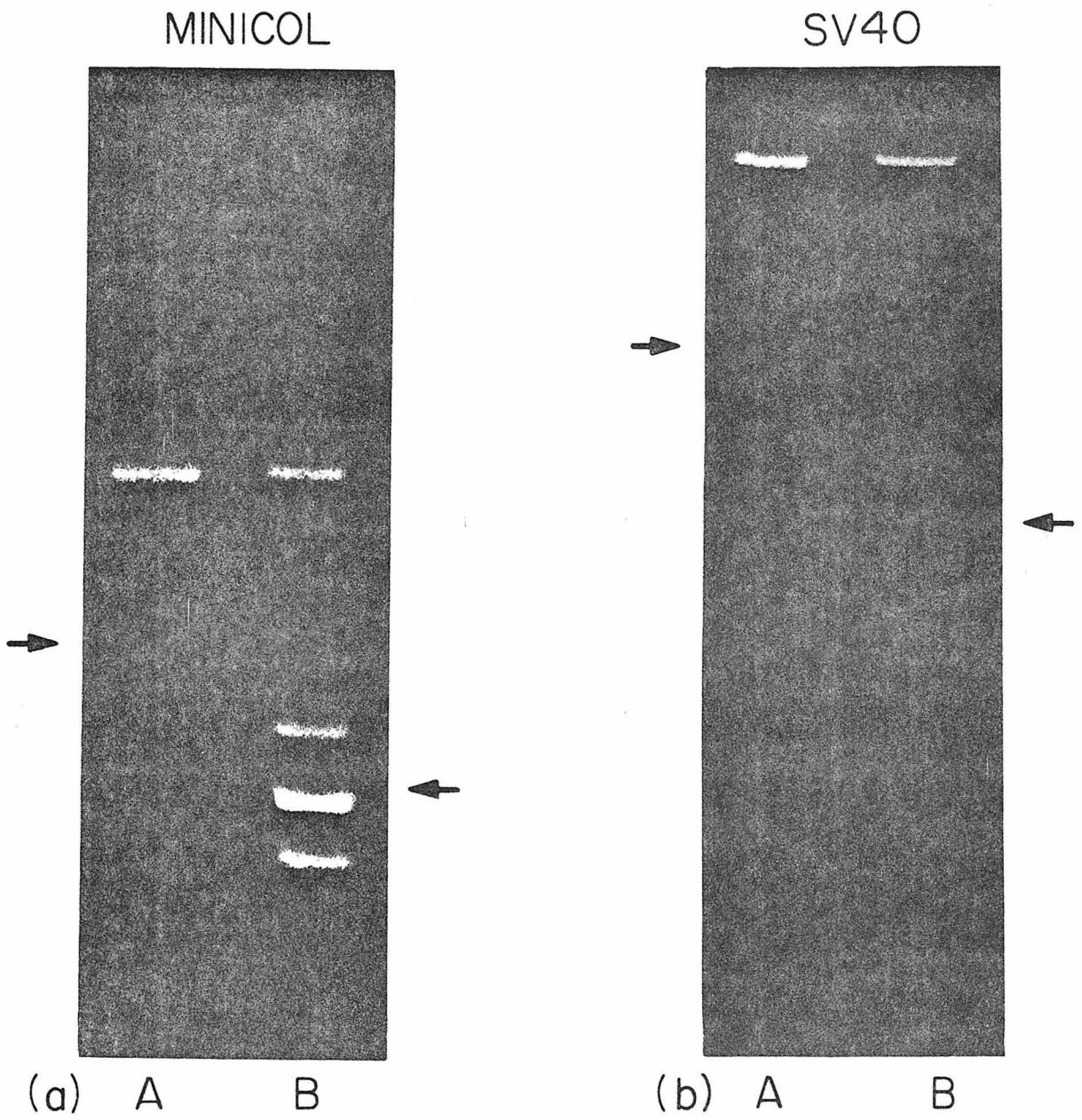

Figure 5. The Effect of Preparing Limit Products in the Presence of 0.2 M NaCl versus 0.001 M $MgCl_2$ on the Center of the Distribution of Multiple Species

(a) The top band in (A) and (B) corresponds to Minicol DNA II. (A) is the limit product (0.001 M Mg^{2+}, 37°C) of the action of polynucleotide ligase on singly nicked Minicol II DNA. (B) is the limit product (0.2 M NaCl, 37°C) of N-C enzyme action on Minicol I DNA.
(b) The top band in (A) and (B) corresponds to SV40 DNA II. (A) is the limit product (0.001 M Mg^{2+}, 37°C) of the action of polynucleotide ligase on singly nicked SV40 II DNA. (B) is the limit product (0.2 M NaCl, 37°C) of N-C enzyme action on SV40 I DNA.
The arrows designate the zero-th band under the reaction conditions.

circular DNA from 0.2 M NaCl to 0.001 M Mg^{2+} at 37°C. The mean value for experiments with both Minicol and SV40 DNAs is calculated to be $\Delta\sigma = -(0.59 \pm 0.05) \times 10^{-2}$.

The Unwinding Angle, ϕ, of Ethidium

An approximate determination of the ethidium unwinding angle can be made by comparing the superhelix densities determined by the band counting method with the superhelix densities determined by a method based upon the unwinding angle for ethidium. The buoyant separation method in dye (either EtdBr or PDI) CsCl gradients has previously been calibrated with an assumed value of 12° for ϕ_{EB} (Gray et al., 1971). The coefficients in the equations relating the separations to the superhelix densities were determined by calibration against superhelix densities determined by the ethidium dye titration-sedimentation velocity method. In PDI–CsCl gradients, the increment in σ_o is calculated with the equation:

$$\Delta\sigma_o = (0.095 \pm 0.005)(\Omega_c^{PDI} - 1), \quad (5)$$

where $\Omega_c = \Delta r/\Delta r^*$ and $\Delta\sigma_o = \sigma_o - \sigma_o^*$; Δr is the distance between the open and closed forms of the DNA of unknown superhelix density; Δr^* is the corresponding separation for the reference DNA with known superhelix density. The above equation is applicable for DNAs of similar base composition. Because the coefficient in the above equation was determined using SV40 I DNA as the reference DNA, and because Equation (5) is an equation of both a difference and a ratio, native virion SV40 DNA is the only DNA which may be used as the standard in measuring Δr^*. In the equation $\Delta\sigma_o = \sigma_o - \sigma_o^*$, $\Delta\sigma_o$ is a simple difference, where σ_o^* is the standard superhelix density of SV40 I DNA and σ_o is the standard superhelix density of another DNA. However, $\Delta\sigma_o$ may also be regarded as being equal to $\sigma - \sigma^*$, with the latter quantities specifying the superhelix densities of the two DNAs under arbitrary nondenaturing conditions.

It was noted (Wang, 1969; Gray et al., 1971) that changing the environment from ligation conditions, 1 mM Mg^{2+}, to 3–5 M CsCl affects the rotation angle of the duplex in such a way as to increase the absolute superhelix density by 5 to 9 $\times 10^{-3}$ units in terms of a 12° ethidium unwinding angle. To avoid the need of introducing corrections for salt and temperature effects on σ, buoyant separation experiments were performed with three forms of the same DNA in one centrifuge tube. The nicked form serves as the fiducial reference. N-C enzyme-relaxed DNA gives the value for Δr with, however, $^{12°}\sigma$ taken to be zero. Native SV40 I DNA serves as the standard reference, giving the value for Δr^*, but serves as the unknown in terms of $^{12°}\sigma^*$ (Figure 6). The superhelix densities ($^{12°}\sigma$) determined in this way for virion SV40 DNA at 37°C were $(-2.6 \pm 0.2) \times 10^{-2}$ and $(-2.9 \pm 0.2) \times 10^{-2}$ in 0.2 M NaCl and 0.001 M $MgCl_2$, respectively.

It has not yet been possible to evaluate analogous experiments performed with Minicol DNA because of the requirement that SV40 I DNA be used to evaluate Δr^*. The analysis of data obtained with Minicol DNA is contingent upon a recalibration of the coefficient in Equation (5).

Comparing these superhelix densities with those determined by band counting, the unwinding angle for ethidium is calculated from the relation:

$$[\sigma(\text{band count})/\sigma(\text{buoyant method})] \times 12° = \phi_{EB} \quad (6)$$

The mean value determined from experiments with SV40 DNA was 23 ± 3°. The preceding data are summarized in Table 1, in which it is apparent that the band counting method (top part) and the buoyant separation method (bottom part) give essentially the same superhelix densities if 23° is used for the unwinding angle of EtdBr. The values of $^{23°}\sigma$ at 37°C are calculated to be $(-4.98 \pm 0.35) \times 10^{-2}$ and $(-5.56 \pm 0.39) \times 10^{-2}$ for SV40 I DNA in 0.2 M NaCl and for SV40 I DNA in 0.001 M Mg^{2+}, respectively. The value of 23° for the unwinding angle of ethidium is somewhat lower than those determined by Wang (1974) (26°) and by Pulleyblank and Morgan (1975) (26–33°).

Discussion

The number of supercoils in a DNA, determined by the band counting method, depends critically upon the conditions under which the fully relaxed DNA was prepared. In the experiments presented above, the relaxed or reference DNA was prepared in 0.2 M NaCl at 37°C. These conditions closely approximate the physiological conditions under which both SV40 virus and Minicol DNA are formed.

We have determined that there are 26 ± 0.5 titratable superhelical turns in SV40 DNA. It has recently been shown that there are 21 ± 1 nucleosomes on SV40 minichromosomes obtained from infected cells (Griffith, 1975) and on SV40 nucleohistone complexes extracted from virions (Germond et al., 1975). We calculate that the average number of superhelical turns per nucleosome is 1.25 ± 0.09. Strictly, the number 1.25 applies to 1/21 of the SV40 genome under the reaction conditions. If, however, the regions of internucleosomal DNA are relaxed, then the number refers to the average number of superhelical turns accommodated within a nucleosome. It is important to note that these

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superhelical turns need not exist within the nucleosome entirely as such. The DNA may be coiled around a central axis either to a greater or lesser extent than this if there were, respectively, local overwinding or underwinding of the primary helix in such a way that the average number of superhelical turns per nucleosome, after deproteinization, equaled approximately 1.25. There is no requirement that the number of superhelical turns in a nucleosome be integral.

Environmental Effects on the Duplex and Topological Winding Numbers

As mentioned earlier, the superhelix density in terms of $\phi_{EB} = 12°$, $^{12°}\sigma_o$, changes by 5–9 $\times$ 10^{-3} units when a closed circular DNA is transferred from a ligase reaction mixture at 30°C and 1 mM Mg^{2+} to 3–5 M CsCl at 20°C. This change results from the effects on the average rotation angle of the DNA duplex. Because ligase conditions are at low salt (0.01–0.03 M Tris), the value 0.009 generally has

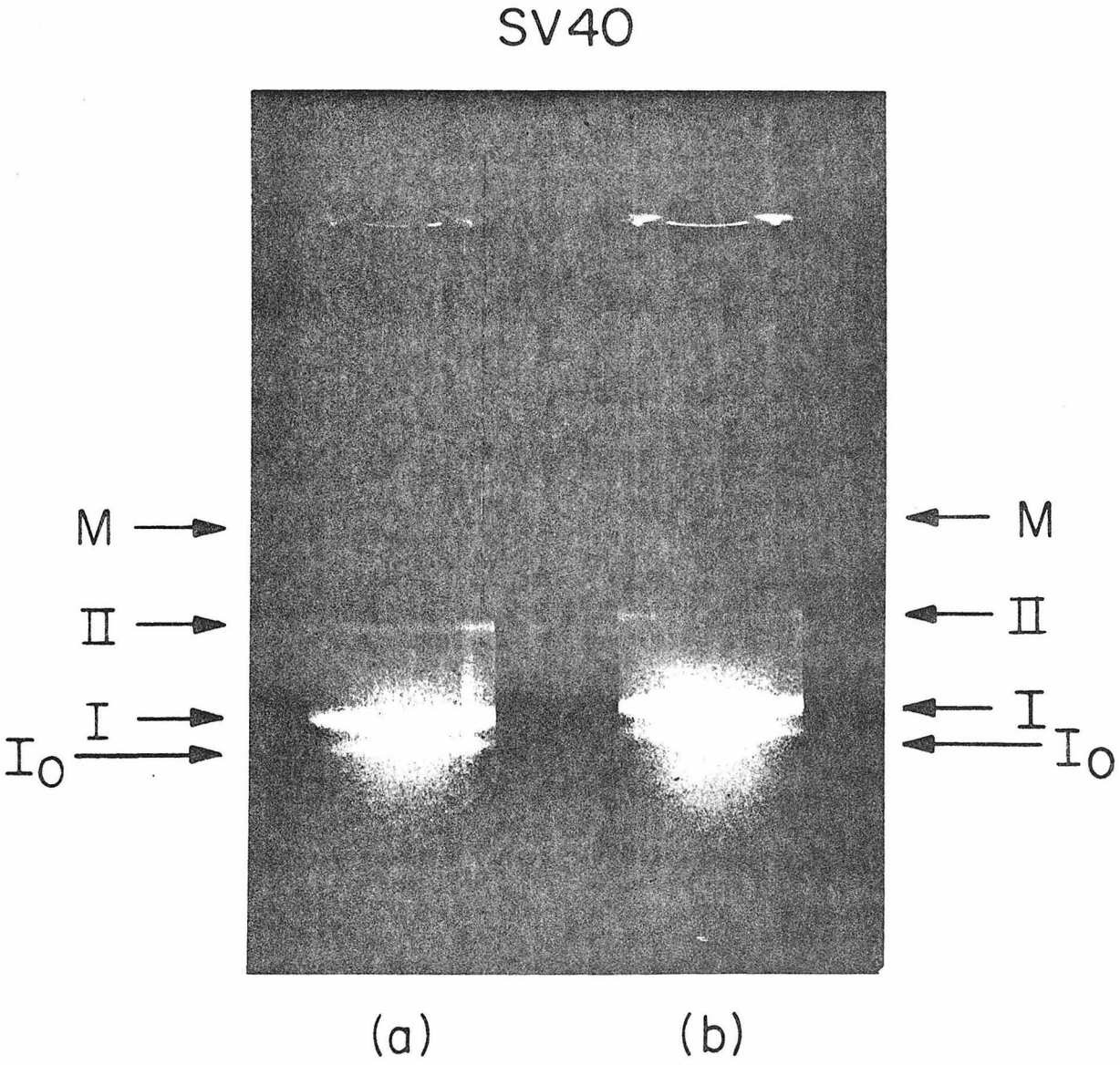

Figure 6. Fluorescent Photographs of Buoyant PDI-CsCl Density Gradients, Each Containing Three Forms of SV40 DNA

(a) I_o, I, II, and M are the limit products (0.001 M Mg^{2+}, 37°C) of the action of polynucleotide ligase on singly nicked SV40 DNA, native SV40 DNA, the nicked form of SV40 DNA, and the meniscus, respectively.

(b) I_o, I, II, and M are the limit products (0.2 M NaCl, 37°C) of the action of N-C enzyme on native SV40 DNA, native SV40 DNA, the nicked form of SV40 DNA, and the meniscus, respectively.

Table 1. The Number of Superhelical Turns in and the Superhelix Densities of SV40 and Minicol DNAs under Various Ambient Conditions

Band Counting Method		
	$\bar{\tau}$	$\sigma \times 10^2$
SV40 I (0.2 M NaCl, 37°C)	−26 ± 0.5	−5.05 ± 0.10
SV40 I (0.001 M $MgCl_2$, 37°C)	−29 ± 0.7	−5.63 ± 0.14
Minicol I (0.2 M NaCl, 37°C)	−19 ± 0.5	−5.76 ± 0.15
Minicol I (0.001 M $MgCl_2$, 37°C)	−21 ± 0.7	−6.31 ± 0.21

Buoyant Separation Method				
	$^{12°}\bar{\tau}$	$^{23°}\bar{\tau}$	$^{12°}\sigma \times 10^2$	$^{23°}\sigma \times 10^2$
SV40 I (0.2 M NaCl, 37°C)	−13.4 ± 1	−25.6 ± 1.8	−2.6 ± 0.2	−4.98 ± 0.35
SV40 I (0.001 M $MgCl_2$, 37°C)	−14.9 ± 1	−28.6 ± 2.0	−2.9 ± 0.2	−5.56 ± 0.39
SV40 I (3–5 M CsCl, 20°C)	−20.0 ± 1	−38.5 ± 1.9	−3.9 ± 0.2[a]	−7.48 ± 0.37

All values of $\bar{\tau}$, the average number of titratable superhelical turns, and σ, the superhelix density, are at 37°C in either 0.2 M NaCl (N–C reaction conditions) or 0.001 M $MgCl_2$ (ligation conditions), except for the values in the bottom line. In this line, the conditions (3–5 M CsCl, 20°C) are "standard" conditions, and therefore the superhelix densities in this line are values for the "standard" superhelix density (σ_o). The superscripts, 12° and 23°, indicate the ethidium unwinding angle used in the calculations.
[a](−3.9 ± 0.2) × 10^{-2} is the value of $^{12°}\sigma_o$ for virion SV40 I DNA determined by Upholt et al. (1971).

been used to correct values of σ_o to low salt conditions. The ligations for which the 0.009 value was determined were performed at 30°C. If the ligations had been carried out at 37°C, the salt correction would have been 0.010, using 0.005°/°C base pair (in terms of ϕ_{EB} = 12°) for the thermal rotation coefficient (Wang, 1969).

The previous value, $^{12°}\sigma_o$, for SV40, against which other superhelix densities were measured was (−3.90 ± 0.2) × 10^{-2} (Upholt et al., 1971). Thus the difference in superhelix density between the limit products of a ligation performed in 0.001 M Mg^{2+} at 37°C and native virion SV40 DNA should be $^{12°}\Delta\sigma$ = (−2.9 ± 0.4) × 10^{-2}, which is in agreement with the values obtained in the present study.

Limit products of the action of nicking-closing enzyme which have been taken to have a value of σ = 0 in the buoyant centrifugation experiments were prepared in 0.01 M Tris, 0.2 M NaCl at 37°C. The superhelix density of native SV40 DNA relative to the limit products of N-C enzyme action on SV40 DNA, $^{12°}\sigma$, determined in the above buoyant experiments is (−2.60 ± 0.2) × 10^{-2}. If instead of using the limit products as a defined zero superhelix density reference, the native DNA had been used as the reference (with $^{12°}\sigma_o$ = −0.039), the absolute superhelix density, $|^{12°}\sigma_o|$, of the limit products would be 0.013. The latter value is therefore the correction to be used for transferring DNA from the low salt conditions of the nicking-closing reaction to 5 M CsCl. The value of 0.013 is only slightly larger than the correction in superhelix density for transfer from ligase conditions to 3–5 M CsCl. All other conditions being equal, ligated DNA has a lower absolute superhelix density (0.010) than does its N-C enzyme relaxed counterpart (0.013).

The difference in superhelix density between the limit products of polynucleotide ligase (0.001 M Mg^{2+}, 37°C) and those of N-C enzyme (0.2 M NaCl, 37°C) has been evaluated from measuring the difference between the centers of the distributions resolved on agarose gels. This method is not dependent upon the knowledge or assumption of an unwinding angle for ethidium and, furthermore, permits differences to be measured to within τ = 0.1. Using both Minicol and SV40 DNAs, the difference in superhelix density between the two limit products $[\sigma_{(0.2\ M\ NaCl,\ 37°C)} - \sigma_{(0.001\ M\ Mg^{2+},\ 37°C)}]$ is −(0.59 ± 0.05) × 10^{-2}.

We therefore conclude that transfer of DNA from 0.2 M NaCl to 1 mM Mg^{2+} overwinds the DNA duplex. This conclusion is consistent with the results obtained with agarose gel electrophoresis systems. As mentioned earlier, it has been found that the limit products of the nicking-closing reaction could be resolved by electrophoresing in the presence of Mg^{2+} (1–5 mM) at 4°C. Both the addition of Mg^{2+} and the decrease in temperature cause overwinding of the duplex and result in the acquisition of $\Delta\tau$ negative superhelical turns by all closed species. In the absence of Mg^{2+}, however, changing the ionic strength of the running buffer by a factor of two has virtually no effect on the mobility or the resolution of closed circular DNAs of different superhelix densities.

From the foregoing, we observe that the mobilities of closed circular DNA in agarose gels are relatively independent of τ in the neighborhood of τ = 0. At higher values, the mobilities are sensitive to changes in τ, but at still higher values of τ, the mobilities again become insensitive to changes in τ. From the above, we infer that the relation between mobility and $|\tau|$ is approximately sigmoidal.

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Experimental Procedures

Enzyme and DNA

Fraction V N-C enzyme was prepared from mouse LA9 cell nuclei (Vosberg and Vinograd, 1976) and was supplied by Dr. H.-P. Vosberg, E. coli polynucleotide ligase was a gift from Dr. H. W. Boyer. Minicol DNA was supplied by Dr. D. E. Pulleyblank and was prepared from bacterial strain PVH51 supplied by Dr. H. W. Boyer.

SV40 DNA was obtained from virus which had been plaque-purified twice. Samples of SV40 DNA were gifts from D. Tang and Dr. H. Kasamatsu.

Relaxation of Closed Circular DNA

10–100 μg/ml of DNA were incubated in 0.2 M NaCl, 0.01 M Tris-HCl, 1 mM EDTA (pH 7.8) with 5–20 units of N-C enzyme per μg of DNA for 24 hr at 37°C. The reactions were terminated after 9 min to prepare partially relaxed material. Reactions were terminated by the addition of SDS to 0.1%. Relaxations in the presence of ethidium ($1.2 \times 10^{-2} - 2.16 \times 10^{-2}$ μg EtdBr per μg DNA) were performed in the dark.

Ligation of Nicked Circular DNA

Singly nicked circular DNA was prepared according to the procedure outlined by Hsieh and Wang (1975). The singly nicked material was purified by banding in CsCl–ethidium bromide gradients (Radloff, Bauer, and Vinograd, 1967), after which the dye was removed from the DNA by repeated extractions with 1-butanol. The samples of DNA were precipitated with ethanol, resuspended in and then simultaneously dialyzed against 0.01 M Tris-HCl, 1 mM EDTA, 2 mM $MgCl_2$ (pH 7.8) (ligation buffer). The counterion in this medium is essentially 0.001 M Mg^{2+}. The samples were adjusted to a final concentration of 50 μg/ml bovine serum albumin and 30 μM DPN by the addition of stock solutions which had been dissolved in ligation buffer. The samples were pre-equilibrated to 37°C, after which approximately 2×10^{-3} units (0.25 μl) of E. coli polynucleotide ligase were added to each sample with a Hamilton microsyringe. After 2 hr, the reactions were quenched by placement at 0°C and the addition of EDTA (pH 8.0) to a final concentration of 10 mM.

Electrophoresis

A vertical slab gel electrophoresis apparatus (Aquebogue) was used. Gel A system: gels contained 2% agarose (Sea Kem), 40 mM Tris, 30 mM NaH_2PO_4, 1 mM EDTA (pH 7.8). Electrophoresis was performed at room temperature (23°C). Gel B system: gels contained 1% agarose (Sea Kem), 40 mM Tris-acetate (pH 7.8), 1 mM EDTA, 5 mM $MgAc_2$. Electrophoresis was performed at 4°C. Modified Gel B system: gels contained 2% agarose (Sea Kem), 40 mM Tris-acetate (pH 7.8), 1 mM EDTA, 1 mM $MgAc_2$. Electrophoresis was performed at 4°C. 2–3 V/cm were applied to the 4 mm analytical gels; and electrophoresis times (72–96 hr) were varied according to voltage gradient, temperature, agarose percentage, and molecular weight of the DNA.

Centrifugation

Preparative equilibrium ultracentrifugation in propidium-diiodide-CsCl gradients was performed as described by Gray et al. (1971), with the exception that the open form, native closed form, and relaxed closed form of the same DNA were present in each centrifuge tube.

Photography of the tubes and measurement of the band centers were performed according to the methods described by Watson, Bauer, and Vinograd (1971) and by Gray et al. (1971), respectively.

Determination of Centers of Masses of DNA Species by Fluorescence Photography

Gels were stained in the dark overnight in 10 mM Tris-HCl (pH 7.8), 2 mM EDTA, 2 μg/ml EtdBr. The gels were illuminated from below with short wavelength ultraviolet light from a trans-illuminator (Ultra Violet Products, Inc.) and photographed on Kodak Plus X film. The films were scanned on a Joyce-Loebl microdensitometer. γ, the slope of the linear region of the characteristic curve, was calculated for each film. Traces were evaluated with a Hewlett Packard 9864A Digitizer Platen and 9820A calculator, according to the method previously outlined (Pulleyblank et al., 1975).

Addenda

Attention is called to a paper by Keller (1975) which appeared after this manuscript was accepted for publication. This author determined the number of supercoils in virion SV40 DNA by similar but nonidentical procedures and obtained a result, $|\bar{\tau}| = 24 \pm 2$, in reasonable agreement with the results presented here.

R. L. Burk and W. Bauer (personal communication) have also shown that virion SV40 I DNA is the only DNA which may be used as a reference in conjunction with the relations determined by Gray et al. (1971). Burk and Bauer have derived equations which permit the use of any nicked (II)/closed (I) DNA pair as a reference provided that the superhelix density of the reference DNA is known.

Acknowledgments

We wish to thank H. W. Boyer (University of California, San Francisco) for providing the bacterial strain harboring the Minicol plasmid. This work was supported in part by grants from the National Cancer Institute and the National Institute of General Medical Sciences. M. S. is a recipient of an NSF graduate fellowship.

Received November 17, 1975; revised February 24, 1976

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CHAPTER III

The Problems of Eukaryotic and Prokaryotic DNA Packaging and *in vivo* Conformation Posed by Superhelix Density Heterogeneity

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

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Received 24 December 1976

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, α. 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 λ 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 (α), is preserved upon isolation. In the present work we examine the heterogeneity in α 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 α, 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. α must be integral and cannot be altered without a nicking-closing event. β, 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 β need not be integral and is dependent upon the environmental conditions. τ is the number of superhelical turns in the molecule and is normally negative for naturally occurring closed circular DNAs; like β it need not be integral, and will vary with β in response to changing environmental conditions. The superhelix density, $\sigma \equiv \tau/\beta^\circ$ (where β° 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 α must be integral, and β 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 α. 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 α; 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 β, and therefore τ, 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 β 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 μg/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

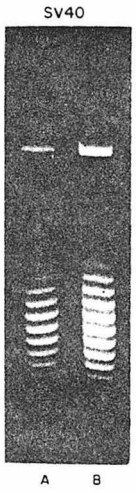

FIGURE 1. The Resolved Species of SV40 Form I DNA after Electrophoresis in the Presence of Ethidium.
The uppermost band in both channels is SV40 form II DNA. In (A) and (B) are virion and intracellular SV40 form I DNAs respectively. The intracellular DNA was prepared by Hirt lysis of infected cells at 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.

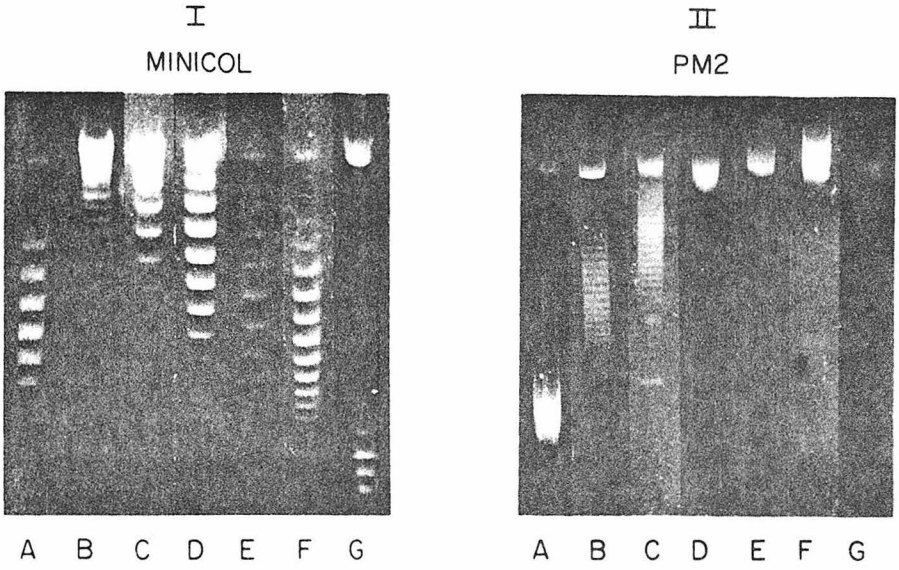

FIGURE 2. Development of the Chloroquine Phosphate Gel Electrophoresis System.
Panel I: Minicol DNA and Panel II: PM2 DNA after electrophoresis in the presence of: (A) 7.5 μg/ml, (B) 15 μg/ml, (C) 25 μg/ml, (D) 50 μg/ml, (E) 75 μg/ml, (F) 100 μg/ml, and (G) 200 μg/ml, chloroquine phosphate. Electrophoresis 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 $\pm$ 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

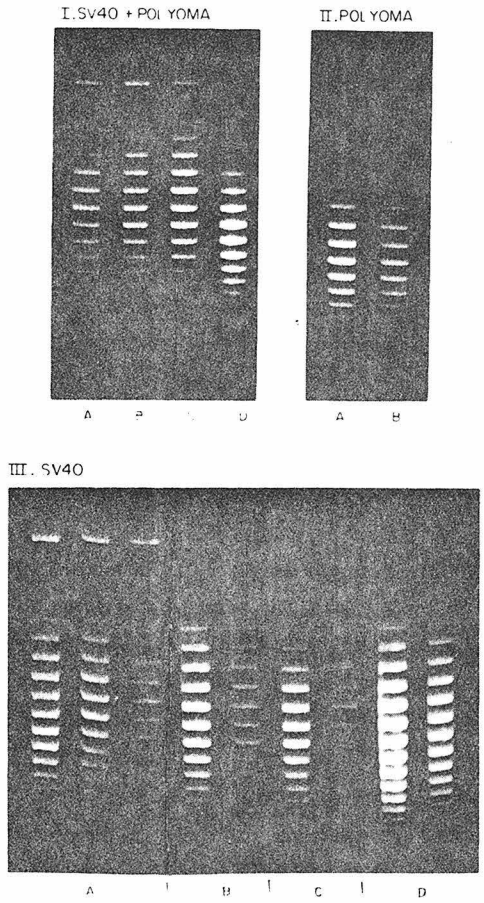

FIGURE 3. Comparison of the Intracellular and Virion Form I DNAs of Polyoma and SV40. The uppermost band in each channel is the nicked circular form (II) of the DNA. Panel I: (A) and (B) contain intracellular SV40 form I DNA prepared by Hirt lysis of infected cells at 23°C and 37°C respectively. (C) 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_α, 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_α, is proportional to the number, N_α, of molecules having a given value of α. Plots of N_α 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:

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

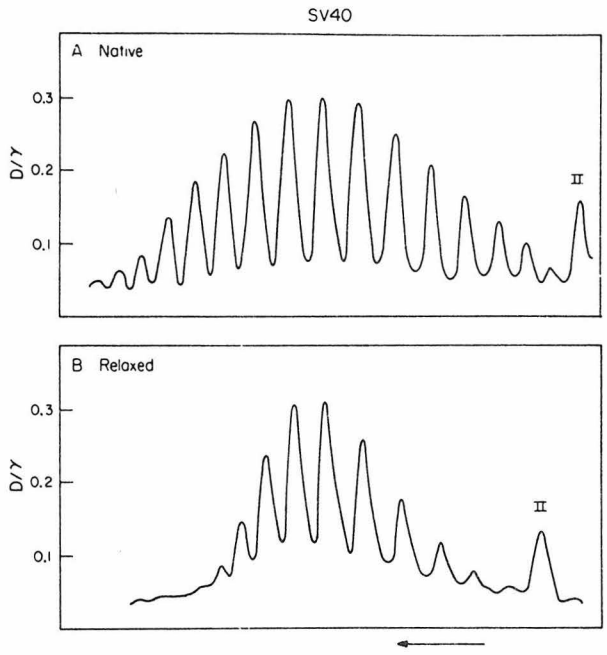

FIGURE 4. Comparison of the Distribution Within Native SV40 Form I DNA and the Thermal Distribution Within the Limit Product of N-C Enzyme Action on SV40 Form I DNA.

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/γ). 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 τ 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 ± 0.004 and 0.050 ± 0.004, respectively, and for virion and intracellular polyoma DNA, 0.053 ± 0.005 and 0.050 ± 0.006, respectively. Since the value of $\bar{\alpha}$ need not be integral, the center of a distribution will

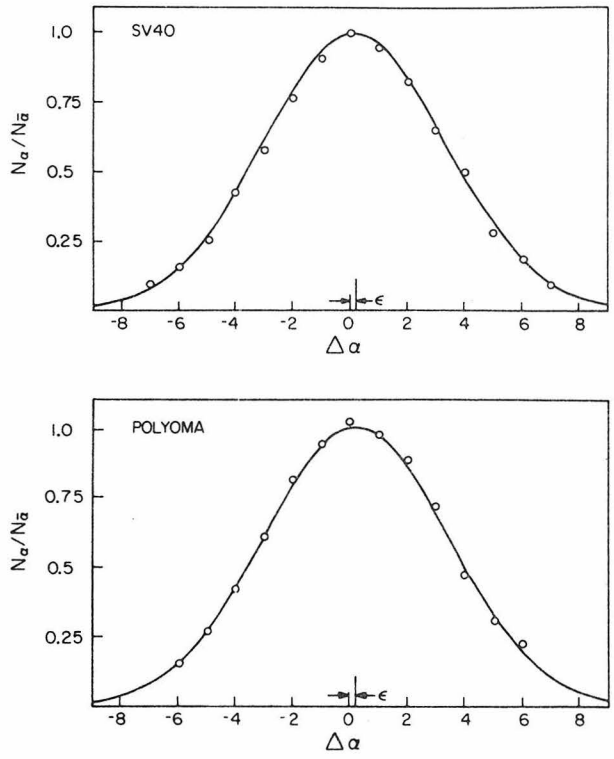

FIGURE 5. The Distributions Present Within SV40 and Polyoma DNAs are Gaussian.

The number (N_α) of molecules having a given value of α 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 ϵ. 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 α and are the most negatively supercoiled.

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

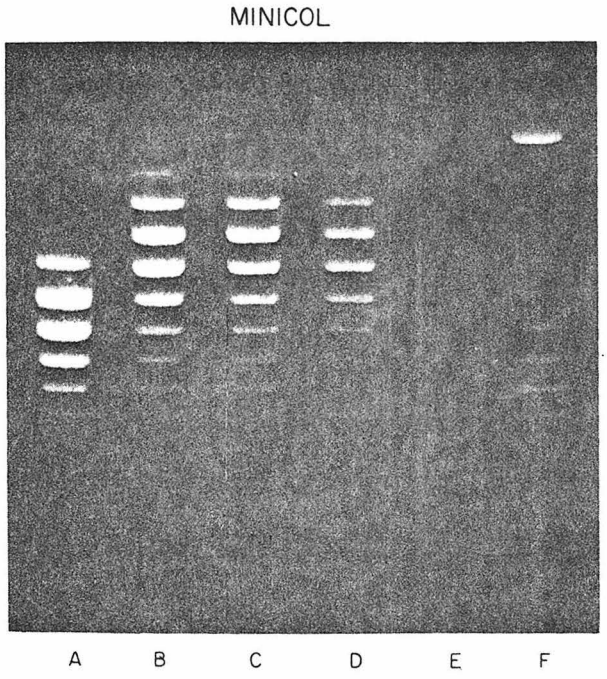

FIGURE 6. The Effect of Incubation Temperature on the Distribution of Species Within Native Form I Minicol DNA.

(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 6.4×10^6 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.

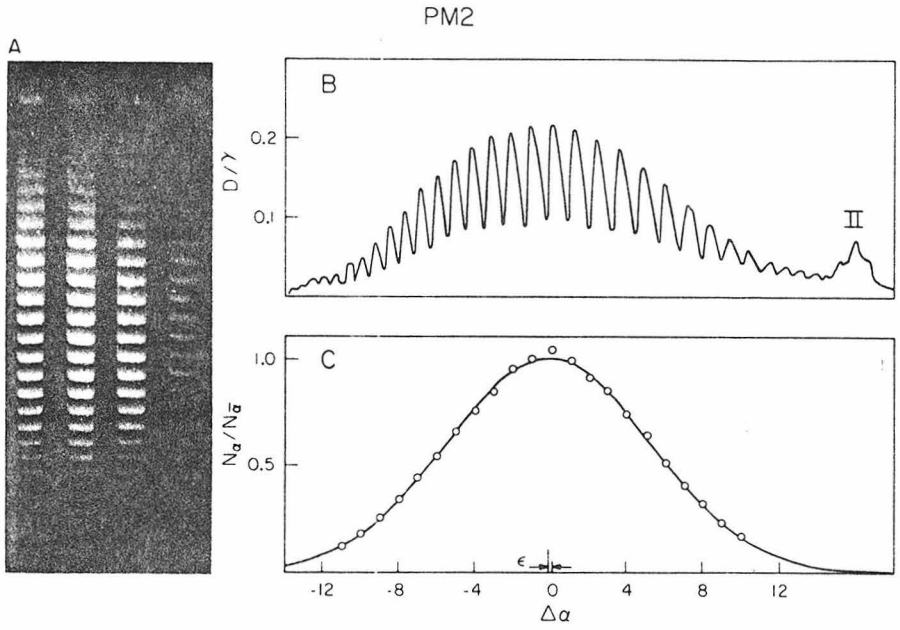

FIGURE 7. The Distribution of Species Within PM2 Form I DNA.
Panel A shows the species present in PM2 form I DNA resolved after electrophoresis at 3 v/cm, for 38 hrs in the presence of 20 μg/ml of chloroquine phosphate in a 1% agarose gel. Panel B is a densitometric trace of one of the channels shown 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_α with respect to $N_{\bar{\alpha}}$ as well as the sign of $\Delta\alpha$ and the determination of ϵ 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 μg/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 ϵ 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	ϵ	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 ϵ 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 2.06×10^3 (22) and the molecular weights of the DNAs were taken to be: SV40, 3.4×10^6 daltons; polyoma, 3.55×10^6 daltons; PM2, 6.4×10^6 daltons; and Minicol, 2.2×10^6 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 ϵ 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 α 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 α.

In considering heterogeneity in α, 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 τ_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 τ_i) is proportional to $\sqrt{M}$. From the molecular weights of SV40 and polyoma DNAs (taken to be 3.4×10^6 daltons and 3.55×10^6 daltons, respectively) and the previously published value for b/RT of 2.06×10^3, 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 9×10^5 daltons for SV40 (i.e. 26% of the SV40 genome) and 1.05×10^6 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 α 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 α in polyoma and SV40 DNAs is a phenomenon associated solely with nucleosomes. In either case, it is probable that the heterogeneity in α 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 ± 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 ϵ is sensitive to small differences in the molecular weight of the DNA (5), the similarity between the SV40 and polyoma ϵ 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 α 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.

ACKNOWLEDGEMENTS

We are grateful to all of those who gave us samples of DNA, virus, and bacterial strains, to M. Kiernan for assistance with tissue culture and virus propagation, and to D. Agard for help with the fitting procedures used. This work was supported in part by grants from the National Cancer Institute and the National Institute of General Medical Sciences. M. S. is a recipient of an NSF graduate fellowship. D. E. P. is a recipient of a Medical Research Council of Canada fellowship. This is contribution no. 5442 from the Division of Chemistry and Chemical Engineering.

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CHAPTER IV

The Quantitation of Fluorescence by Photography

The quantitation of fluorescence by photography

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Received 1 January 1977

ABSTRACT

A method based on theory has been developed for the photographic quantitation of fluorescent substances. DNA stained with ethidium in agarose gels is used as an example of an application of this method. In the course of developing this method we have demonstrated that the empirical methods employed by other authors can give rise to large systematic errors. We have also developed an approximate method based on photographic theory, avoiding the use of digital integration which is required by the rigorous method.

INTRODUCTION

Detection methods based upon fluorescence are widely used in the chemical sciences because they offer sensitivities several orders of magnitude higher than can be obtained by other optical techniques. Of particular current importance is the use of enhanced ethidium fluorescence (1) for the detection of nanogram quantities of nucleic acids after gel electrophoresis.

Photography, which provides the simplest method of recording such data, suffers from a serious deficiency in not permitting simple quantitation of the species present. We describe here the steps that can be taken in order to achieve accurate quantitation from fluorescence photographs. Furthermore we show that two of the empirical procedures that have been employed by other authors (2, 3, 4, 5) can give only approximate values since they fail to account for the logarithmic nature of the photographic process.

The photographic characteristic curve, a plot of the optical density of the developed film against the logarithm of the exposure, (Figure 1) may be considered to have three domains:

A. Underexposure: In this region the curve turns sharply upwards with increasing exposure.

B. Correct exposure: In this region there is a linear relationship between the optical density of the developed film and the logarithm of the exposure. The slope of this region of the curve is denoted by γ, which is the index of contrast of the photograph.

C. Overexposure: In this region further increments in exposure lead to decreasing increments in the optical density of the developed film.

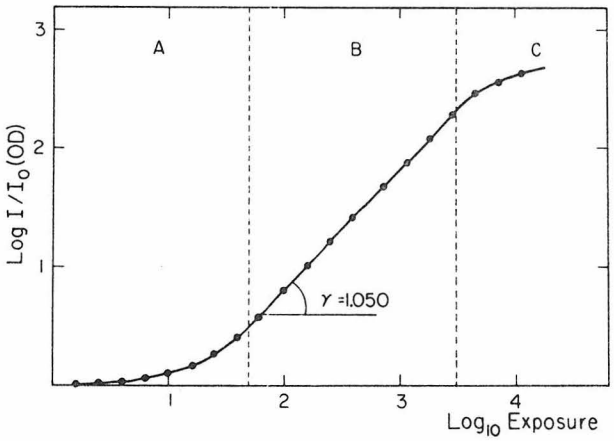

FIGURE 1. The characteristic curve of Kodak Plus-X film measured from a microdensitometer tracing of the image of a calibrated step wedge. Development was for 5' in Kodak D11 at 20°C.

From a consideration of Figure 1, it is clear that photographic quantitation of fluorescence intensity can be conveniently achieved only if both the background absorbance of the film and the maximum absorbance in the most intense peak fall within the linear region of the curve (Figure 1B). Since numerous factors can affect the precise shape of the characteristic curve, it is necessary to evaluate this curve whenever quantitation of fluorescence by photography is attempted. It is important to emphasize that both axes of the curve are logarithmic, necessitating the introduction of an exponent when relating the optical density of the developed film to the intensity (J) of the fluorescence (Equation 1).

$$J \propto 10^{D/\gamma} - 1 \qquad 1$$

D is the optical density of the developed film above background.

Under ideal conditions the intensity of the fluorescence is directly proportional to the quantity of the fluorescing species. For a band on an electrophoresis gel, where the concentration of a species is not uniform across the width of the band, it is necessary to integrate Equation 1 nu-

merically over the width of the band in order to determine the total quantity (Q) of the fluorescing species (Equation 2).

$$Q \propto \int_{\text{band}} J\,dx \approx \sum_{\text{band}} (10^{D/\gamma} - 1)\,\Delta x \qquad 2$$

Digital integration of fluorescence intensity by the procedure described above has been used in previous work (6, 7) to quantitate DNA in electrophoresis gels after staining with ethidium.

By comparison with fluorescent species, the photographic quantitation of absorbing species (e.g. in electrophoresis gels stained with an absorbing dye such as Coomassie Blue) is straightforward since it does not require the introduction of the exponential term $10^{D/\gamma}$. This follows from the fact that the intensity of the transmitted light (I) is logarithmically related to the amount of the absorbing species in the light path by the Beer-Lambert Law

$$\log_{10} (I_o/I) = \epsilon\, c l \qquad 3$$

where c is the concentration, l is the path length, ϵ is the extinction coefficient and I_o is the intensity of the incident light. Since within the linear range, the optical density of the developed film is proportional to the logarithm of the exposure (see Figure 1) the amount of absorbing material is directly proportional to the difference (E) between the background absorbance and the absorbance within the image of the absorbing species. Peak area measurement is therefore the correct method of quantitation (Equation 4).

$$P \propto \int_{\text{band}} E\,dx \approx \sum_{\text{band}} E\,\Delta x \qquad 4$$

P is the total quantity of the absorbing species in the band.

EXPERIMENTAL

DNA Restriction Fragments

30 μg PM2 DNA (gift of R. Watson) were digested with 30 units of HindIII restriction endonuclease (New England BioLabs) at 45°C for 4 hours. Partial digestion products were not observed to be present in the sample.

Electrophoresis

A vertical slab gel electrophoresis apparatus (Aquebogue) was used. Gels (4 mm thick) contained 1% agarose (Sea Kem) in 40 mM Tris-acetate pH 7.8, 5 mM sodium acetate, 0.1 mM EDTA, 1 μg/ml ethidium bromide (8).

Prestaining of the electrophoresis gels is necessary when very small restriction fragments are present since these may be partially

eluted during staining after electrophoresis. Electrophoresis gels containing high molecular weight DNAs can be stained overnight in 10 mM Tris-HCl, 1 mM EDTA pH 8, 1-2 μg/ml ethidium bromide. The concentrations of ethidium used for quantitative photography are high so as to ensure that the background fluorescence results in photographs that are within the linear range.

Photography

Gels and step density wedges (Kodak #2) were illuminated from below using short wavelength ultraviolet light from a transilluminator (Ultra-Violet Products, Inc.).

A 6"x 6" x 1/4" quartz plate (Amersil T08) was placed between the transilluminator and the electrophoresis gel in order to minimize local variations in illumination. The step density wedge was placed over a 1" x 6" x 1/8" strip of orange fluorescent plexiglass, lightly sandblasted on one side and taped at the edges to minimize stray light. This evenly illuminates the step wedge with red light.

The step density wedge was calibrated on an absolute scale using a Syntex AD-1 microdensitometer. The Syntex calibration disagreed by 25% with a calibration previously determined by Kodak, but agreed closely with an independent calibration performed by placing the wedge in the light path of a Gilford spectrophotometer.

Photographs were taken on 4" x 5" Kodak Plus X or Ilford FP4 sheet film through a Wratten 23-A filter. Development was for 5' at 20°C in Kodak D11 with continuous agitation.

Evaluation

Photographs were traced on a Joyce-Loebl microdensitometer. The characteristic curve of each film was determined by measurement of the step heights on the trace of the calibrated step wedge (see Figure 3b). If either the background absorbance or the maximum peak absorbance fell outside the linear range of the film response, the film was not further evaluated. Traces of correctly exposed photographs were digitized on a Hewlett Packard 9864A digitizer platen and evaluated on a Hewlett Packard 9820A calculator (Figures 2, 3).

RESULTS

For the purpose of illustrating the quantitative procedures employed we have used the HindIII fragments of PM2. The molecular weights of the seven fragments determined by a subtractive procedure (9) are listed in Table 1 (see also 10).

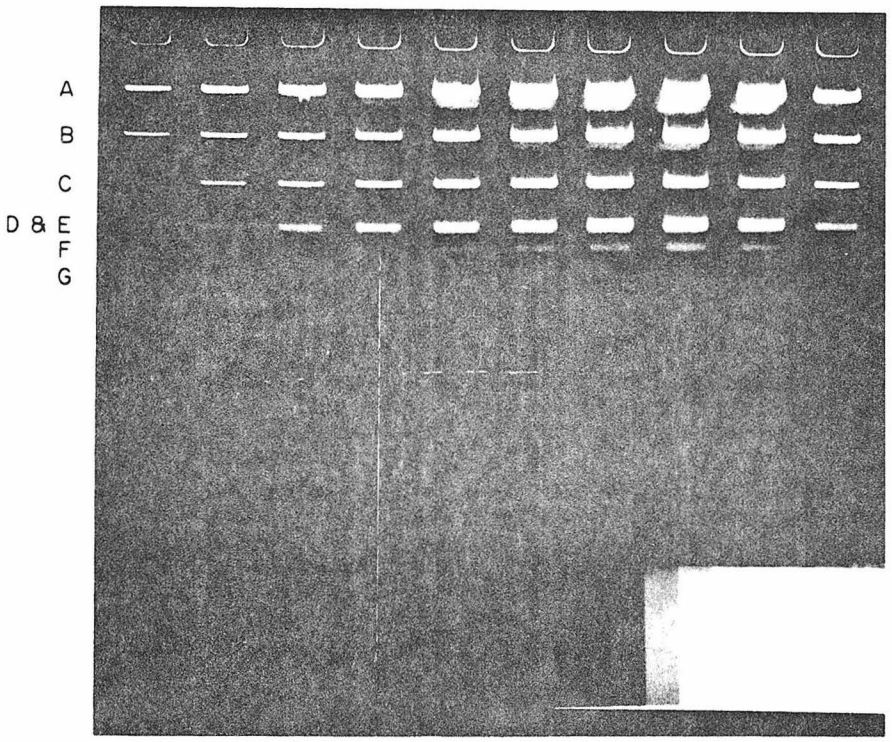

FIGURE 2. A quantitative photograph showing the resolution of species present in PM2 HindIII digests. Electrophoresis was for 2 hours at 2v/cm in a 1% agarose gel containing 40 mM Tris-acetate, 5 mM sodium acetate, 0.1 mM EDTA, 1 μg/ml ethidium bromide. Under these conditions fragments D and E migrate as a single species. In some places the images appear double because of reflections from the lower surface of the quartz plate. This did not interfere with the quantitation procedures.

Since fragment A is large compared to all of the other fragments it cannot be accurately quantitated in the same electrophoresis channel as the smaller fragments (see Figure 3a). It has therefore been excluded from this discussion. Deliberate use has been made of the small difference in mobility between fragments D and E to obtain photographs in which these species are not resolved.

The relative masses of the fragments B through G were determined from two independent sets of traces by the integrated transform procedure outlined in the introduction. These are listed in Table 2, column 1. Excellent correlation with the relative masses determined subtractively (Table 1, column 2) can be seen to hold for all but the smallest fragments, where the errors in both methods are maximal. The apparent relative

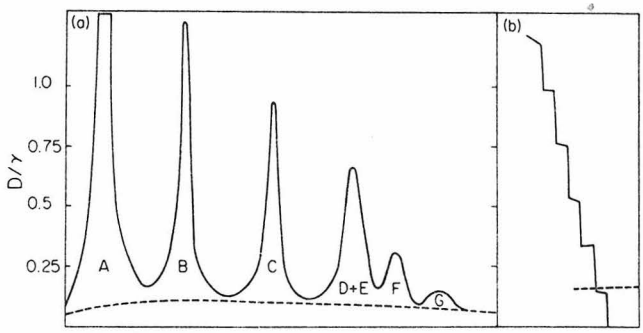

FIGURE 3(a). Microdensitometer tracing of a channel from the photograph shown in Figure 2. The vertical scale has been expressed in units of D/γ. For the purpose of quantitation, the background absorbance (---) was subtracted as indicated.

FIGURE 3(b). Microdensitometer tracing of the image of the wedge shown in Figure 2. The non-flat appearance of the steps is due to minor variations in the intensity of illumination of the wedge. Vertical step heights are measured at the edges of each step. Each step on this image represents 0.20 optical density units on the calibrated wedge, and is therefore equivalent to 0.2 D/γ units on the developed film.

TABLE 1. Molecular Weights of PM2 HindIII Fragments

Fragment	MW x 10^{-6} daltons	Fraction of (Total - Fragment A)
A	3.5	--
B	1.34	0.502 ± 2%
C	0.61	0.222 ± 5%
D	0.34)	0.194 ± 5%
E	0.27)	
F	0.15	0.057 ± 20%
G	0.06	0.025 ± 50%

masses were also determined by measuring peak heights and by area integration. The results of these measurements are listed in Table 2, columns 2 and 3 respectively. In each case the results obtained by these measurements differ significantly from the results obtained by the integrated transform method. Peak area, which at first sight would appear to be the more suitable measurement, actually shows greater errors overall. Peak height measurement suffers from the obvious defect of neglecting the effects of band shape. In the present example the use of peak height measurement could lead one to the incorrect conclusion that

TABLE 2. Photographically Determined Relative Mass of PM2 HindIII Fragments

Fragment	1* $\Sigma(10^{D/\gamma}-1)\Delta x$ band	2* Height	3* Area
B	0.514 ± 3.4%	0.430 ± 8.8%	0.386 ± 5.2%
C	0.227 ± 5.6%	0.278 ± 2.7%	0.246 ± 8.8%
D and E	0.192 ± 4.3%	0.192 ± 8.9%	0.250 ± 6.1%
F	0.049 ± 7.4%	0.076 ± 14%	0.084 ± 11.9%
G	0.017 ± 20.3%	0.024 ± 18.7%	0.034 ± 16.3%

*Averages and standard deviations are based on 10 measurements of separate channels in 2 electrophoresis gels.

the peak containing fragments D and E, in fact, contained a single species. The standard deviations on the peak height and peak area measurements are significantly higher than those on the integrated transform. This results from the systematic effects of sample loading on the apparent relative masses determined by either peak height or peak area measurements.

The Parabolic Approximation

Peak area and peak height are shown to be unsuitable measurements for any but the most approximate estimates of the relative concentrations of species in a resolved mixture. Since digital integration facilities are not universally available, we have developed an alternative approximate method based upon the assumption that the image of a peak has a parabolic height to width relationship (see Figure 4).

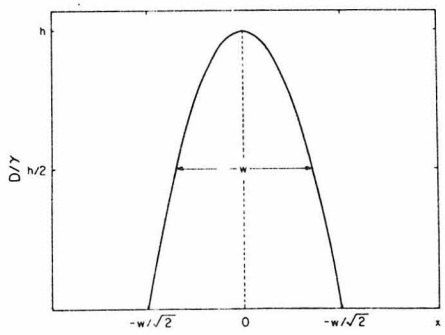

FIGURE 4. A parabola of height, h, and width, w, at half height is taken to represent the vertical height/width relationship of a band image for the purposes of approximation. The main deviation from this relationship usually occurs in the tails of a band profile which make only a small contribution to the integral.

Calibration of the photograph remains an essential feature of this method since it is necessary to show that both the baseline absorbance and the maximum peak absorbance lie within the linear range of the film response. Calibration is also necessary for measurement of the peak height, h, which must be expressed in units of D/γ. The width of the peak, w, is measured at half height and is in arbitrary units. The transform of the parabola has the form

$$I = \int_{-w/\sqrt{2}}^{w/\sqrt{2}} \{ 10^{(h - 2h x^2/w^2)} - 1 \} \, dx \qquad 5$$

This integral (I) has been calculated for values of $0 < h \leq 1$ (this corresponds to the useful range of the linear regions for most photographic materials) and has been shown to closely fit the polynomial (Equation 6) over this range.

$$I = w (2.28 h + 0.93 h^2 + 3.327 h^3) \qquad 6$$

Equation 6 has been evaluated for each of the PM2 HindIII fragments from photographs such as that shown in Figure 2. The normalized values for fragments B through G are listed in Table 3.

TABLE 3. Estimates of Relative Mass of PM2 HindIII Fragments by Parabolic Approximation

Fragment	$w(2.28 h + 0.93 h^2 + 3.327 h^3)$
B	0.498 ± 6.9%
C	0.223 ± 7.3%
D and E	0.206 ± 8.2%
F	0.053 ± 8.7%
G	0.019 ± 15.4%

Comparison of these values with those in Table 2, column 1 shows excellent agreement for all of the fragments. The larger standard deviations on the individual measurements are due primarily to random errors associated with peak width measurements.

DISCUSSION

It has been shown that proper evaluation of fluorescence photographs can give accurate values for the relative concentrations of the fluorescent species. However, the full procedure as outlined here does require digital integration and therefore, will prove inconvenient for many laboratories wishing to make use of fluorescence photographs for quantitation. The parabolic approximation has been developed to overcome the

need for digital integration. It provides a more suitable measurement than either peak area or peak height since full consideration is given to the logarithmic nature of the photographic response. It remains an approximation however, and as such should be applied with caution when the peak profile deviates markedly from that of a parabola.

Additional factors that must be considered when quantitating fluorescence photographs are: 1) The concentration of the fluorescing species must be sufficiently low to avoid attenuation of the exciting light. This is a potential problem when weakly fluorescent species are being estimated. 2) Differing affinities for a fluorescent stain, and differing quantum efficiencies of the fluorophor must be taken into account when estimating the relative amounts of diverse species. This is not a significant problem when estimating the amount of linear double stranded DNA by ethidium fluorescence, but becomes a potential problem when single stranded or synthetic polynucleotides are being quantitated (11).

Because of the strong effect of superhelix density on the binding of ethidium to closed circular DNA, there is a potential problem when estimating the relative amounts of closed circular DNAs. We have overcome this difficulty in previous work by photolytic nicking of the closed circular DNA in stained gels by exposure to high intensity ultraviolet light for 1-2 minutes prior to restaining and quantitative photography (6, 7).

Some attention should be paid to the factors that affect the suitability of a photograph for quantitation. High contrast photographs (large values of γ) will generally give more accurate values than low contrast photographs (low values of γ). This consideration, together with generally irreproducible results led us to abandon Polaroid negative films for quantitative use. Maximum contrast is obtained upon prolonged development of medium speed films in vigorous developers such as D11. Other factors affecting contrast include the film type, storage conditions, development temperature and exposure time (reciprocity failure). If format sizes smaller than 4" x 5" are to be used, attention must be paid to the contribution of adjacency effects that occur when the dimensions of the image are small. Such effects are minimized by prolonged development with continuous agitation.

The considerations discussed in this work apply equally to fluorography where attempts have been made to quantitate by peak area measurement (12). Prefogging of the film used by these authors has the effect of bringing the background into the linear region of the characteristic curve,

but does not overcome the need for exponential integration. At the present time we do not see any convenient method for measuring the characteristic curve of a fluorographic image since exposure time plays an important role in determining this function. In the absence of such a measurement, the most accurate method of quantitation must still be excision of the radioactive species followed by direct counting.

ACKNOWLEDGEMENTS

We wish to thank Dr. M. Ross and M. Klymkowsky for their help in calibrating the step density wedges. This work was supported in part by USPHS, National Institutes of Health Grants GM15327 and CA 08014. D. E. P. is a recipient of a Medical Research Council of Canada fellowship. M. S. is a recipient of an N. S. F. graduate fellowship. This is contribution No. 5426 from the Division of Chemistry and Chemical Engineering.

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CHAPTER V

The Structure of Virion SV40 DNA *in situ*: An Examination by Chemical Modification with Dimethylsulfate

SUMMARY

The structure of virion SV40 DNA in situ has been probed using dimethylsulfate (DMS). Virions are permeable to DMS and remain physically intact during the reaction, while the DNA (specifically, the bases adenine and guanine) within the virus particles becomes methylated. This general approach permits the examination of possible specific protein-DNA interactions within SV40 in the absence of artifacts of isolation and reconstitution.

DMS reactions were done to an extent that an average of one nucleotide in 250 became methylated. After quenching the methylation reaction, the DNA was purified from the virus particles, and the purified methylated DNA was treated with a given restriction endonuclease depending upon which position of the SV40 genome was to be examined. The resultant fragments were labeled at their 5' termini using [γ-^{32}P]ATP and T4 polynucleotide kinase, and the kinased DNA was then digested with one or more different restriction endonucleases to produce fragments having only one of the two strands labeled. Such fragments were eluted from gels after preparative electrophoresis. Each purified fragment was depurinated at the methylated bases, following which the DNA was hydrolyzed at depurinated sites. This entire process resulted in a series of DNA fragments, which had the same labeled 5' terminus but which differed from one another in length by adjacent purines.

The cleaved products were fractionated on a denaturing gel under conditions where differences in length of a single nucleotide could be distinguished. The cleaved products obtained from the methylation of DNA within intact virus were electrophoresed adjacent to those obtained from a parallel treatment of purified naked SV40 DNA.

A total length of DNA of approximately 3600 nucleotides (35% of the nucleotides in SV40 DNA) was scanned using this method. A total of 15 segments of the SV40 genome (obtained by kinasing 15 different 5' termini produced by treatment with different restriction endonucleases) was screened. The fragments represented both strands of the DNA and were chosen to obtain samples representative of the entire genome, with a particular emphasis on fragments covering and surrounding the origin(s) of DNA replication and both late and early RNA transcription. No major or convincing differences were ever observed in the purine patterns obtained from corresponding samples derived from bare DNA and from virus.

ABBREVIATIONS

Ac,	acetate
BME,	2-mercaptoethanol
BPB,	bromophenol blue
ddH_2O,	double-distilled H_2O
DMS,	dimethylsulfate
dT,	deoxythymidine
DTT,	dithiothreitol
EDTA,	[ethylenedinitrilo]-tetraacetic acid
EtdBr,	ethidium bromide
EtOH,	ethanol
Lys,	L-lysine
Pi,	orthophosphate
pfu,	plaque-forming units
SDS,	sodium dodecylsulfate
Tris,	Tris(hydroxymethyl)aminomethane
XCFF,	xylene cyanol FF

Enzyme	Bacterial origin	Recognition sequence and Points of cleavage*	
Alu I	Arthrobacter luteus	5' ... AG↓CT ... 3' 3' ... TC↑GA ... 5'	
Bam HI	Bacillus amyloliquefaciens HI	5' ... G↓GATCC ... 3' 3' ... CCTAG↑G ... 5'	
Eco RI	Escherichia coli RY13	5' ... G↓AATTC ... 3' 3' ... CTTAA↑G ... 5'	
Hae III	Haemophilus aegyptius	5' ... GG↓CC ... 3' 3' ... CC↑GG ... 5'	
Hha I	Haemophilus haemolyticus	5' ... GC↓GC ... 3' 3' ... C↑GCG ... 5'	
Hind III	Haemophilus influenzae Rd	5' ... A↓AGCTT ... 3' 3' ... TTCGA↑A ... 5'	
Hinf I	Haemophilus influenzae Rf	5' ... G↓ANTC ... 3' 3' ... CTNA↑G ... 5'	N = any nucleotide
Hpa I	Haemophilus parainfluenzae	5' ... GTT↓AAC ... 3' 3' ... CAA↑TTG ... 5'	
Hpa II	Haemophilus parainfluenzae	5' ... C↓CGG ... 3' 3' ... GGC↑C ... 5'	
Taq I	Thermus aquaticus YT-I	5' ... T↓CGA ... 3' 3' ... AGC↑T ... 5'	

*See Roberts (1976).

INTRODUCTION

Questions concerning protein-DNA interactions have been of interest for some time. There are three broad categories into which these types of interactions may fall: (1) enzymatic, (2) regulatory, (3) structural. The first of these is perhaps the most obvious and often the least complex, as, in some cases, it may involve only a single protein and a single or relatively few substrate(s) (e.g., the DNA and perhaps ribo- or deoxyribonucleoside triphosphates). Examples of this are numerous, and include both DNA and RNA polymerases, ligases, kinases, phosphatases, exo- and endonucleases, as well as enzymes which alter the topology of DNA such as gyrase and ω from prokaryotes and nicking-closing enzymes isolated from mammalian cells.

The second of these types of interactions is exemplified in prokaryotes by repressor-operator interactions in bacteria and phage. The discovery, purification, and elucidation of the function(s) of such repressors were greatly facilitated by the availability of mutants and by the ability to perform genetic experiments with the organisms. To date, no such specific repressors have been characterized in eukaryotes, although this may be more an indication of the difficulty in working with such complex organisms than of an absence of a similar phenomenon. In the category of regulatory interactions must also be mentioned the specific recognition of DNA sequences (promoters) by RNA polymerase (aside from the actual enzymatic activity of the polymerase with the DNA as a template) as well as the specific recognition of DNA sequences by restriction endonucleases (aside from the actual nucleolytic activity of the enzymes).

The third type of protein-DNA interaction, involving structural proteins, has largely been studied by physical methods such as neutron, electron, or X-ray

diffraction (e.g., studies of histone-DNA interactions in nucleosomes; the determination of the structure of tobacco mosaic virus), as well as by electron microscopy (e.g., the determination of the capsid structures of papovaviruses; studies of nucleosome spacing and packing in chromatin).

Examples of recent work in all three categories of protein-DNA interactions can be found in the volume edited by Vogel (1977).

Dimethylsulfate (DMS) was initially used by Mirzabekov (Mirzabekov and Melnikova, 1974; Mirzabekov and Kolchinsky, 1974) to study the interactions of histones and DNA in both chromatin and intact nuclei. By measuring the amount of methylated purines (actually the ratios of m^7G/m^3A and m^7G/m^1A) liberated after complete hydrolysis of DNA from chromatin or nuclei which had been treated with dimethylsulfate, it was concluded (1) that histones in chromatin lie partly inside the major groove and partly out of the grooves, but leave the minor groove open, and (2) that the major groove of DNA in chromatin is protected by only 10-20% against methylation with DMS. Since the minor groove is not protected, the overall difference in levels of methylation of bare DNA vs. DNA in chromatin can be expected to be quite small.

More recently, in conjunction with the DNA sequencing technique of Maxam and Gilbert (1977), dimethylsulfate has been used to probe the specific contacts between lac repressor and operator DNA (Gilbert, Maxam and Mirzabekov, 1976), between λ repressor and the left and right operators of λ DNA (Humayun, Kleid and Ptashne, 1977), and between λ cro protein and λ operator DNA (Johnson, Meyer and Ptashne, 1978). This method has also been used to elucidate the recognition sequences of certain Class II restriction endonucleases which cleave at sites

upstream or downstream from the specific DNA sequences recognized (Kleid et al., 1976).

It is clear that many small molecules may serve as useful probes for studying protein-DNA interactions. The advantage of purine methylation with dimethylsulfate is that in conjunction with both modern sequencing techniques and a defined genome or DNA fragment, the exact bases involved in the interaction can be determined.

Simian virus 40 (SV40) is a papovavirus which produces lytic infections in cultured monkey cells (Tooze, 1973; Fried and Griffin, 1977; Fareed and Davoli, 1977). The genome of SV40 is a double-stranded supercoiled DNA of approximately 5200 base pairs whose entire sequence has recently been determined by the laboratories of both Weissman and Fiers (Reddy et al., 1978; Fiers et al., 1978) Within both intact virions and within the nuclei of infected cells, SV40 DNA is found complexed with the four nucleosomal histones: H2a, H2b, H3, and H4 (White and Eason, 1971; Huang, Estes and Pagano, 1972; Hall, Meinke and Goldstein, 1973; Lake, Barban and Salzman, 1973; Meinke, Hall and Goldstein, 1975; Pett, Estes and Pagano, 1975). In addition, in early stages of infection, the SV40 minichromosomes (SV40 DNA complexed with the four core histones) contain H1 (Varshavsky et al., 1976, 1977; Christiansen and Griffith, 1977; Müller et al., 1978). A small genome, structural similarities to eukaryotic chromatin (Griffith, 1975; Bellard et al., 1976; Cremisi et al., 1976) and the ease with which both intact virus particles, as well as viral DNA and intracellular SV40 chromatin, can be physically separated from host cell components have made SV40 an ideal model system for studying questions concerning eukaryotic genome organization, expression, and regulation.

Much work on nucleosome structure, both histone-histone interactions and histone-DNA interactions, has been done using (1) histones purified from animal cells, (2) bulk chromatin extracted from nuclei, or (3) intact nuclei. The proximity of different histones relative to one another as well as the number of histones and gross subunit arrangement in nucleosomes were questions approached via both chemical (specifically, protein cross-linking studies) and physical methods using purified histones (D'Anna and Isenberg, 1974; Kornberg and Thomas, 1974; Roark, Geoghegan and Keller, 1974; Bonner and Pollard, 1975; Chalkley, 1975; Martinson and McCarthy, 1975; Thomas and Kornberg, 1975; Weintraub, Palter and Van Lente, 1975). The elucidation that chromatin structure is based upon repeating subunits came from both electron microscopic and biochemical studies using nuclei, extracted chromatin, and chromatin which had been reconstituted from purified DNA and purified histones (Hewish and Burgoyne, 1973; Barrett, 1974; Burgoyne, Hewish and Mobbs, 1974; Kornberg, 1974; Noll, 1974a; Olins and Olins, 1974; Felsenfeld, 1975; Olins, Carlson and Olins, 1975; Oudet, Gross-Bellard and Chambon, 1975). Isolation of mononucleosomes and oligomers from bulk chromatin or from nuclei permitted initial characterization in terms of hydrodynamic properties (Rill and Van Holde, 1973; Sahasrabuddhe and Van Holde, 1974; Shaw et al., 1974; Van Holde et al., 1974; Bakayev et al., 1975). Evidence that in nucleosomal chromatin, the DNA is wrapped around a core of proteins (histones) to a large extent, rather than being buried within, came from the early experiments on the gross nuclease sensitivity of chromatin (Clark and Felsenfeld, 1971), from experiments utilizing a combination of nucleases and proteases (Weintraub and Van Lente, 1974), as well as from neutron diffraction studies (Baldwin et al., 1975; Carpenter et al., 1976). More recently, there has been both hydrodynamic

and electron microscopic evidence for higher order structure in chromatin (Bellard et al., 1976; Finch and Klug, 1976; Christiansen and Griffith, 1977; Varshavsky et al., 1977; Müller et al., 1978). Using cellular chromatin, nuclease digestion and techniques of gel electrophoresis, nucleosome repeat lengths were determined and "internucleosome" distances estimated. (For reviews on chromatin structure see Elgin and Weintraub, 1975, and Kornberg, 1977.) Later, extension of these techniques revealed internal nucleosomal DNA structure as evidenced by the appearance of DNA fragments of reasonably discrete lengths differing by approximately 10 bases or 10 base pairs, depending upon whether the chromatin or nucleosomes (1) were digested with DNase I (pancreatic DNase) and the products electrophoresed on denaturing gels or else (2) were digested with micrococcal nuclease and the products electrophoresed on native gels, respectively (Axel et al., 1974; Noll, 1974b; Sollner-Webb, Melchior and Felsenfeld, 1978).

The discovery that the DNA and histones in the SV40 nucleoprotein complexes isolated from virions and from infected cells (late in infection) were physically similar to H1-depleted nuclear chromatin, however, greatly facilitated the elucidation of other aspects of nucleosome structure and arrangement. The fact that SV40 DNA is homogeneous in size, small, and closed circular, made it possible to estimate compaction ratios by electron microscopy (Griffith, 1975; Cremisi et al., 1976). In addition, it was possible to perform experiments in vitro which demonstrated that the supercoiling in native SV40 DNA is a combined consequence of the binding of the four histones, H2a, H2b, H3, and H4, and the action of a nicking-closing enzyme (Germond et al., 1975). Since native SV40 DNA is negatively supercoiled, the results of such experiments indicated that

the binding of nucleosomal histones to DNA resulted in an unwinding of the helix or else in a tertiary structure which is topologically equivalent.

Another important question which was approached using SV40 minichromosomes concerned the sequence specificity and phasing of nucleosomes. Similar experiments were performed in several laboratories using restriction endonucleases in conjunction with both viral minichromosomes and those extracted from the nuclei of infected cells (Polisky and McCarthy, 1975; Cremisi et al., 1976; Cremisi, Pignatti and Yaniv, 1976; Ponder and Crawford, 1977). From the observed accessibility of enzymes to various restriction sites in the DNA of minichromosomes, it was concluded that in a population of minichromosomes, nucleosomes are positioned randomly rather than at fixed points on the SV40 genome.

Objections (regarding the intactness of the preparations of minichromosomes) can be made to the above experiments. This is especially true in the case of complexes derived from virions, since it has been shown that the alkaline conditions used to disrupt the virus yield a population of nucleoprotein complexes largely deficient in histones (Christiansen et al., 1977). Other objections to such experiments concern the fact that it is unknown whether or not the histones can move during the extraction and/or enzyme incubation steps. In addition the intactness of the minichromosomes (in terms of number of nucleosomes) often was not monitored either during or following the enzyme digestion(s).

Due to the possible artifacts inherent in such approaches, it seemed reasonable to utilize alternate methods which would be capable of answering questions concerning DNA-protein interactions without perturbing the structure of the DNA-protein complex. As has been previously mentioned, dimethylsulfate has been used successfully to determine the protein-purine contacts in various

operator-repressor systems (Gilbert, Maxam and Mirzabekov, 1976; Humayun, Kleid and Ptashne, 1977; Johnson, Meyer and Ptashne, 1978). The use of various operator mutants greatly facilitated the interpretation of the results, and more importantly, yielded data on protection or enhancement of methylation in the presence of bound repressor which correlated well with both the genetic and the physical characterizations of the mutants (including the sequence of each mutant operator). Results of these experiments indicated that the methylation patterns did indeed reflect the true binding of repressor to operator and were not artifactual.

The above results combined with Mirzabekov's DMS experiments (Mirzabekov and Melnikova, 1974; Mirzabekov and Kolchinsky, 1974) on bulk chromatin, suggested that DMS might be used to probe the structure of virion SV40 DNA in situ. A distinct advantage to this approach is that virions are permeable to DMS and remain intact while the DNA within becomes chemically modified (see Results). This permits the detection of specific protein-DNA interactions in the absence of possible artifacts of isolation and reconstitution. Three types of protein-DNA interactions in SV40 virions could be examined using this method: (1) interactions involving virion capsid proteins, (2) interactions involving histones, (3) interactions involving any of several proteins which have been located at or near the origin of DNA replication and RNA transcription, and which are found bound (noncovalently in some cases and covalently in others) to the DNA either after disruption of virions by special methods or upon treatment of intracellular nucleoprotein complexes in certain ways (Griffith, Dieckmann and Berg, 1975; Kasamatsu and Wu , 1976a; Kasamatsu and Wu, 1976b).

RESULTS

SV40 Virions are Permeable to DMS and Remain Intact while the DNA within Becomes Chemically Modified

Before attempting to compare methylation patterns obtained after treatment of intact virions with DMS with those obtained after parallel treatment of naked DNA with DMS, it was essential to determine both (1) whether the virions were permeable to DMS (i.e., whether or not the DNA within virions became methylated) and (2) whether or not the virions remained physically intact during and after the methylation reaction. Over a period of time, several similar experiments were performed to answer these questions. The basic approach was to determine first whether the buffer in which the methylations were done disrupted (osmotically) the virions, and then to determine whether or not the virions were disrupted during the DMS treatment.

The experiments performed were based on two pieces of information. (1) Intact wild-type SV40 virions have a buoyant density in CsCl of approximately 1.32 gm/cc. (2) DNA within intact virus particles is resistant to treatment with DNase; and after DNase treatment, virions retain a buoyant density of 1.32 gm/cc in CsCl (i.e., the virion protein and the virion DNA co-band).

To determine whether or not DMS buffer caused osmotic disruption of virions, aliquots of SV40 virus (labeled with ^{3}H-Lys and ^{14}C-dT) were dialyzed into both DMS buffer (50 mM Na cacodylate pH 8.0, 10 mM $MgCl_2$, 0.1 mM EDTA) and into TD buffer (0.14 M NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 25 mM Tris HCl pH 7.4), a buffer which is routinely used in various stages of virus preparation, purification, and storage. $MgCl_2$ was added to the suspension of virus in TD

buffer to a final concentration of 10 mM and both preparations were incubated with crude pancreatic DNase at a final concentration of 200 μg/ml (at 37°C for 30 minutes). The samples were then placed on ice and EDTA was added to a final concentration of 50 mM to quench the reactions. An aliquot of the same DNase stock solution used above was added to a sample of purified SV40 DNA and this mixture was incubated alongside the virus samples. After quenching the reaction, the DNase-treated DNA was electrophoresed adjacent to another aliquot of the same SV40 DNA which had been incubated at 37°C but without DNase. Electrophoresis was through a 1% agarose gel in the presence of 0.5 μg/ml EtdBr. Under the electrophoresis conditions employed, a DNA fragment of approximately 200 base pairs in length would have migrated one-third of the way down the gel. The results (not shown)* indicated that the DNase was quite active and digested the DNA to nucleotides or very small oligonucleotides in view of the fact that no fluorescent material was visible in the channel in which the DNase-treated SV40 DNA had been electrophoresed. The control DNA (SV40 DNA incubated at 37°C but without DNase) migrated as two components: the supercoiled form (I) and the nicked circular form (II), a small amount of which was already present in this preparation.

Each DNase-treated virus sample was then adjusted to a final density of 1.32 gm/cc by the addition of solid CsCl. Two-thirds of each sample were

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*The same control experiment to monitor the activity of the DNase was performed in the set of experiments (described later) designed to determine whether or not the methylation reaction disrupted the overall structure of the virions. The results of this control are shown in Figure 2 where it can be seen that (unmethylated) SV40 DNA incubated at 37°C but without DNase migrates as forms I and II (Ch. c), but that the channel in which the DNase-treated SV40 DNA had been electrophoresed (Ch. b) is completely devoid of fluorescent material.

placed in separate 5 ml cellulose nitrate centrifuge tubes and the remaining thirds of each sample were mixed and placed in a third centrifuge tube. The volume of each gradient was adjusted to 3.5 ml with a CsCl solution of density 1.32 gm/cc, and the gradients were centrifuged in a Beckman SW50.1 rotor at 35,000 rpm at 20°C for 36 hours. The gradients were fractionated from the bottom and aliquots of each fraction were counted for ^{3}H (lysine) and ^{14}C (dT). The profiles (not shown) of all three gradients were indistinguishable, indicating that incubation of the virus in DMS buffer did not render the DNA within accessible to DNase and therefore did not disrupt the overall virion structure.

Although in this particular experiment it was not determined whether the DNA within the virus was intact (by deproteinization of aliquots of each of the bands of virus and subsequent examination of the resultant DNA by electrophoresis), this was done in the next experiment to be described. As can be seen in Figure 2 (Ch. g), aliquots of virus which had been incubated in DMS buffer, treated with DNase, banded in CsCl and then deproteinized, yielded largely form I (supercoiled) SV40 DNA and a small amount of form II (nicked circular) SV40 DNA. The presence of nicked circular DNA did not result from treatment of the virions with DNase, but rather was due to the age of the particular virus preparation used.

To determine whether or not the methylation reaction disrupted the virus, an experiment similar to the previous one was performed. Purified virus was dialyzed into DMS buffer and the final volume was adjusted with DMS buffer to a final virus concentration such that the final concentration of DNA (within virus) was 50 μg/ml. The sample was divided equally between two tubes and these, along with a tube of dimethylsulfate, were placed on ice for at least 30 minutes

to chill to 0°C. After chilling, DMS was quickly added to one virus sample to a final concentration of 53 mM (1 µl 10.7 M DMS/200 µl reaction). The sample was mixed and was then replaced on ice. The other tube of virus served as an unmethylated control. After 1.5 hours at 0°C*, samples were extracted three times with ether. In this experiment, ether was used to quench the methylation reaction rather than the prescribed DMS stop solution. DMS is much more soluble in ether than in aqueous solutions (Merck Index, eighth edition) and therefore should be efficiently removed by this procedure. In addition, because the capsid of SV40 virions does not contain lipid, ether extraction of virus does not result in disruption (Tooze, 1973; and results of tests not shown). Previous attempts to perform this experiment using the stop solution prescribed by Maxam and Gilbert (1977) resulted in partial disruption of the virus, presumably because addition of the stop solution to the reaction mixture brings the sample to a final concentration of 0.2 M mercaptoethanol, resulting in reduction of disulfide bonds in proteins within the virion capsid.

Residual ether was evaporated by placing the tubes (open) at 37°C for 10-20 minutes. The samples were then dialyzed against TD buffer, adjusted to a final concentration of 10 mM $MgCl_2$, and incubated at 37°C for 30 minutes with crude pancreatic DNase at a final concentration of 200 µg/ml. At the same time, two aliquots of SV40 DNA were incubated, one with DNase and one without. At the end of 30 minutes the virus samples were placed on ice and adjusted to a final concentration of 50 mM EDTA. The DNA samples were deproteinized with phenol and were placed at -20°C until subsequent analysis.

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*This experiment has also been done using longer methylation reaction times (4 hours, 6 hours). The profiles of the CsCl gradients (not shown) were indistinguishable from those shown in Figure 1, indicating that SV40 virus is not disrupted by prolonged treatment with DMS.

Following treatment with DNase, each virus sample was divided in two. Solid CsCl was added to one-half of each sample to a final density of 1.32 gm/cc and to the other half to a final density of 1.70 gm/cc. (SV40 DNA has a base composition of 41% G+C [Tooze, 1973], corresponding to a buoyant density in neutral CsCl of 1.701 gm/cc, if the buoyant density of E. coli DNA is taken as 1.710 gm/cc.) The latter was done to ensure detection of any free DNA which could have resulted from disruption of virus followed by DNase treatment. The gradients were centrifuged to equilibrium in an SW50.1 rotor (20°C, 35,000 rpm, 112 hours). Following centrifugation, in both tubes of $\rho_{average}$ = 1.32 gm/cc two opalescent bands could be seen in the central regions of the gradients. These correspond to virus (lower band) and a small amount of "shells" or empty capsids (upper band) which were initially present in this virus preparation. In both tubes of $\rho_{average}$ = 1.70, only one opalescent band was visible. This was located at the tops of the gradients, at the interface between the CsCl solution and the mineral oil. All four gradients were fractionated by dripping from the bottoms of the tubes. Ten-drop fractions (approximately 100 µl/fraction) were collected. Aliquots of 30 µl each were taken from each fraction and counted in Aquasol (New England Nuclear) for the presence of both ^{3}H and ^{14}C.

The profiles of the gradients (shown in Fig. 1) are indistinguishable for virus samples regardless of whether or not they had been treated with DMS. The small peaks of ^{3}H in both Figures 1a and 1c are the empty capsids mentioned in the preceding paragraph. Peaks of ^{14}C are absent from the bottoms of both gradients of $\rho_{average}$ = 1.32 [Fig. 1, (a) and (c)] and from the middles of both gradients of $\rho_{average}$ = 1.70 [Fig. 1, (b) and (d)], indicating that DMS did not make the virion DNA accessible to DNase. In addition, the background of ^{14}C

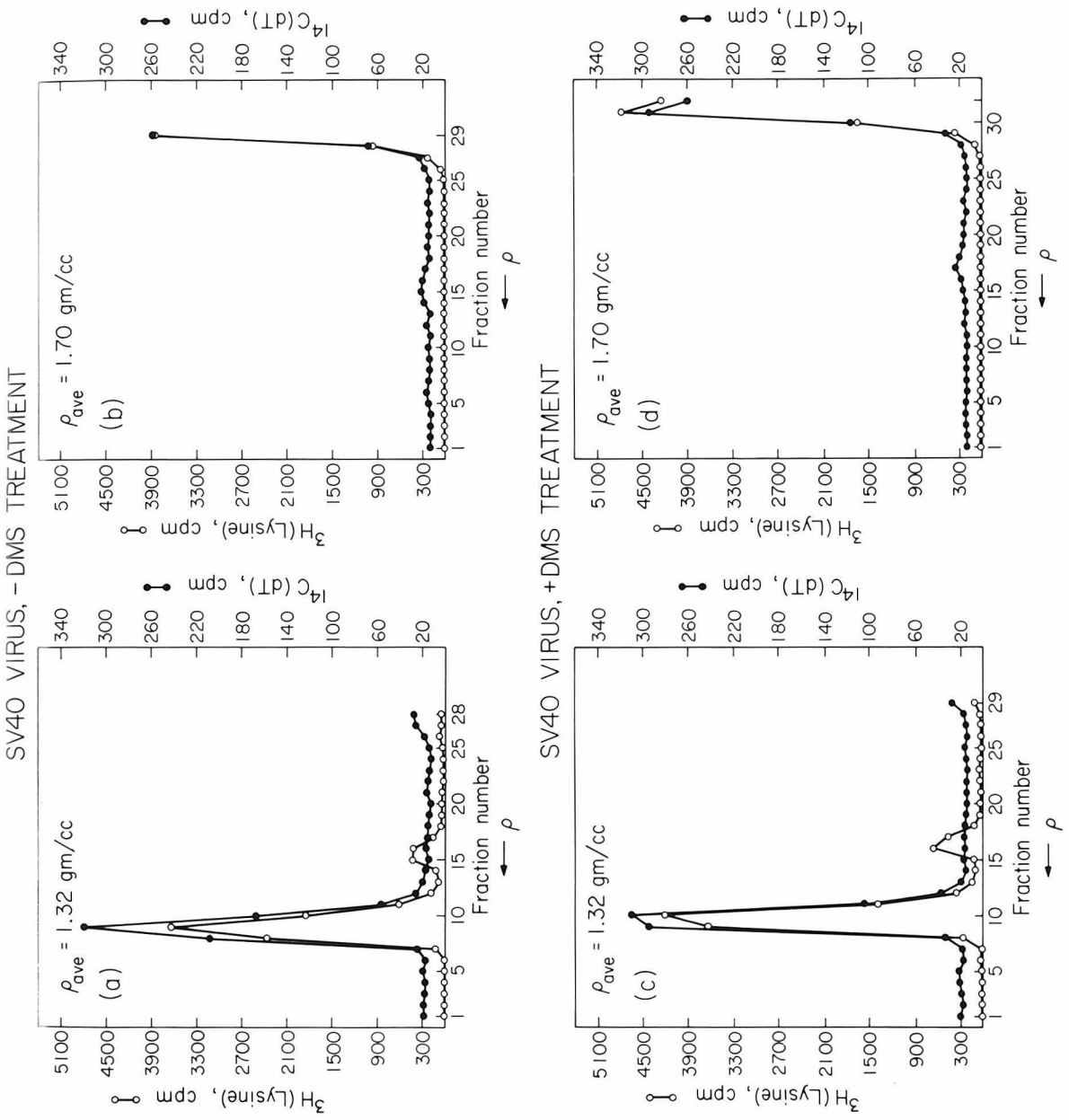
FIGURE 1. Profiles of CsCl Density Gradients of SV40 Virus Labeled with ^{3}H-Lysine (Open Circles) and ^{14}C-dT (Filled Circles).

Sections (a) and (c): Samples of virus were incubated for 1.5 hours at 0°C both in the absence and in the presence, respectively, of 53 mM DMS, following which they were treated with DNase and were then centrifuged to equilibrium in CsCl gradients having an average density (ρ_{ave}) of 1.32 gm/cc. The small peaks of ^{3}H of lower buoyant density are "shells" or empty capsids, a small amount of which were present in this preparation of virus.

Sections (b) and (d) contain aliquots of the same samples as do sections (a) and (c), respectively. Centrifugation was, however, in CsCl gradients of ρ_{ave} = 1.70 gm/cc.

In all four sections, density increases from right to left.

Experimental details are given in the Results section.


SV40 VIRUS, - DMS TREATMENT
SV40 VIRUS, + DMS TREATMENT
ρ_{ave} = 1.32 gm/cc
ρ_{ave} = 1.70 gm/cc
(a)
(b)
(c)
(d)
^{3}H (Lysine), cpm
^{14}C (dT), cpm
Fraction number
ρ

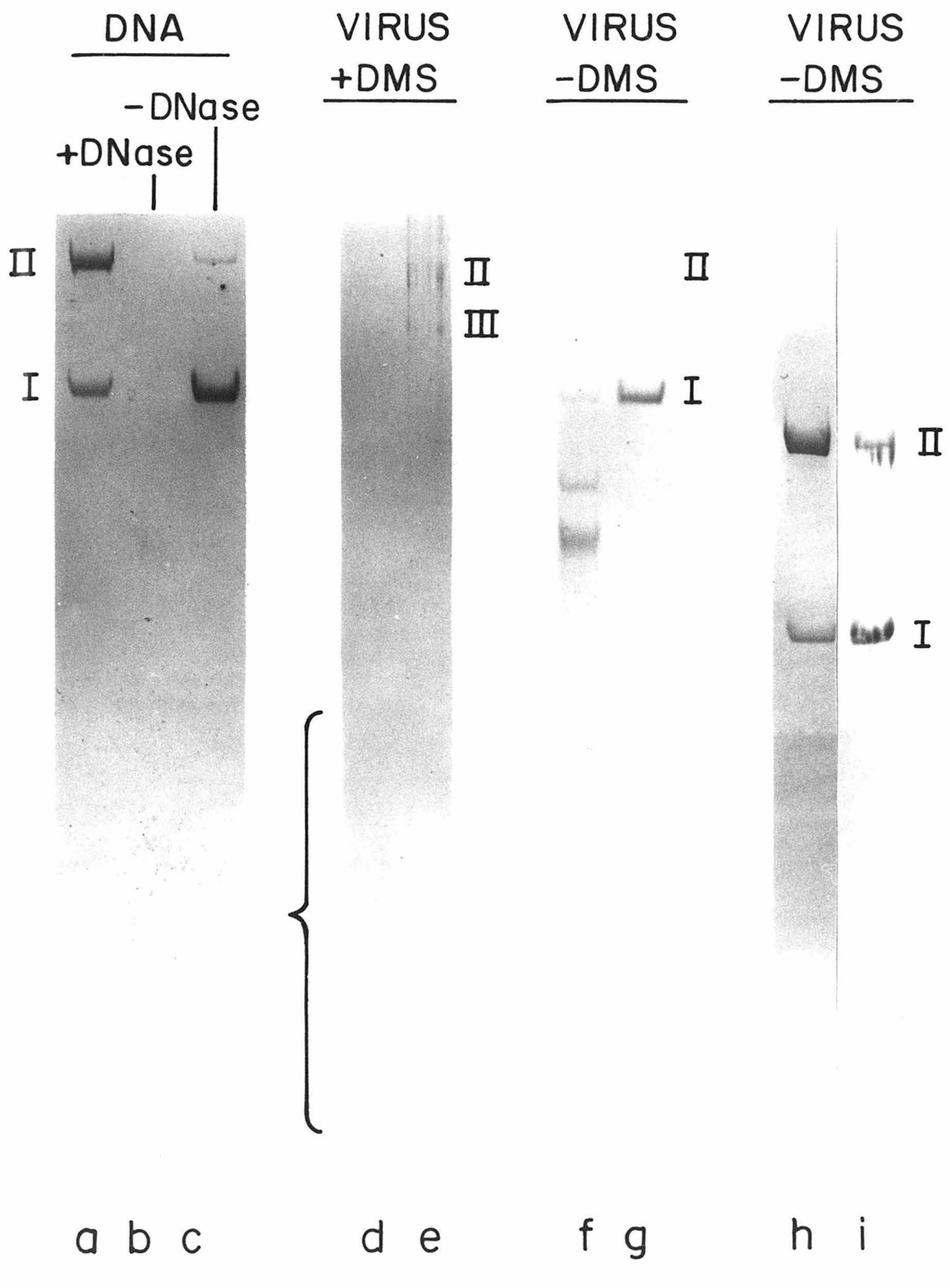
is essentially the same in all four gradients. If treatment with DMS had rendered the DNA within the virus particles accessible to DNase and if the DNase treatment then resulted in the production of nucleotides or in very short oligonucleotides, the background of ^{14}C (dT) would have been expected to increase.

The remainder of the peak fractions of virus from each gradient were pooled, dialyzed against TD buffer, and deproteinized by multiple extractions with phenol. The DNA was precipitated twice with ethanol and each sample was finally resuspended in 10 mM Na phosphate pH 7.0, 1 mM EDTA. Halves of each sample were withdrawn and subjected to the procedures for neutral depurination at methylated bases, followed by procedures for alkaline hydrolysis at depurinated sites (see Experimental Procedures). The remaining halves of the samples were left on ice. Following termination of the reactions, all samples were diluted with a mixture of glycerol plus dyes. The control samples of SV40 DNA that had been incubated previously (at 37°C) with and without DNase were thawed and were also diluted with glycerol plus dyes. Aliquots of each were electrophoresed through a 1.5% agarose gel. As can be seen in Figure 2 (Ch.g), virus which had not been treated with DMS yielded intact SV40 DNA (both the supercoiled form [I] and a small amount of the nicked circular form [II]) after deproteinization. DNA from the same sample which then underwent the depurination and hydrolysis steps (Fig. 2, Ch.f) migrated as three components: (1) supercoils and (2) two components migrating faster than supercoils. The slower of these corresponds to single strands of SV40 DNA and the faster to a compact denatured form probably analogous to denDNA I (Grossman, Watson and Vinograd, 1974), produced by treating the supercoiled DNA with alkali and loading directly onto the gel without a renaturation step. Following a renaturation step, however,

FIGURE 2. Agarose Gel Electrophoresis of Samples of DNA Derived from Virus which had been Incubated with and without DMS.

Samples in channels (a-g) were electrophoresed through a 1.5% agarose gel. Samples in channels (h) and (i) were electrophoresed through a discontinuous 1% (top half)-1.5% (bottom half) agarose gel. Electrophoresis was in the presence of 0.5 μg/ml EtdBr. I, II, and III refer to the native supercoiled, the nicked circular, and the linear forms of SV40 DNA respectively.

Channels (a) and (h) contain a mixture of unmethylated SV40 DNA I plus II electrophoresed as markers. Channel (b) contains SV40 DNA incubated at 37°C in the presence of DNase. Channel (c) contains the same sample of SV40 DNA incubated at 37°C but without DNase. Channel (e): DNA from DMS-treated virus. Channel (d): An aliquot of the same sample as in (e), which was subjected to the conditions for neutral depurination and alkaline hydrolysis. The bracket indicates a broad region of faintly fluorescent material (low molecular weight denatured DNA). Channel (g): DNA from virus which had not been treated with DMS. Channel (f): An aliquot of the same sample as in (g) which was subjected to the conditions for neutral depurination and alkaline hydrolysis. From top to bottom, respectively, the bands in (f) correspond to form I SV40 DNA, single strands of SV40 DNA, and a compact form of SV40 DNA analogous to denDNA I (Grossman, Watson and Vinograd, 1974). Channel (i): An aliquot of the same sample as in (f) which, after exposure to the conditions for depurination and hydrolysis, was ethanol precipitated, resuspended, and then incubated to permit renaturation.


DNA
VIRUS
+DMS
VIRUS
-DMS
VIRUS
-DMS
-DNase
+DNase
II
I
II
III
II
I
II
I
a b c
d e
f g
h i

this DNA sample did migrate as native intact SV40 DNA (forms I and II) as can be seen in Figure 2, Ch.i. Virus which had been treated with DMS yielded intact SV40 DNA after deproteinization (Fig. 2, Ch.e); however, the DNA was largely in the nicked circular and linear forms (II and III, respectively). After the depurination and hydrolysis steps the same sample migrated as a smear of small molecular weight material (Fig. 2, Ch.d, bracket), indicating that the DNA within the virus had indeed been methylated. In Figure 2, the channel (Ch.b) in which the DNase-treated SV40 DNA had been electrophoresed shows no fluorescent material, indicative that the DNase used to treat the virus samples was active. The control SV40 DNA (Fig. 2, Ch.c) which had been incubated (at 37°C) at the same time but without DNase, is undegraded.

From these experiments it is clear that treatment of SV40 virus with dimethylsulfate leaves the virus physically intact, and the viral DNA within does become methylated.

Determination of the Frequency of Methylation

In order to scan as much of the SV40 genome as possible it was desirable to methylate the DNA to an extent so that as many purines as possible could be resolved and detected on any one sequencing gel. Initially, a very rough determination of the extent of methylation was made by methylating both virus and naked DNA for varying times (2, 4, 6, 8, 10, 12, and 13.5 hours) at 0°C, followed by depurination, hydrolysis, and subsequent sizing of the resultant populations of fragments on denaturing gels. This provided only a very rough estimate of the extent of methylation for several reasons: (1) the populations of DNA were detected by ethidium staining, and the binding of ethidium to single-stranded

nucleic acids is not quantitative, (2) because of the first reason, no attempt was made to take quantitative photographs of the gels, and (3) as a result of the two preceding reasons, the centers of the distributions of intensity of ethidium fluorescence were judged by eye, rather than by densitometry. Having thus obtained a rough estimate, it would be possible to later correct or modify the amount of methylation based upon the degree of resolution obtained when the experiment was actually performed with the first set of restriction fragments.

Initially, however, many attempts to obtain a methylated restriction fragment, labeled with ^{32}P at only one 5' end, resulted in severe degradation of the DNA at one of the points before the purified fragment could be excised from a preparative gel. Experiments using combinations and permutations of the various incubation conditions indicated that the degradation was not a necessary consequence of any of them*. For whatever reason(s) the DNA was falling apart, it seemed desirable to reduce its fragility to some extent by reducing the extent of methylation. It therefore became necessary to determine more accurately the average degree of methylation.

Parallel samples of virus and of naked SV40 DNA were methylated at 0°C (see Experimental Procedures) for 1, 2, 4, 6 and 7 hours. The reactions were quenched; the samples were deproteinized by extraction with phenol, precipitated with ethanol, and subjected to conditions for neutral depurination followed by alkaline hydrolysis at depurinated sites. The samples were labeled at their 5'

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*Evidence has since accumulated that the severe degradation of the methylated samples often observed in the earlier experiments was largely a result of freezing the solutions of methylated DNA. No degradation was ever observed in any sample of methylated DNA which was stored either (1) at 0-4°C for up to 24 hours as a buffered aqueous solution or else (2) at -20°C for up to 2 weeks as an ethanol precipitate (i.e., the precipitated DNA was stored in the ethanol).

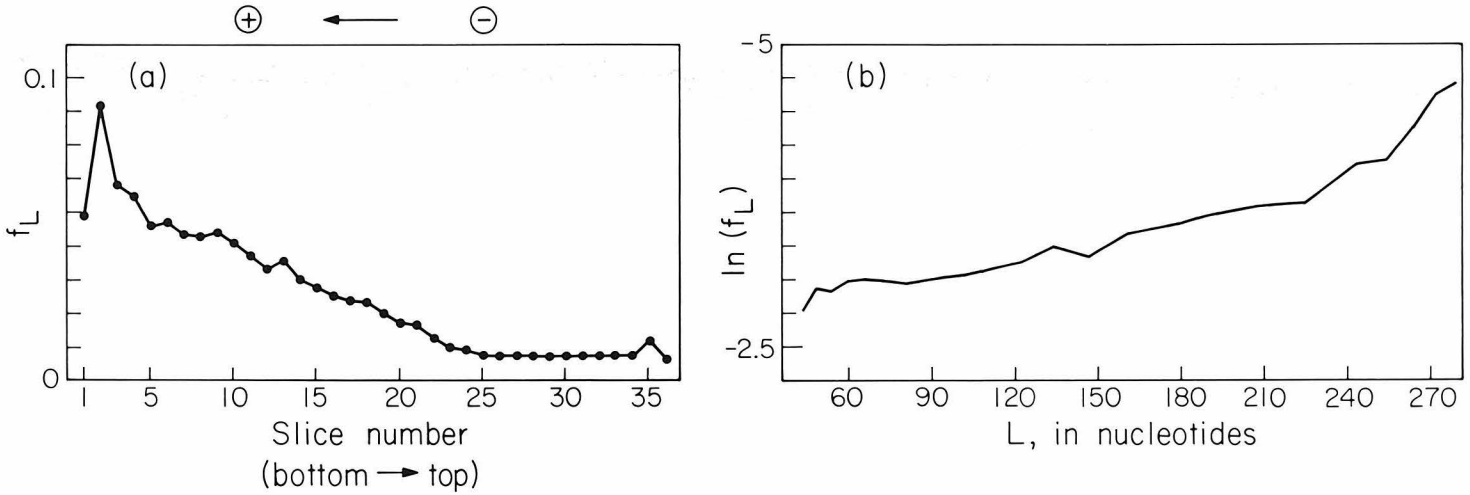
ends using [γ-^{32}P]ATP and T4 polynucleotide kinase according to the method of Berkner and Folk (1977). The samples were ethanol precipitated, resuspended in 5 mM Tris borate pH 8.3, 0.1 mM EDTA, 7 M urea, 0.05% XCFF, 0.05% BPB, and electrophoresed on a 1.5 mm x 33 cm x 40 cm 10% polyacrylamide (1:20 crosslink) gel containing 7 M urea and 2 x TBE (100 mM Tris borate pH 8.3, 2 mM EDTA) adjacent to kinased length standards (ØX174 RFI DNA digested with Hae III, ØX174 RFI DNA digested with Hind II, and SV40 DNA digested with Hae III). Electrophoresis was carried out at 800 V until the xylene cyanol (XCFF) marker reached the bottom of the gel. Therefore, fragments of approximately 35 nucleotides and less would have been electrophoresed off of the gel, in addition to any [γ-^{32}P]ATP and free ^{32}Pi (orthophosphate) remaining after ethanol precipitation of the kinased samples.

Following electrophoresis, several autoradiographs were made of the gel. While the kinased restricted fragments appeared as discrete bands on the gel, the fragments resulting from methylation of the whole SV40 genome appeared as smears of radioactivity in all cases. This is not unexpected considering the exponential increase in sequence heterogeneity with increase in length for any given length of DNA, when the DNA is degraded by a random process. (For a DNA of length L, the number of possible different sequences is equal to 4^L.)

The size standards were located and the channels containing the fragments from the various methylated samples were sliced and counted for Cerenkov radiation. For each sample, a calculation was made of the fraction of total counts in the channel contained within each slice (f_L). Examples of plots of f_L vs. gel slice for corresponding DNA and virus samples are shown in Figure 3 (a and c, respectively). The average size range covered by each gel slice was determined

FIGURE 3. Examples of Data from which the Frequency of Methylation was Determined.

Sections (a) and (b) and sections (c) and (d) represent material derived from samples of naked SV40 DNA and SV40 virus, respectively, which had been treated with DMS for 4 hours at 0°C. (a) and (c) are plots of f_L (the fraction of ^{32}P counts within a channel present in each gel slice) vs. slice number and hence, vs. average length L. The average length of DNA, L, in nucleotides represented by each slice was determined from size standards, such as those shown in Figure 4. (b) and (d) are plots of $\ln(f_L)$ vs. L, from which values for Xn, the number average molecular weight, were determined.



SV40 VIRUS, +DMS, 4 HRS, 0°C

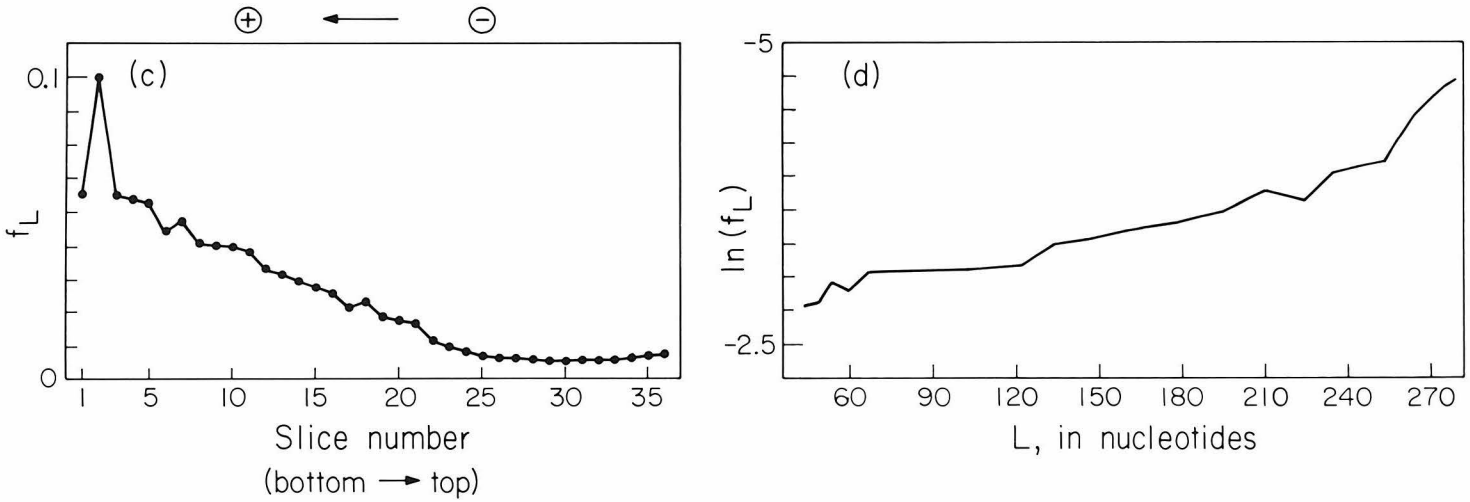
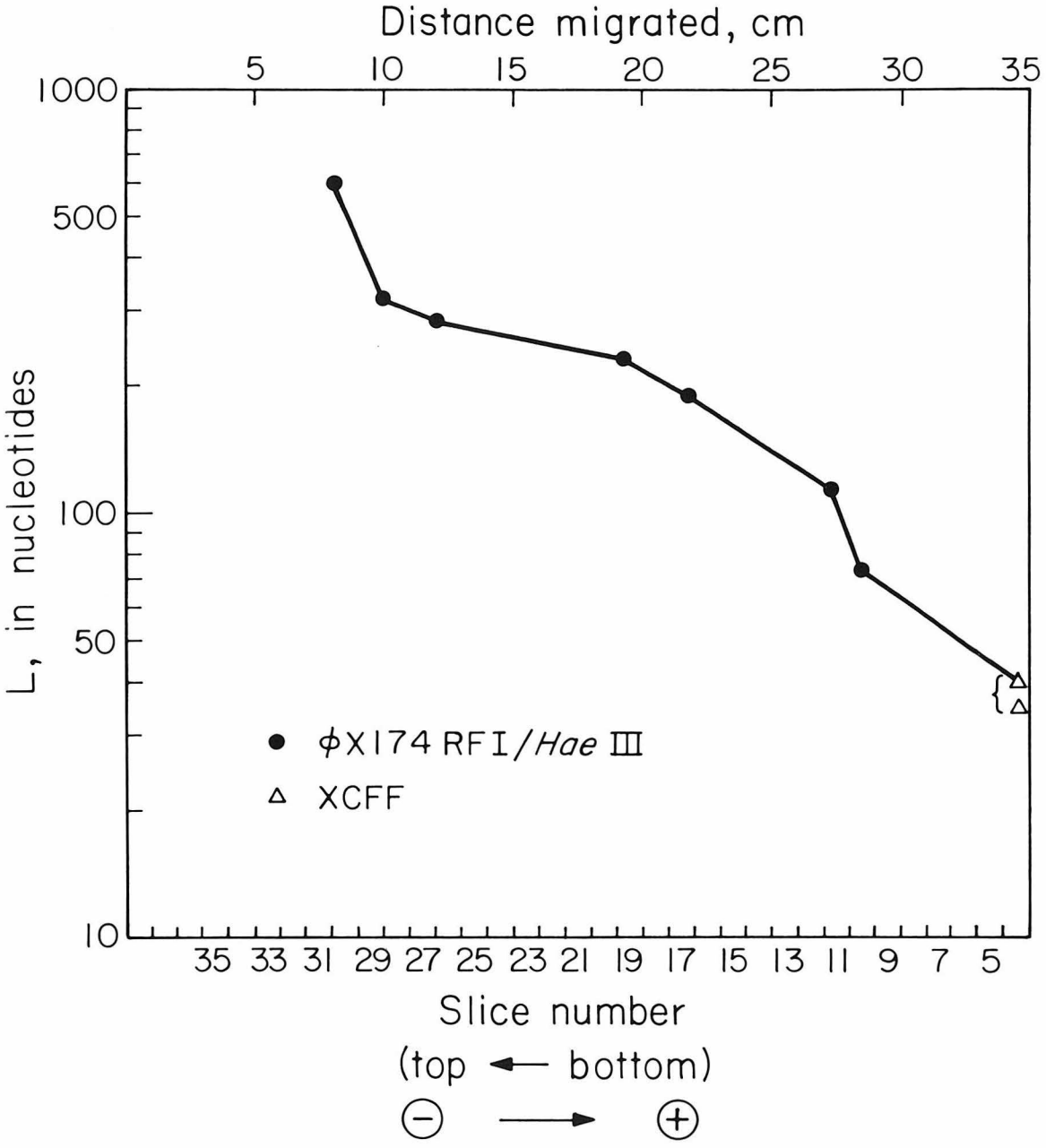


FIGURE 4. Graph of Length vs. Distance Migrated and vs. Slice Number for Size Standards Used in the Determination of the Frequency of Methylation.

The filled circles indicate ØX174 Hae III fragments (Lee and Sinsheimer, 1974; Sanger et al., 1977). The open triangles indicate the position of the XCFF dye which, under these electrophoresis conditions, comigrates with fragments approximately 35 nucleotides in length (A. Maxam, personal communication). Electrophoresis was at 800 V for 9 hours through a 10% polyacrylamide (1:20 crosslink) gel containing 7 M urea and 2 x TBE.


Distance migrated, cm
5
10
15
20
25
30
35
1000
500
100
50
10
L, in nucleotides
● ϕX174 RF I/Hae III
△ XCFF
35 33 31 29 27 25 23 21 19 17 15 13 11 9 7 5
Slice number
(top ← bottom)
⊖ ⟶ ⊕

from the size standards, whose length vs. slice number (and distance migrated) were plotted on a semi-logarithmic scale (Fig. 4). The plot was not linear; therefore, adjacent points were merely connected by straight lines and values for the average length of a DNA fragment in each slice were determined by interpolation.

Because the DNA fragments were end-labeled after depurination and hydrolysis, if it is assumed that the efficiency of kinasing is the same for all fragments regardless of sequence or size, a plot of the logarithm of the fraction of counts per slice ($\ln[f_L]$, which corresponds to the fraction of counts in fragments of length L) vs. the average chain length per slice (L), should yield a line having a slope of -1/Xn and a y intercept of ln (1/Xn), where Xn is the number average molecular weight (in nucleotides) of the population of fragments. This follows from the relationship for number average lengths of polymers given in the following equation:

$$fi = \frac{1}{Xn} e^{-Xi/Xn}$$

In the present case, fi is equivalent to f_L and Xi is equivalent to L. Such graphs were plotted for all samples; examples of these are shown in Figure 3b and 3d.

Values for Xn were determined in each case. No attempt was made to do least square linear fits of the points to a line; rather, the slopes were merely determined for lines drawn (by eye) through the points. The number average molecular weights (in nucleotides) for DNA from both virus samples and bare DNA samples are listed in Table I. As can be seen, the rate of methylation is similar for bare DNA samples and for virus samples which have been treated with DMS for the same amounts of time. In addition, the frequency of methylation does not increase proportionately with increasing reaction times. This is most

Table I

Methylation time (hours)	Xn (nucleotides)		Methylation frequency (x100) = % of nucleotides methylated ($\frac{100}{Xn}$)	
	Bare DNA	DNA within virus	Bare DNA	DNA within virus
1	308	284	0.32	0.35
2	213	208	0.47	0.48
4	156	144	0.64	0.69
6	121	123	0.83	0.81
7	97	sample lost	1.03	sample lost

probably due to the relative instability and hydrolysis of DMS in aqueous solutions (Merck Index, eighth edition) even at low temperature, although it could reflect a decrease in the number of purines available in the DNA for methylation.

Scanning the SV40 Genome: Purine Patterns Derived after Methylation

Based on the results of the previous experiment, an average methylation frequency of approximately 1 in 250 nucleotides was chosen, corresponding to a methylation time of 90 minutes at 0°C for both DNA and virus samples. Specific methylation conditions as well as the specific conditions for all subsequent preparative and analytical procedures are given in Experimental Procedures.

Parallel samples of naked SV40 DNA and SV40 virus were methylated, and the purified methylated DNAs were treated with a restriction endonuclease. The resulting fragments were labeled at their 5' termini with [γ-^{32}P]ATP and T4 polynucleotide kinase, and were then incubated with a second restriction endonuclease. The products were electrophoresed on a preparative gel (either agarose or polyacrylamide; exact electrophoresis conditions for each set of fragments are given in the legend to Figure 5), and the fragments which were labeled at only one 5' terminus, due to treatment with the second restriction enzyme, were excised and eluted from the gel. These fragments were then subjected to treatment for either neutral or acid depurination of methylated bases, followed by alkaline hydrolysis at depurinated sites. Resulting degradation products were then electrophoresed on polyacrylamide gels in the presence of 7 M urea, after which the gels were autoradiographed to locate the fragments. Examples of such autoradiographs are shown in Figures 6-11. In each pair of channels, adjacent samples were derived from corresponding restriction fragments prepared from

FIGURE 5. Summary of the Portions of the SV40 Genome Screened.

(1) The filled circle indicates the Eco RI cleavage site (0 on the physical map of SV40 DNA).

(2) The filled triangle indicates the origin of DNA replication which is 0.663-0.674 genome units clockwise from the RI site on the physical map. The numbers 0.663 and 0.674 are based on the published sequences of Fiers et al. (1978) and of Reddy et al. (1978), respectively.

(3) The thick lines indicate relevant positions of cleavage with the primary restriction endonuclease, and the thin lines indicate positions of cleavage with the secondary restriction endonuclease(s). The positions of cleavage indicated are not necessarily all of the sites for any enzyme; the only positions indicated are the ones relevant to the actual fragments screened. The capital letters in the headings above diagrams refer to the relevant fragment(s) cleaved. For information concerning the maps of SV40 DNA derived for various restriction enzymes see Morrow and Berg (1972); Mulder and Delius (1972); Danna, Sack and Nathans (1973); Sack and Nathans (1973); Sharp, Sugden and Sambrook (1973); Lebowitz, Siegel and Sklar (1974); Subramanian et al. (1974); Roberts (1976); Yang, Van de Voorde and Fiers (1976a, 1976b); Subramanian et al. (1977); Fiers et al. (1978); Reddy et al. (1978).

(4) The asterisks indicate the position of the 5'-^{32}P end-label.

(5) Concentric lines outside and inside the circles refer to the ^{32}P-labeled DNA strand as being complementary to late and to early SV40 mRNA, respectively.

In the following pages are the conditions (present in the format below) for preparative gel electrophoresis for the different digests.

Type of gel and percentage

Dimensions: thickness x width x length

Voltage, time, temperature

Unless otherwise stated, all polyacrylamide gels were cast using a ratio of Bis acrylamide/total acrylamide of 1:30, w/w (i.e., 1:30 crosslink).

Following the electrophoresis conditions are the lengths of the excised fragments (in nucleotides) which are based on the sequence published by Reddy et al. (1978) for SV40 strain 776. cE and cL refer to the ^{32}P-labeled DNA strand as being the strand complementary to early and to late SV40 mRNA, respectively.

Eco RI/Hae III B

7% polyacrylamide

0.4 cm x 15 cm x 30 cm

150 V,23 hours, 2-4°C

large Eco RI/Hae III B : 478 : cE

small Eco RI/Hae III B : 278 : cL

Eco RI/Bam HI

1% agarose

0.8 cm x 15 cm x 15-16 cm

50 V, 17 hours, 2-4°C

large Eco RI/Bam HI : 4493 : cL

small Eco RI/Bam HI : 751 : cE

Bam HI/Hae III C

7% polyacrylamide

0.4 cm x 15 cm x 30 cm

150 V, 23 hours, 2-4°C

large Bam HI/Hae III C : 277 : cL

small Bam HI/Hae III C : 267 : cE

The values for the lengths above are opposite in magnitude to the actual situation for SV40 strain 777 used in the present experiments. Because there are no sequence data available for strain 777, the absolute lengths of the two fragments are not known; however in the DNA used for these experiments, the large Bam HI/Hae III C fragment represents the DNA strand complementary to early SV40 mRNA (cE) and the small Bam HI/Hae III C fragment represents the strand complementary to late SV40 mRNA (cL).

Bam HI/Eco RI

1% agarose

0.8 cm x 15 cm x 15-16 cm

50 V, 16.5 hours, 2-4°C

large Bam HI/Eco RI : 4493 : cE

small Bam HI/Eco RI : 751 : cL

Hpa II/Hind III C

4% polyacrylamide

0.4 cm x 15 cm x 15-16 cm

100 V, 11.5 hours, 2-4°C

large Hpa II/Hind III C : 699 : cE

small Hpa II/Hind III C : 399 : cL

Hpa II/Eco RI

1% agarose

0.8 cm x 15 cm x 15-16 cm

50 V, 17 hours, 2-4°C

large Hpa II/Eco RI : 3807 : cL

small Hpa II/Eco RI : 1435 : cE

Taq I/Alu I B

7% polyacrylamide

0.4 cm x 15 cm x 30 cm

150 V, 23 hours, 2-4°C

large Taq I/Alu I B : 388 : cE

small Taq I/Alu I B : 97 : cL

Hha I/Hpa I + Eco RI

discontinuous agarose: upper two-thirds (∿10 cm), 1% agarose; lower third (∿5-6 cm), 2% agarose

0.8 cm x 15 cm x 15-16 cm

75 V, 6.5 hours, 2-4°C

large Hha I/Hpa I B : 1573 : cL (redundant to both large Hpa II/Eco RI and small Hpa II/Hind III C)*

Hha I/Eco RI : 947 : cE (equivalent to large Hha I/Hpa I A)

small Hha I/Hpa I A : 312 : cL

small Hha I/Hpa I B : 155 : cE (redundant to both small Hpa II/Eco RI and large Hpa II/Hind III C)*

Hinf I (A + D)/Hpa II + Taq I

discontinuous polyacrylamide: upper two-thirds[(a)] or half[(b)] (∿20 cm[(a)] or 15 cm[(b)]), 7% polyacrylamide (1:30 crosslink); lower third[(a)] or half[(b)] (∿10 cm[(a)] or 15 cm[(b)]), 15% polyacrylamide (1:20 crosslink)

0.4 cm x 15 cm x 30 cm

150 V, 32 hours[(a)] or 50 hours[(b)], 2-4°C

small Hinf I A/Hpa II : 437 : cE

large Hinf I D/Taq I : 397 : cL

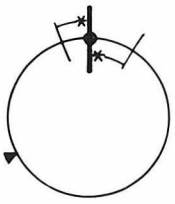
small Hinf I D/Taq I : 172 : cE

---

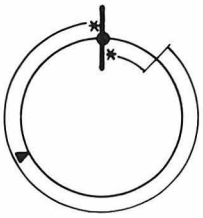
*See Discussion.

In the DNA from strain 777 used in the DMS experiments, the large Hinf I D/Taq I fragment is slightly larger than the small Hinf I A/Hpa II fragment.

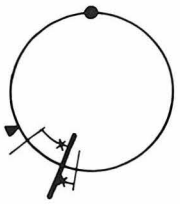
Eco RI/*Hae* III B



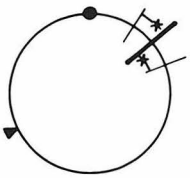
Eco RI/*Bam* HI



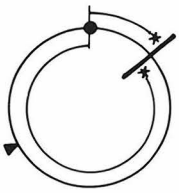
Taq I/*Alu* I B



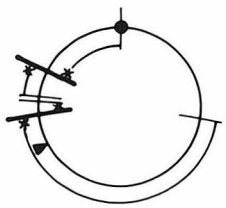
Bam HI/*Hae* III C



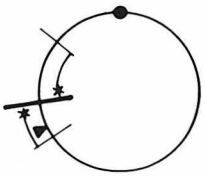
Bam HI/*Eco* RI



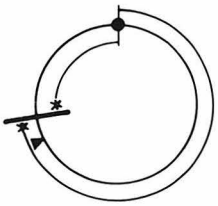
Hha I/*Hpa* I + *Eco* RI



Hpa II/*Hin* d III C



Hpa II/*Eco* RI



Hin f I (A + D)/*Hpa* II + *Taq* I

- ● *Eco* RI site, O on physical map
- ▲ Origin of DNA replication, 0.663–0.674 on physical map
- * 5' ^{32}P end label
- ——— Primary restriction cut (s)
- — Secondary restriction cut (s)

FIGURES 6-11. Purine Patterns Derived from Corresponding Samples of DMS-Treated DNA and DMS-Treated Virions.

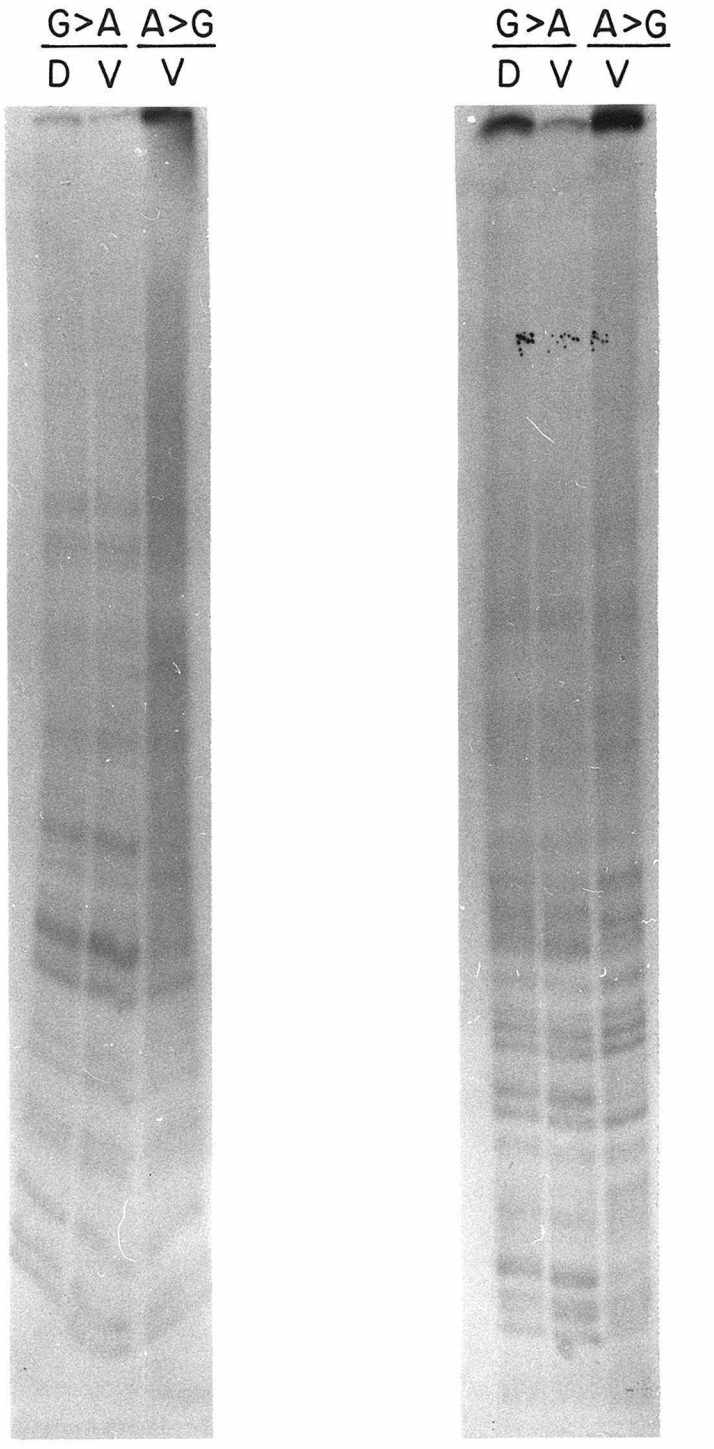
V and D indicate that the sample was derived from naked DNA and from virions, respectively. G>A and A> G refer to relative intensities of bands following neutral and acid depurination at methylated bases, respectively.

In Figures 10 and 11, 5'-^{32}P end-labeled fragments (determined by sequencing to be 187 and 111 nucleotides in length) were gifts from J. Posakony.

FIGURE 6. Hpa II/Eco RI.

Electrophoresis was through a 10% polyacrylamide gel at 1000 V until the XCFF reached the bottom of the gel (7 hours).

Hpa II / *Eco* R I



cE

Small fragment

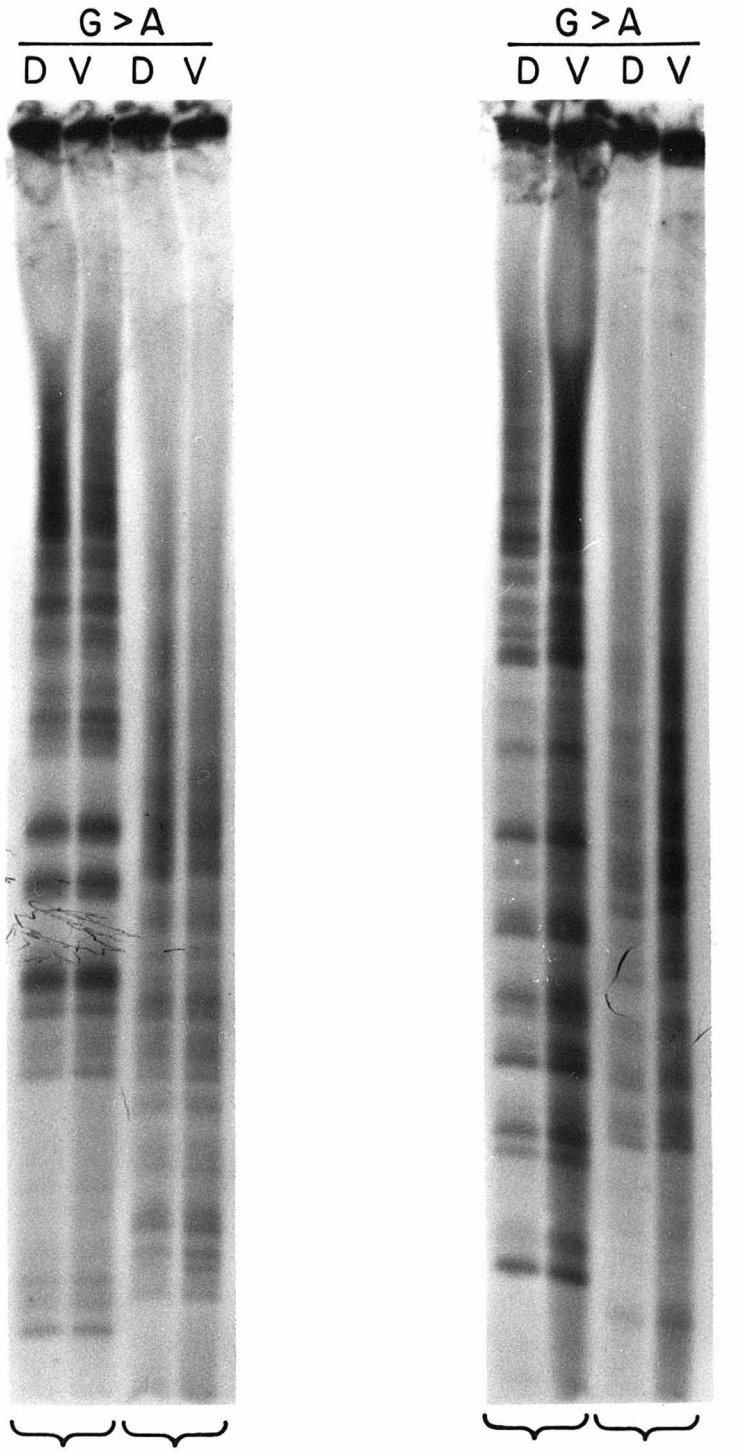
cL

Large fragment

FIGURE 7. Hpa II/Hind III C.

Electrophoresis was through a 10% polyacrylamide gel at 800 V. Aliquots of the samples were applied at two different times such that electrophoresis was for 11 hours and 6 hours.

Hpa II/*Hin* d III C



cL

Small fragment

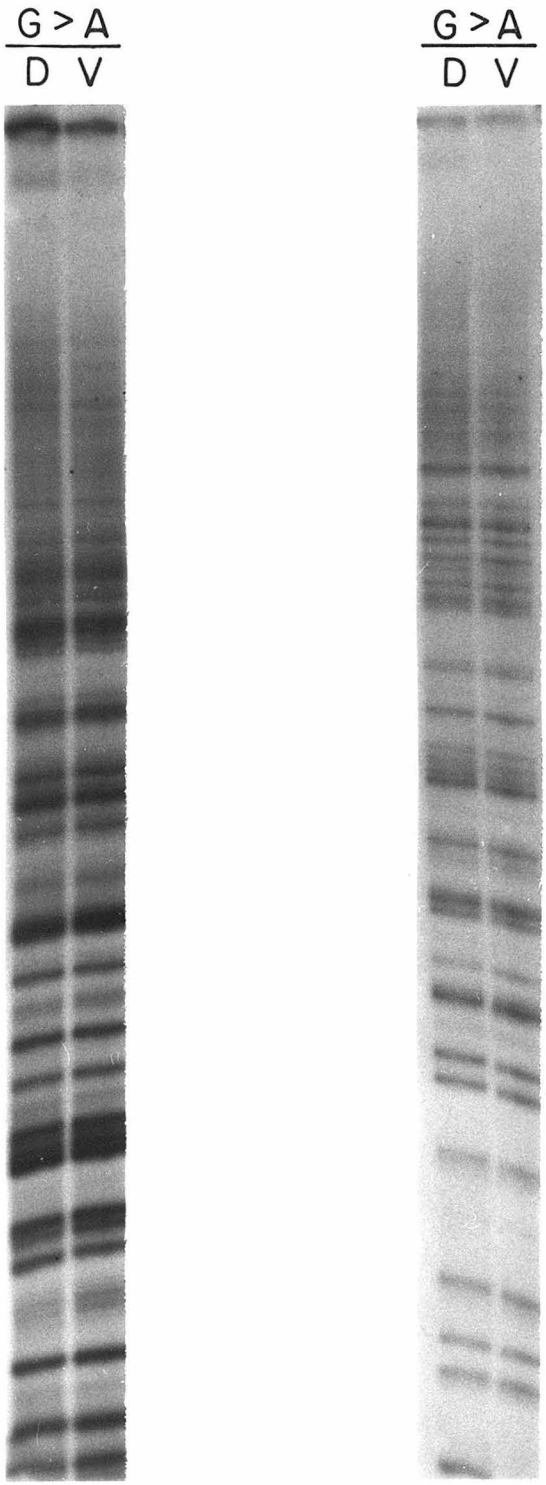
cE

Large fragment

FIGURE 8. Eco RI/Bam HI.

Electrophoresis was through a 10% polyacrylamide gel at 1000 V until the XCFF reached the bottom of the gel (7 hours).

Eco R I / *Bam* H I



cE

Small fragment

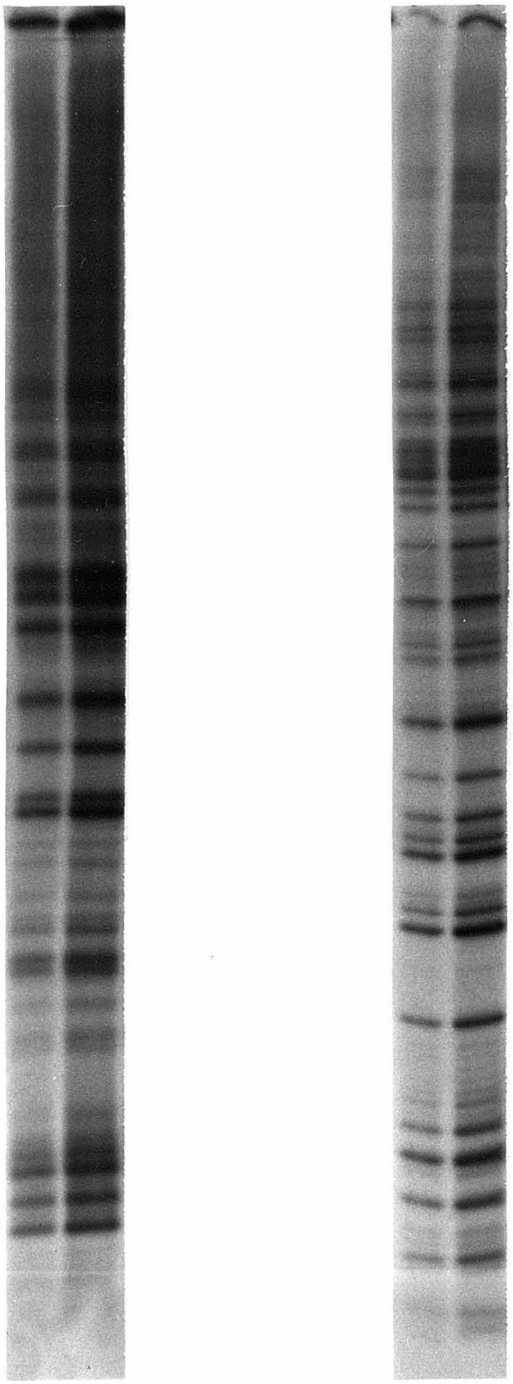
cL

Large fragment

FIGURE 9. Bam HI/Eco RI.

Electrophoresis was through a 10% polyacrylamide gel until the XCFF was approximately 4-5 cm from the bottom (8-8.5 hours).

Bam HI/*Eco* RI



cL cE

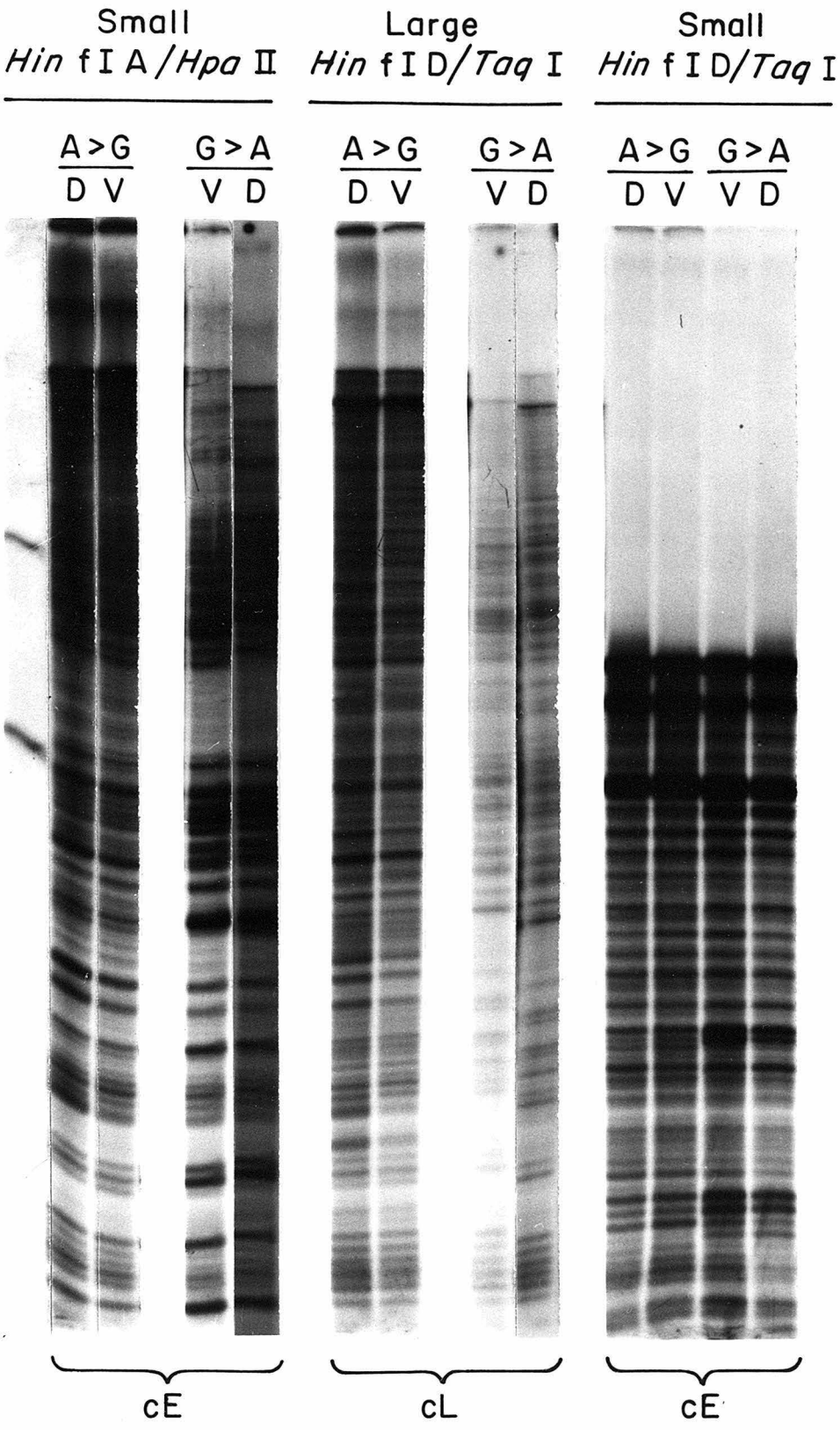
Small fragment Large fragment

FIGURE 10. Hinf I (A + D)/Hpa II + Taq I.

Electrophoresis was through a 10% polyacrylamide gel at 800 V until the XCFF was 4-5 cm from the bottom (8-8.5 hours).

The very left-hand slot contains ^{32}P-labeled fragments of 187 and 111 nucleotides in length (upper and lower, respectively).

Although the patterns are the same in the 4th and 5th slots from the left (i.e., small Hinf I A/Hpa II, G>A, V and D), the bands do not line up exactly because the samples were electrophoresed under identical conditions but on two different gels. The same is true for the samples in the 8th and 9th slots from the left (i.e., large Hinf I D/Taq I, G>A, V and D).



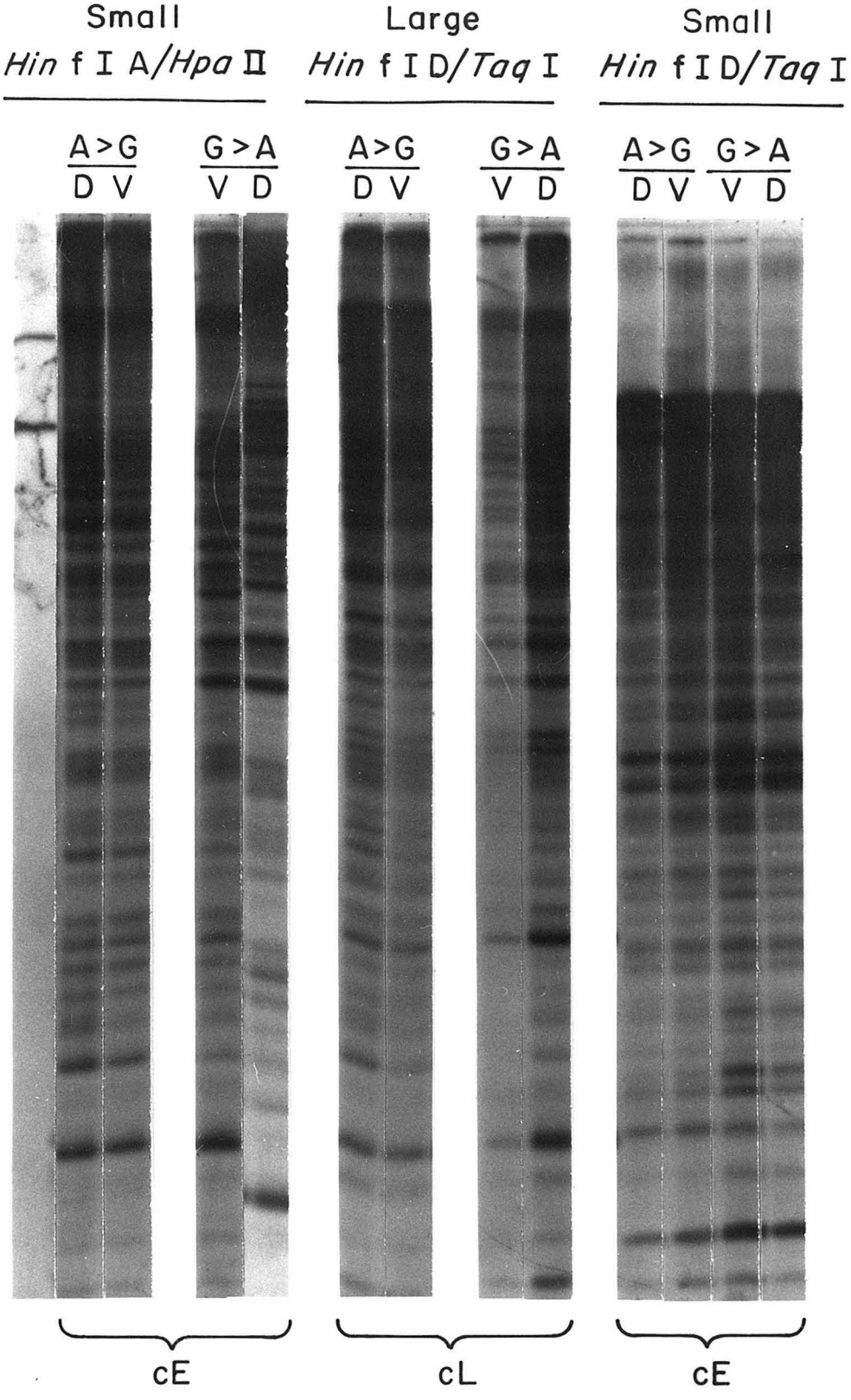
10% Acrylamide

FIGURE 11. Hinf I (A + D)/Hpa II + Taq I.

Electrophoresis was through a 20% polyacrylamide gel at 1000 V until the BPB was approximately 1 cm from the bottom of the gel (9-9.5 hours).

The end slot on the left contains ^{32}P-labeled (kinased) fragments of 187 and 111 nucleotides in length (top and bottom, respectively).

Although the patterns in the 4th and 5th slots from the left (i.e., small Hinf I A/Hpa II, G>A, V and D) are the same, the bands are not perfectly aligned because the samples were electrophoresed under the same set of conditions but on different gels.



20% Acrylamide

aliquots of bare SV40 DNA and SV40 virus which had been methylated and subsequently treated in parallel.

It can be seen in Figures 6-11 that there are no apparent differences between methylation patterns obtained from corresponding restriction fragments prepared from parallel samples of naked SV40 DNA and from intact virions. A total of 15 segments of the SV40 genome (obtained by kinasing 15 different 5' termini produced by digestion of the DNA with different restriction endonucleases) was screened using this method. The fragments represented both strands of the DNA and were chosen to obtain samples representative of the entire genome, with a particular emphasis on fragments covering and surrounding the origin(s) of DNA replication and both late and early RNA transcription. Diagrams of the sites are shown in Figure 5. In no case were any major differences in methylation patterns observed, although increases or decreases in the relative levels of methylation which resulted in as low as a 2-fold difference in the relative intensity of a band could have been detected easily for any but the very minor bands in a purine pattern.

DISCUSSION

An Estimate of the Proportion of the SV40 Genome which was Screened

On different occasions, when the DNA was kinased at the same restriction site but was digested subsequently with different secondary restriction endonucleases, the purine patterns obtained after depurination and hydrolysis were essentially the same and usually could be easily aligned on the autoradiographs of the respective sequencing gels (e.g., Bam HI/kinase/Hae III vs. Bam HI/kinase/Eco RI, and Eco RI/kinase/Hae III vs. Eco RI/kinase/Bam HI). Alignment of the Hpa II patterns shown

in Figure 6 (Hpa II/kinase/Eco RI) and in Figure 7 (Hpa II/kinase/Hind III C) however, is hindered by the fact that in both cases the sequencing gels ran anomalously.

Occasionally, there were minor differences in the purine patterns observed after processing corresponding fragments obtained from samples of methylated naked DNA and methylated virus. Such differences were either (1) the presence or absence of very faint bands, (2) slight intensity differences in the corresponding bands in parallel DNA and virion samples, or (3) a smear of material which occurred in a region of the purine pattern in only the DNA or the virus sample, but not in both. These differences, however, were not reproducible from experiment to experiment. In addition, when experiments were done using restriction fragments which overlapped, any slight differences which might have appeared in patterns derived from one set of fragments were not present in the patterns derived from the other set of overlapping fragments and therefore were probably artifactual. Major differences were never observed in purine patterns from corresponding DNA and virion samples.

Length standards (^{32}P-labeled DNA fragments of known size) were sometimes electrophoresed on the sequencing gels adjacent to the depurinated and hydrolyzed samples (e.g., Figs. 10 and 11). From these, it can be estimated that lengths up to approximately 300 nucleotides from the ^{32}P end-label could be screened using both a 20% and a 10% polyacrylamide gel, although resolution decreased markedly in the region of the gel displaying lengths of roughly 250-300 nucleotides. If only a 10% gel were used, electrophoresis conditions resulted in loss of approximately the first 20-30 nucleotides proximal to the ^{32}P end-label. Several restriction fragments covered the same region of the SV40 genome. Only 4 fragments (two sets of two), however, both (1) covered the same region

of the genome in the first 300 nucleotides from the ^{32}P end-label and (2) represented the same strand of the DNA. These fragments are the ones generated by kinasing the single Hpa II site and the Hha I site which is proximal to the origin of DNA replication. In fact, in the published sequence (Fiers et al., 1978; Reddy et al., 1978) of SV40 DNA (strain 776) it can be seen that these 2 restriction sites overlap as follows:

```
                                       _______ Hpa II
                              Hha I _______
Origin of                  5' ... G C G C C G G ...  3'  cE
DNA replication  ......    3' ... C G C G G C C ...  5'  cL
```

The strand labeled cL is the strand complementary to late SV40 mRNA. The strand labeled cE is complementary to early mRNA; the 5' terminal G in this strand is nucleotide 261 in the sequence published by Reddy et al. (1978), and nucleotide 324 in the sequence of the late region published by Fiers et al. (1978).

A derivative of SV40 strain 777 was used in the DMS experiments reported here. Since there are apt to be sequence differences between strains based upon the observation of occasional differences in patterns obtained after digestion with some restriction enzymes, the exact positions of the Hpa II site and the nearest Hha I site may differ slightly in strain 777 from those published for strain 776. Patterns obtained after gel electrophoresis of SV40 DNA which had been digested with the above enzymes, however, indicates that the two sites are quite close (if not overlapping) in strain 777 (personal observations).

Three of the 15 restriction fragments employed were smaller than 300 nucleotides. One of these, however, was one of the two redundant Hha I fragments mentioned above. Assuming that 300 nucleotides of each fragment were resolved by the sequencing gels, as well as eliminating the two Hha I fragments which exhibited redundancy to the two Hpa II fragments, and using the actual lengths

of the two remaining fragments which were smaller than 300 nucleotides, it can be calculated that a total length of approximately 3600 nucleotides was examined. Because either of the two strands of the DNA could be involved in an interaction with a protein, SV40 DNA must be considered to have a genome size of approximately 10,400 nucleotides rather than one of 5200 base pairs. In light of this, the experiments employing DMS reported here have screened 35% of the SV40 genome.

Possible Interpretations

Thus far, in situations where there is both physical and genetic evidence that a protein binds specifically to a DNA sequence, striking differences in the purine patterns have been observed after methylation of a DNA fragment containing that sequence in the presence and absence of bound protein. The differences occur within the sequence itself, and mutations which alter the binding of the protein to the DNA have been shown in some cases to correlate well with changes in the comparative methylation (purine) patterns. Successful experiments of this type have been done using a purified DNA fragment containing an operator sequence(s) (i.e., the E. coli lac operator, the left and right operators of bacteriophage λ) to which the corresponding purified repressor (i.e., the lac repressor, the λ repressor or the λ cro protein) has been bound in vitro (Gilbert, Maxam and Mirzabekov, 1976; Humayun, Kleid and Ptashne, 1977; Johnson, Meyer and Ptashne, 1978). The λ repressor (cI gene product) and the λ cro protein act in an antagonistic manner (see Lewin, 1977) in the determination of the mode of growth of λ (i.e., the establishment or maintenance of lysogeny vs. lytic infection or the induction of a lysogen). Both proteins act by binding to the left and right

operators of λ DNA. Methylation studies (Humayun et al., 1977; Johnson, Meyer and Ptashne, 1978) have revealed that the cro protein binds to a subset of the operator sequences to which the cI protein binds, and therefore results of these experiments should prove to be quite helpful in fully understanding mechanisms of regulation at the molecular level.

In light of the positive results obtained from experiments employing DMS (mentioned above), since no convincing differences were observed between the purine patterns from any of the corresponding restriction fragments derived from naked SV40 DNA and from intact SV40 virions, it seems unlikely that any of the proteins within the virion interact with the virion DNA in a sequence-specific manner. This implies that although histones may be arranged on the DNA in a regular fashion in terms of spacing, they are not bound to a set of 20-21 specific 140-200 base-pair-long DNA sequences within the whole population of virus particles. This implication is in agreement with conclusions reached from experiments in which isolated SV40 minichromosomes were treated with various restriction endonucleases (Polisky and McCarthy, 1975; Cremisi et al., 1976; Cremisi, Pignatti and Yaniv, 1976; Ponder and Crawford, 1977), despite the fact that objections can be raised to drawing conclusions based upon the results of some of these experiments in terms of various possible artifacts.

Experiments have been done which indicate that, in vitro, SV40 T antigen binds to SV40 DNA (either specifically or preferentially) at or near the origin of DNA replication. The most elegant of these experiments used a protein (related to SV40 T antigen) isolated from cells infected with an adeno-SV40 hybrid virus (Tjian, 1978). When the protein was bound to purified SV40 DNA in vitro, three contiguous specific segments of the DNA near the origin of replication were

protected from digestion with DNase. Evidence to date, however, indicates that SV40 T antigen is not present within SV40 virus particles. Therefore, differences in methylation pattern due to bound T antigen cannot be expected to result after treating SV40 virions with DMS.

There have been several publications concerning proteins which are found bound (both covalently and noncovalently) to SV40 DNA after disruption of virions by particular methods. In all cases, the protein(s) was (were) located in the vicinity of the origin of DNA replication. Mapping of the bound protein(s) was done using a combination of restriction endonucleases and electron microscopy or else using a combination of restriction endonucleases and gel electrophoresis. The complex (protein bound noncovalently to supercoiled SV40 DNA) reported by Griffith et al. (1975), to be salt-stable but SDS-sensitive was localized at 0.7 ± 0.05 map units clockwise from the Eco RI cleavage site on the physical map of SV40 DNA. The complex (protein bound covalently to nicked circular SV40 DNA) reported by Kasamatsu and Wu (1976a, 1976b) to be stable in solutions of high salt, SDS, alkali, 4 M guanidine HCl, 3.86 M hydroxylamine (pH 4.23), and 98% formamide was localized at 0.67 genome units clockwise from the Eco RI site. Based upon structures observed in the electron microscope following denaturation and subsequent renaturation of the DNA to which the protein was attached, Kasamatsu and Wu (1976b) concluded that within the population of complexes, the scission in the DNA could be on either strand of the DNA but that the position of the scission on one strand was displaced from that on the other strand by a few hundred nucleotides. The sequence of SV40 DNA in the vicinity of the origin of replication contains both perfect and imperfect tandem repeats, a palindrome and several sequences which have 2-fold rotational symmetry (Subramanian,

Dhar and Weissman, 1977; Subramanian, Reddy and Weissman, 1977). It is therefore quite possible that the protein interacts with one of these special sequences, although the halves of the sequences which are known to possess 2-fold rotational symmetry are closer together than the observed spacing of the staggered nicks reported by Kasamatsu and Wu (1976b).

Although much effort was placed on screening the purines in the region of the SV40 genome including and flanking the positions at which the various protein complexes were localized, and therefore covering a broad portion of the genome surrounding and including the origins of DNA replication and both late and early RNA transcription, no differences in methylation patterns were ever observed. It would appear, therefore, that within intact virions, the proteins found in the above complexes do not interact with the DNA in a specific manner. Because the identities and function(s) of these reported proteins are largely unknown, both from a physical and from a genetic standpoint, the possibility cannot be eliminated that the isolated protein-DNA complexes are artifactual. It is possible that there is a specific interaction within the virion but that the bound protein does not interact with the DNA in a manner which would alter the methylation pattern. It is also possible that the interactions between these proteins and the DNA occur only upon lysis of the virus, but not within intact virions.

The reported bound proteins were localized only roughly, i.e., they were localized to a region of the DNA but were never shown (in a rigorous biochemical manner) to be bound to a specific unique DNA sequence. If, in fact, it were the case that a protein or a complex of proteins were always bound to a certain portion of the genome, but not to a unique sequence within that portion, it is unclear what results could be expected from methylation experiments. This

reservation applies equally to the interpretation of methylation patterns in terms of the proteins reported by Griffith et al. and by Kasamatsu and Wu, as well as to the interpretation of methylation patterns in terms of histones and the question of nucleosome-phasing.

The results of Ponder and Crawford (1977) concerning the accessibility of restriction sites in polyoma (and in SV40) minichromosomes are germane to this reservation. From the gross accessibility of sites, they concluded that nucleosomes are positioned randomly overall. Experiments were also performed in which minichromosomes were first digested with micrococcal nuclease to produce monomers (~140 base pairs of DNA plus the histone octamer), following which the monomers were deproteinized and the resultant DNA was then incubated with a restriction endonuclease which cleaved the viral genome only once. This resulted in the appearance of 10 fragments, each shorter than the length of the monomer DNA. The authors concluded from this that nucleosomes can occupy a small number of distinct alternative positions. In addition, the authors reported that micrococcal nuclease has preferred cleavage sites within naked DNA, resulting in the appearance of discrete bands after electrophoresis. They concluded, however, that the site preference exhibited by micrococcal nuclease was not the explanation for the 10 fragments derived from nucleosomes.

If nucleosomes do in fact occupy a distinct number of alternative sites within the DNA, differences between the purine patterns obtained after DMS treatment of both virus and naked DNA might have been expected to occur at each of the possible sites.

If, however, the total of all of the possible sites included the entire genome, and if histones protected all of the purines within each site to an equal extent, the methylation patterns would appear random.

A random pattern would again have been expected even if the sites did not include the entire genome, but if at each site there were different possible nucleosome conformations in which the histones interacted with the same DNA sequence but were in contact with different sets of purines depending upon the conformation.

EXPERIMENTAL PROCEDURES

Foreword

The methods employed in the growth and purification of nondefective SV40 virus as well as in the purification of virion SV40 DNA are those published by Tai et al. (1972).

The procedures described in detail in the following sections for the methylation reactions, for the elution of DNA from preparative polyacrylamide gels, for both the neutral (G>A) and the acid (A>G) depurination reactions, for alkaline hydrolysis at depurinated sites, and for the preparation and electrophoresis of sequencing gels are those published by Maxam and Gilbert (1977) or else are adaptations thereof.

References to protocols for the purification of various restriction endonucleases, in addition to information concerning enzyme digestion conditions, can be found in the review by Roberts (1976), as well as in the current catalogues from both New England Biolabs, Inc. (Beverly, Mass.) and Bethesda Research Laboratories, Inc. (Rockville, Md.).

Growth and Purification of Virus

TC-7 cells were grown on 9 cm Petri dishes in the presence of 1 μCi/ml

^{3}H-Lys (New England Nuclear, see below) and 10% dialyzed fetal calf serum (Irvine Scientific). The medium (TC-7) in which the cells were grown contained only half the normal amount of cold lysine. When the cultures reached approximately 95% confluence, they were infected at a multiplicity of 0.01 pfu/cell with a stock of twice plaque-purified SV40 (small plaque strain, sp12, a derivative of strain 777). At approximately 24-36 hours, 48-60 hours, and 72-84 hours post-infection a mixture of ^{3}H-Lys (L-lysine monohydrochloride [4,5-^{3}H(N)]-, 60-80 Ci/mMole, New England Nuclear) and ^{14}C-dT (thymidine [2-^{14}C]-, >50 mCi/mMole, New England Nuclear) was added to the infected cultures to final concentrations (per addition) of 0.33 μCi/ml and 0.034-0.067 μCi/ml, respectively. Virus was harvested when a full cytopathic effect was observed (10-14 days post-infection at 37°C). The medium and cell debris from each dish were scraped into Erlenmeyer flasks. The lysates were then frozen (-70°C, dry ice/ethanol bath) and thawed (37°C) four times. After the final thaw, the lysates were centrifuged at 2300 rpm in an IEC centrifuge at room temperature for 10 minutes to pellet the cell debris. The supernatants were decanted and discarded, and the pellets (containing most of the virus) were resuspended in TD buffer (0.14 M NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 25 mM Tris HCl pH 7.4) using approximately 1 ml of buffer per initial dish of cells. To disperse clumps, aliquots of the resuspended debris (approximately 25 ml each) were homogenized in a 40 ml Dounce homogenizer, using 10-20 strokes with a B (tight-fitting) pestle. The suspensions were then adjusted to a final concentration of 1% (w/v) Na deoxycholate, and were incubated for 10-20 minutes at room temperature with occasional mixing, after which solutions of crude pancreatic DNase (Sigma Chemical Co.) and ribonuclease A (Sigma Chemical Co.) were added to final concentrations of 200 μg/ml and 500 μg/ml, respectively.

Incubation was continued at room temperature for 30 minutes. The suspensions were then centrifuged at 10,000 rpm, 4°C, for 15 minutes in a Sorvall SS-34 rotor to remove large particulate material. The supernatants were decanted and were warmed to room temperature to prevent precipitation of the KBr. Approximately 23-24 ml of supernatant plus additional TD buffer were then layered onto a 15 ml cushion of a solution of saturated KBr in TD buffer. Six of these step gradients (made in cellulose nitrate SW27 tubes) were used for every 100 Petri dishes of infected cells. Gradients were centrifuged in a Beckman SW27 rotor at 20°C, 23,000 rpm for 3 hours. Following centrifugation, three opalescent bands were visible within approximately one centimeter below the buffer/KBr interface. The lowest band corresponds to wild-type SV40 virions; the middle band corresponds to a small amount of defective virions produced during virus growth and multiplication; and the uppermost band corresponds to "shells" or empty virion capsids. The band of virus was collected by puncturing the bottoms of the tubes. Pooled virus was dialyzed at 4°C against TD buffer. The sample was then adjusted to a final concentration of 10 mM $MgCl_2$ and crude (pancreatic) DNase I was added to a final concentration of 200 μg/ml. Digestion was at 37°C for 30 minutes, after which the sample was placed on ice and EDTA (pH 8.0) was added to a final concentration of 50 mM.

Solid CsCl was then added to a final density of 1.32 gm/cc and the samples were placed in cellulose nitrate tubes to facilitate visualization of viral bands following centrifugation. Centrifugation was in a Beckman Ti50 rotor, at 35,000 rpm, 16-20°C for 36-48 hours. Following centrifugation, opalescent bands were visible. The one of greatest buoyant density, corresponding to intact virus particles, was collected by dripping the tubes from the bottoms. Yields of virus varied

between 230 μg to 400 μg of virus per dish of infected cells. Specific activities ranged between 700-1500 cpm/μg for total virion protein (^{3}H) and between 270-870 cpm/μg for viral DNA (^{14}C). Purified virus was stored in CsCl at 2-4°C.

Purification of DNA

Aliquots of the purified virus preparation were dialyzed against TD buffer. 20% SDS was then added to the sample to a final concentration of 1% and the sample was incubated at 37°C for 30 minutes to permit complete disruption of the virions. Solid CsCl was added to a final concentration of 1 M, after which the sample was placed at 0°C (on ice) for a minimum of 30 minutes to facilitate precipitation of both the Cs dodecylsulfate and protein dodecylsulfate complexes. The precipitate was removed by centrifugation for 20 minutes at 4°C, 15,000 rpm in a Sorvall SS-34 rotor. The supernatant, containing the DNA, was carefully decanted and allowed to warm to room temperature, after which additional solid CsCl was added to a final density of 1.55 gm/cc and a solution of ethidium bromide (EtdBr, Calbiochem) was added to a final concentration of 300 μg/ml (Radloff, Bauer and Vinograd, 1967). Centrifugation was at 35,000 rpm, 18-20°C, in a Beckman SW50.1 rotor for 36-48 hours. Gradients were visualized with a long-wavelength ultraviolet light. In each gradient the band of supercoiled DNA (having the highest buoyant density) was collected by puncturing the bottom of the tube. Essentially all of the DNA in each gradient was supercoiled (form I); upper bands consisting of forms II and III (nicked circles and linears, respectively) were barely detectable when the DNA was extracted from freshly purified virus. EtdBr was removed from the pool of supercoiled DNA by multiple (∿5) extractions with water-saturated n-butanol containing approximately 1% (w/v) Na sarkosyl. CsCl

was then removed by dialysis against 10 mM Tris HCl pH 7.8, 1 mM EDTA, after which the DNA was stored at -20°C.

Enzymes and [γ-^{32}P]ATP

Bacteria (Arthrobacter luteus, Escherichia coli RY13 endo$^-$, Haemophilus aegyptius, Haemophilus influenzae Rd exo$^-$ and Thermus aquaticus YT-1) were grown in preparative quantities from strains obtained from R. J. Roberts. Restriction endonucleases Alu I, Hae III, Hind III, and Eco RI were prepared (with occasional modifications) according to protocols devised or adapted at Cold Spring Harbor Laboratories (see Roberts, 1976). In addition, aliquots of Hind III were purchased from New England Biolabs. Taq I was prepared (with slight modifications) according to a procedure obtained from M. Kamoramy. Hinf I was a gift from A. Efstratiadis. All other restriction endonucleases were purchased from New England Biolabs.

Polynucleotide kinase (from T4-infected E. coli) was purchased either from PL Biochemicals or from Boehringer-Mannheim.

[γ-^{32}P]ATP was synthesized by the method of exchange reactions described by Glynn and Chappell (1964), as detailed in the sequencing protocols of Maxam and Gilbert (1977). Both crystalline rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (GAPDH, A grade, in 2.5 M ammonium sulfate) and crystalline yeast 3-phosphoglycerate kinase (3-PGK, A grade, in 2.7 M ammonium sulfate, 0.04 M Na_4 pyrophosphate) were purchased from Calbiochem. Following synthesis, ATP was not purified away from unincorporated orthophosphate (^{32}Pi).

Methylation

Aliquots of purified SV40 DNA and of purified SV40 virus containing

an equivalent amount of DNA were dialyzed into DMS buffer (50 mM Na cacodylate pH 8.0, 10 mM $MgCl_2$, 0.1 mM EDTA) at 4°C. The amount of bare DNA was determined from the A_{260}; the amount of virus was determined from the relationship that an A_{260} of 10 corresponds to approximately 2 mg/ml of total viral protein and approximately 0.36 mg/ml of total viral DNA. The samples (one of DNA and one of virus) were then placed in polypropylene tubes and the volumes were adjusted (with additional DMS buffer) to give a final concentration of DNA (or of DNA within virus) of 50 µg/ml. The tubes of DNA and of virus, in addition to a tube of 10.7 M DMS (99 + %, Gold Label, Aldrich Chemical Co., Inc.) were placed on ice for at least 30 minutes to permit thorough chilling. The DMS was quickly added to each sample to a final concentration of 53 mM (1 µl DMS per 200 µl reaction mixture). Each sample was mixed and replaced on ice for the prescribed methylation time (anywhere from 1-18 hours). Ultimately, 1.5 hours at 0°C was chosen for all methylations of DNA and of virus used in actual comparisons of purine patterns from corresponding restriction fragments.

Methylation reactions were terminated by the addition of 0.25 volumes of ice-cold 5X DMS stop solution (1.0 M Tris Ac, 1.5 M NaAc, 1.0 M 2-mercaptoethanol, 50 mM $MgAc_2$, 1 mM EDTA, pH 7.5). The samples were immediately extracted two times with equal volumes of buffer-saturated phenol (containing 0.1% 8-hydroxyquinoline); the aqueous phases were extracted with water-saturated ether to remove traces of phenol, after which 2.5 to 3 volumes of 95% EtOH were added. Each sample was mixed, placed at -20°C for at least 30 minutes, and the precipitated DNA was then collected by centrifugation at 0-4°C. Precipitates were resuspended in 0.3 M NaAc, 10 mM Hepes (Sigma Chemical Co.) pH 7.5, 1 mM EDTA and the DNA was again precipitated with ethanol. The samples

were stored at -20°C as the second ethanol precipitate until just prior to digestion with the primary restriction endonuclease.

The precipitates were collected by centrifugation (as above). Residual ethanol was evaporated by leaving the tubes containing the pellets open at room temperature. The dried precipitates were then resuspended in 10 mM Hepes pH 7.5, 1 mM EDTA to give a final DNA concentration of 100-700 μg/ml.

Restriction Endonuclease Digestions

Small volumes of concentrated stock solutions (containing buffer, salts, and reducing agents) were added to the resuspended DNAs to yield the following final concentrations of components required for digestion with the respective restriction endonuclease:

Alu I: 6 mM $MgCl_2$, 6 mM BME, 6 mM Hepes pH 7.5

Bam HI: 0.1 M NaCl, 7 mM $MgCl_2$, 6 mM BME, 5-10 mM Hepes pH 7.5

Eco RI (or RI): 50 mM NaCl, 7 mM $MgCl_2$, 0.1 M Tris HCl, pH 7.4

Hae III: 6 mM $MgCl_2$, 6 mM BME, 6 mM Hepes pH 7.5

Hha I: 50 mM NaCl, 6 mM $MgCl_2$, 6 mM BME, 6 mM Hepes pH 7.5

Hind III: 60 mM NaCl, 7 mM $MgCl_2$, 5-10 mM Hepes pH 7.5

Hinf I: 50 mM NaCl, 6 mM $MgCl_2$, 6 mM BME, 6 mM Hepes pH 7.5

Hpa I: 6 mM $MgCl_2$, 6 mM BME, 6 mM Hepes pH 7.5

Hpa II: 6 mM $MgCl_2$, 6 mM BME, 6 mM Hepes pH 7.5

Taq I: 6 mM $MgCl_2$, 6 mM BME, 6 mM Hepes pH 7.5

All digestions were at 37°C for 60-90 minutes. Longer incubation times were avoided as were higher temperatures in view of the fragility of the methylated

DNA samples. Most digestions were done using roughly a 2-fold excess of enzyme/DNA in terms of units/μg except in cases where the site density in SV40 DNA for a given enzyme was grossly different from the site density in λ DNA, on which most commercially obtained enzymes are titered. (One unit equals the amount of enzyme needed to obtain complete digestion of 1 μg of λ DNA at 37°C in 1 hour.)

In the case of Hind III from New England Biolabs, it was necessary to use at least a 10-fold excess of enzyme/unmethylated* SV40 DNA in order to obtain complete digestion. Because the dinucleotide, 5'...CpG...3', is infrequent in most eukaryotic DNAs (Josse, Kaiser and Kornberg, 1961; Swartz, Trautner and Kornberg, 1962; Subak-Sharpe et al., 1966; Morrison et al., 1967), the density of Taq I sites in SV40 DNA is low. SV40 DNA has only one Taq I site (Roberts, 1976; Fiers et al., 1978; Reddy et al., 1978), while the number of sites in ØX174

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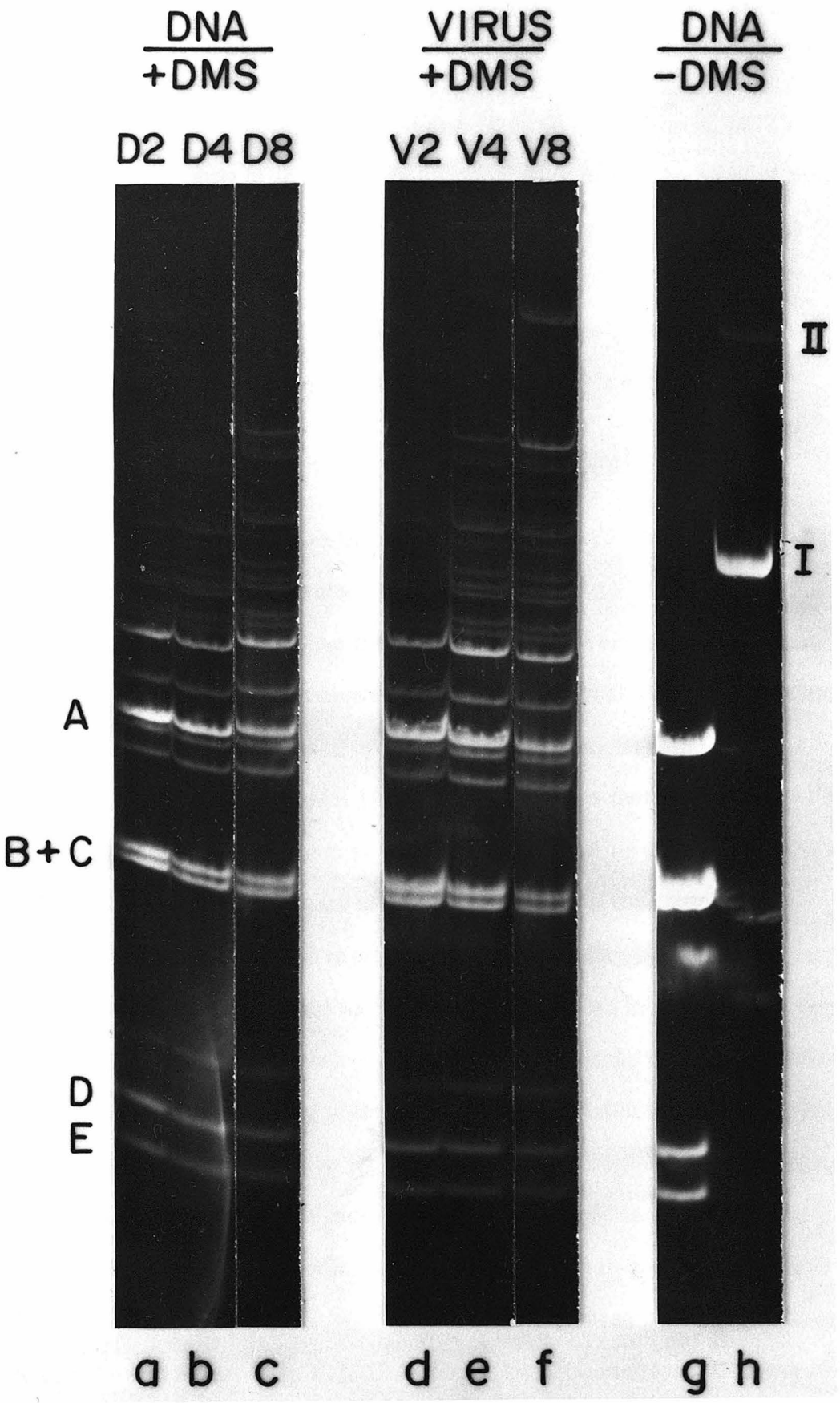
*Before use enzymes were usually titered using unmethylated SV40 DNA as a substrate. When an amount of enzyme, which was known to yield complete digests of unmethylated DNA, was incubated with the same quantity of methylated DNA, partial digestion products were obtained. The amount of partial digestion products present increased with increasing degrees of methylation (i.e., increasing DMS reaction times). An example of this is shown in Figure 12. No attempt was made to quantitate the proportion of partials present in any sample. However, the following calculation can be made to estimate the fraction of any particular restriction enzyme site which, due to methylation, is no longer available for digestion.

Assume, for example, that most methylations are of guanines rather than of adenines. If SV40 DNA is treated with DMS to yield an average fragment size of 250 nucleotides following depurination and hydrolysis, this corresponds to methylation of $1/(250/4) = 0.016 = p$ of all G's in the DNA. The fraction of G's in the DNA which are not modified is therefore equal to $(1-p)$. A Hind III site contains two G's, one on either strand of the DNA. If modification of either of the G's in a Hind III site is sufficient to block that site from digestion, then the probability of inactivating a particular Hind III site is equal to the sum $(2p-p^2)$ of the fraction of that site in which only one G is modified $[2(1-p)p]$ plus the fraction of that site in which both G's are modified (p^2). For low values of p, therefore, the probability of inactivating a particular Hind III site is approximately equal to 2p.

FIGURE 12. The Effect of Methylation of DNA on the Degree of Digestion by Restriction Endonucleases.

Channel (h) contains supercoiled (I) and nicked circular (II) SV40 DNA. Channel (g) contains a sample of unmethylated SV40 DNA digested with Hind III. A, B + C, D, and E are the five largest SV40 Hind III fragments; the smallest fragment (F) was electrophoresed off of the bottom of the gel. Channels (a-f) contain samples (1 μg each) of methylated DNA which had been incubated with an amount of Hind III capable of completely digesting approximately five times as much unmethylated SV40 DNA under the same incubation conditions. D2, D4, and D8 (channels a, b, and c) contain samples of DNA which had been methylated at 0°C for 2, 4, and 8 hours, respectively. V2, V4, and V8 (channels d, e, and f) contain samples of DNA from virus which had been methylated at 0°C for 2, 4, and 8 hours, respectively.

Electrophoresis was through a 1% agarose gel in the presence of 0.5 μg/ml EtdBr.


DNA
+DMS
VIRUS
+DMS
DNA
-DMS
D2 D4 D8
V2 V4 V8
II
I
A
B+C
D
E
a b c
d e f
g h

and λ DNAs are 10 and > 30, respectively (Sato, Hutchison and Harris, 1977). Despite this, vast excesses of Taq were required when digesting samples of methylated SV40 DNA because the incubations were at 37°C where the activity of Taq is at least 10-fold lower than it is at 70°C (personal observations).

Following digestion, the samples were first extracted with phenol and then with ether, after which the DNA was precipitated with ethanol.

End-labeling with T4 Polynucleotide Kinase

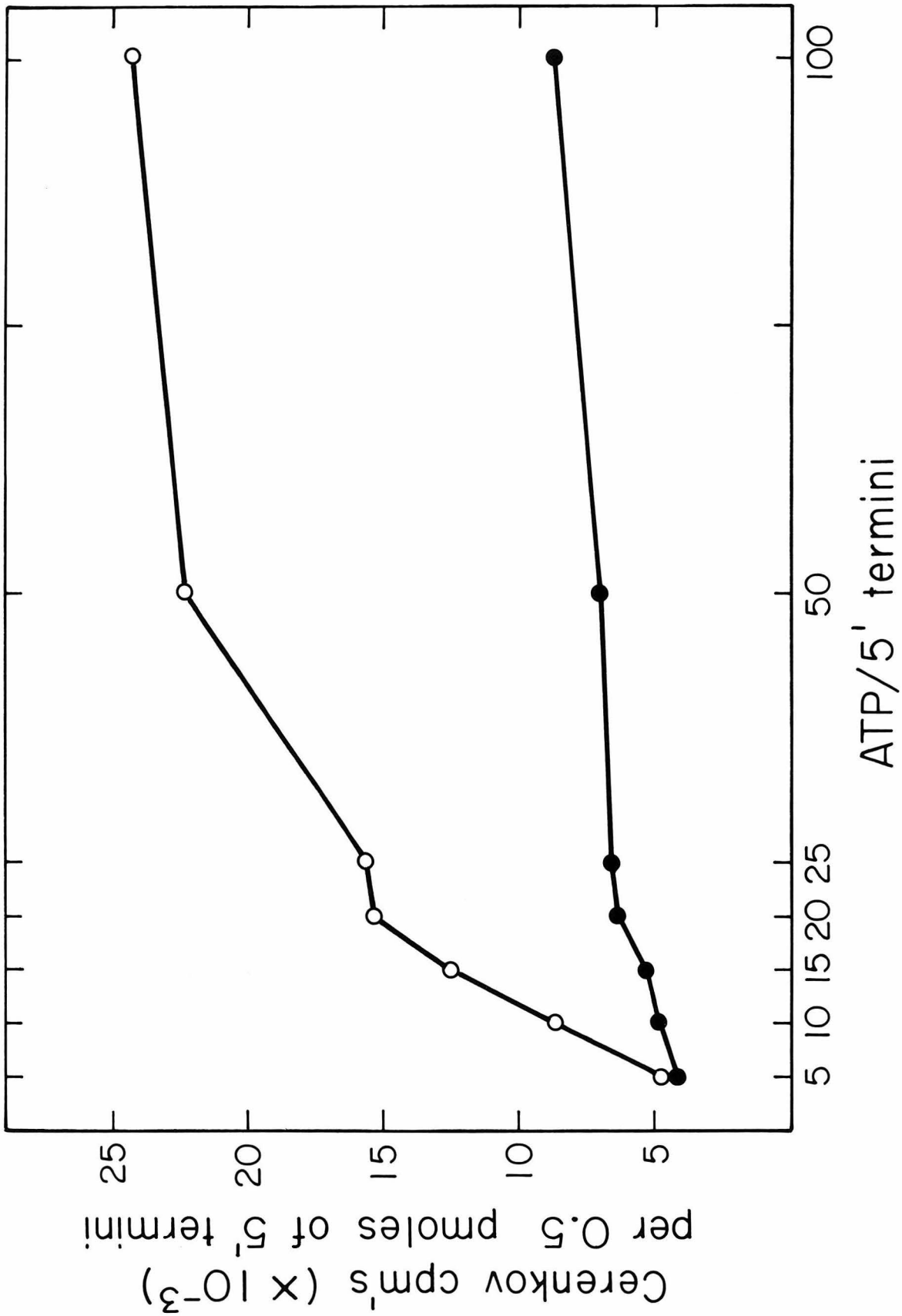
DNA fragments were end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase using conditions for the exchange reaction published by Berkner and Folk (1977). End-labeling was done using the kinase-exchange reaction, rather than by direct (forward) kinasing after treatment of the DNA with bacterial alkaline phosphatase. This choice was based upon reported data (A. Maxam, personal communication; Maxam and Gilbert, 1977) which indicated that (1) incorporation obtained using the forward kinase reaction for duplex DNA or undenatured RNA was not very high and that (2) levels of incorporation using the direct kinase reaction could be increased if the sample were first denatured. Due to the fact that the samples to be end-labeled were already methylated (and therefore were somewhat fragile), denaturation using either heat or alkali did not seem prudent.

In a series of tests using plasmid DNA (pSM1; Mickel and Bauer, 1976) which had been linearized with Eco RI, the conditions of Berkner and Folk gave significantly (3- to 5-fold) higher incorporation of ^{32}P onto the RI ends than the kinase exchange reaction conditions of Chaconas, van de Sande and Church (1975) when the same amounts of [γ-^{32}P]ATP and RI-treated plasmid DNA were used under both conditions (Fig. 13). Under both sets of conditions, incorporation

FIGURE 13. Comparison of Conditions for End-Labeling Using the Kinase Exchange Reaction.

Plasmid DNA, psM1 (Mickel and Bauer, 1976) which had been linearized with Eco RI, was end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase under two different sets of conditions for the kinase exchange reaction. Parallel sets of reactions were conducted using varying ratios of ATP/5' termini. After terminating the reactions, identical (0.85 µg = 0.5 pmoles of 5' termini) amounts of DNA were withdrawn from each sample and were electrophoresed through a 1% agarose gel in the presence of 0.5 µg/ml EtdBr to separate the DNA from the unincorporated [γ-^{32}P]ATP and free ^{32}Pi. Following electrophoresis, the gel was illuminated with UV light and the band of DNA in each channel was excised and was then counted for Cerenkov radiation.

Open circles indicate samples kinased using the conditions of Berkner and Folk (1977): 50 mM Imidazole HCl pH 6.6, 50 mM KCl, 20 mM $MgCl_2$, 5 mM DTT, 100 µg/ml autoclaved gelatin, 320 µM rADP, 10 µM ATP. Filled circles indicate samples kinased using conditions similar to those of Chaconas, van de Sande and Church (1975): 50 mM Tris HCl pH 7.6, 6 mM $MgCl_2$, 10 mM DTT, 10 µM rADP, 10 µM ATP. Reactions were at 37°C for 90 minutes in the presence of 40 units/ml T4 polynucleotide kinase.


Cerenkov cpm's (× 10⁻³)
per 0.5 pmoles of 5' termini
25
20
15
10
5
5 10 15 20 25
50
100
ATP/5' termini

increased with increasing ratios of ATP/5' termini in the DNA. This effect, however, was more pronounced when the conditions of Berkner and Folk were employed.

The precipitated DNA resulting from incubation with the first restriction endonuclease was collected by centrifugation and was then resuspended in a small volume (20-50 µl) of 10 mM Tris HCl pH 7.4, 1 mM EDTA. Water and a stock of concentrated kinase reaction mixture were added to adjust the sample to the following final concentrations:

50 mM Imidazole HCl pH 6.6

50 mM KCl

20 mM $MgCl_2$

5 mM DTT

100 µg/ml autoclaved gelatin

320 µM rADP

The entire sample was then used to resuspend a dried aliquot of [γ-^{32}P]ATP such that the final ATP concentration was at least 10 µM and such that the ratio of ATP/5' ends in the DNA was at least 15-fold, but preferably 50-fold or greater (see preceding paragraph). Typical reaction volumes were 50-250 µl for anywhere from 15-60 µg of restricted DNA (equivalent to 8.82-35.2 pmoles of 5' ends, depending upon the number of fragments generated by treatment with the primary restriction endonuclease). T4 polynucleotide kinase was added to a final concentration of 40 units/ml and the samples were incubated for 15 minutes at 37°C. Reactions were terminated by the addition of SDS to a final concentration of 0.1%. The samples were then passed over either G-50 fine or G-25 superfine Sephadex columns (11 cm long x 0.7 cm internal diameter, equilibrated in 10 mM Tris HCl

pH 7.8, 1 mM EDTA) to purify the kinased DNA away from the bulk of the unincorporated [γ-^{32}P]ATP and the free ^{32}Pi which was present in the sample of ATP. The excluded peaks were collected and the volume of each was reduced to approximately 200-300 µl by extraction with sec-butanol (Stafford and Bieber, 1975) after which each concentrated sample was precipitated with ethanol.

Secondary Restriction Enzyme Digestions

After collecting the precipitated DNA, each kinased sample was incubated with one or more restriction endonucleases to produce DNA fragments having only one 5' terminus labeled with ^{32}P. Digestions were carried out as described previously. If more than one restriction enzyme was used for this secondary digestion, incubation was done simultaneously if the reaction mixtures for the enzymes were similar, or else sequentially if the reaction mixtures were quite different. Between sequential reactions, the sample was extracted with phenol and then with ether, after which the reaction mixture was corrected to conditions required by the next enzyme by the addition of needed components.

Reactions were terminated by extracting the samples with phenol. Traces of phenol were removed by extraction with ether. Residual ether was allowed to evaporate by placing the tubes (open) at 37°C for several minutes. The samples were adjusted to a final concentration of 10 mM EDTA (pH 8.0), after which a mixture of glycerol plus dyes (50% glycerol, 0.05% BPB, 0.05% XCFF) was added.

Corresponding samples obtained from bare DNA and from virus were layered either onto parallel preparative slots on one gel or else onto preparative slots of two identical gels. An aliquot of cold unmethylated SV40 DNA which

had been restricted with the same enzymes as the actual samples was usually electrophoresed in an analytical slot adjacent to the preparative slot(s) on each gel. This served as a reference when excising bands from the gel(s).

Preparative Gel Electrophoresis

Modified vertical slab gel electrophoresis apparati (Aquebogue or Watson Products) were used for gels either 15 cm or 30 cm in length. Preparative gels were cast from either agarose (Seakem ME, Marine Colloids, Inc.) or polyacrylamide (Bio-Rad) depending upon the sizes of the DNA fragments to be resolved. When polyacrylamide gels were used, an even surface of polyacrylamide ($\leq$2 cm below the top of the shorter gel plate) was made by layering H_2O-saturated iso-butanol on top of the solution of acrylamide after it had been poured. Following polymerization of the gel, the iso-butanol was removed and the polyacrylamide surface was rinsed with H_2O and blotted dry. The very top of the gel (into which the slot-former was inserted) was cast from 1-1.5% agarose to facilitate formation of even preparative slots.

Both the gel and the electrophoresis buffer contained 40 mM Tris acetate pH 7.8, 5 mM NaAc, 1 mM EDTA. Electrophoresis was at 2-4°C (to minimize exposure of the methylated DNA to unnecessarily high temperatures) and at 3-10 V/cm for varying lengths of time depending upon the porosity of the gel and the sizes of the DNA fragments. Gels were usually pre-electrophoresed for several hours (1-3 hours) under conditions identical to those ultimately employed after applying the samples. Electrophoresis buffer was recirculated in all cases; therefore, DEAE cellulose paper (Whatman Chromatography Paper, DE 81) was placed in the bottom buffer reservoir (anode) to adsorb any [γ-^{32}P]ATP and free ^{32}Pi remaining in the kinased samples.

Specific electrophoresis conditions for each set of fragments (including the type of gel, voltage, and electrophoresis times) are given in the legend to Figure 5.

Following electrophoresis, bands were localized (1) by autoradiography (at 4°C or at room temperature) of the wet gel using Kodak No-Screen film or Kodak XR-5 film and/or (2) by visualization of the gel(s) with ultraviolet light after staining them in a solution containing 1 μg/ml EtdBr. Fragments which were labeled at only one 5' terminus, due to digestion with the secondary restriction enzyme(s), were excised from each gel. The content of ^{32}P in each fragment was determined by counting each excised gel slice for Cerenkov radiation.

Elution of DNA Fragments from Gels

Slices of agarose containing DNA fragments were frozen and thawed three times. The agarose was then homogenized with a Dounce homogenizer using a B (tight fitting) pestle. During homogenization, a solution of 0.5 M NH_4Ac, 10 mM $MgAc_2$, 0.1% SDS, 0.1 mM EDTA (gel elution solution) was added to each sample. The homogenate was transferred to a 30 ml polycarbonate tube and additional elution solution was added so that the ratio of the volume of elution solution/initial volume of the agarose slice was at least 5. At this point either tRNA in double-distilled H_2O (∿50 μg) or sonicated calf thymus DNA in double-distilled H_2O (∿40 μg) was added to and mixed in with each suspended homogenate. These amounts of carrier were used for volumes of homogenate plus solution in the range of 6-12 ml.

The capped tubes were placed in a 37°C warm-room for at least 12 hours to permit the DNA to diffuse out of the agarose. The bulk of the agarose

was then pelleted by centrifugation in a Beckman Type 30 rotor at 22,000 rpm at 20°C for 1 hour. The supernatants were decanted into polyallomer SW27 tubes after which 2.3-3 volumes of ice-cold 95% EtOH were added to each.

Slices of polyacrylamide containing DNA fragments were placed in plastic 5 ml pipettor tips which had been plugged with silanized glass wool after sealing the pointed end of each tip with a small flame. The gel slice was crushed to a "not-too-fine paste" (A. Maxam, personal communication) using a silanized fire-polished glass rod with a rounded end. Gel elution solution was then added to each crushed gel slice so that the ratio of the volume of added solution to the volume of the gel slice was at least 6. At this point, carrier (either tRNA or sonicated calf thymus DNA) was added to each homogenate as described for the homogenates of agarose gel slices. Each sample was stirred. The pipettor tips were sealed with parafilm and were then placed in a 37°C warm-room for at least 12 hours to permit the DNA to diffuse out of the polyacrylamide. Following incubation at 37°C, the flame-sealed point of each tip was punctured with a hot syringe needle. The liquid was then forced through the plug of glass wool and the paste of polyacrylamide by one of two procedures. (1) Each tip was placed in a tube into which only the lower part of the tip fit. This assembly was centrifuged for approximately one hour at 2300 rpm at room temperature in an IEC centrifuge. (2) Each tip was clamped above a tube and pressure was applied to the liquid within by connecting the top of each pipettor tip to a stream of filtered air. In either case, the polyacrylamide paste and glass wool were rinsed by placing additional gel elution solution (approximately 1/3-1/2 the volume used initially) in each tip and forcing it through by one of the two methods described above.

Each initial eluate and the corresponding rinse were placed in a polyallomer SW27 tube, after which 2.3-3 volumes of ice-cold 95% EtOH were added.

Following the steps above for elution out of either type of gel, each sample was mixed and placed at -20°C for at least 1 hour. Precipitated kinased fragments (plus carrier tRNA or carrier calf thymus DNA) were collected by centrifugation at 0°C for 2 hours at 23,000 rpm in a Beckman SW27 rotor. After drying each pellet, the DNA was resuspended in 100-200 µl of a solution of 10 mM Tris HCl pH 7.8, 1 mM EDTA. The resuspended samples were transferred to 1.5 ml polypropylene tubes and each was then counted for Cerenkov radiation. Recovery of DNA from the gel was in the range of 30-70%, depending upon both the size of the DNA fragment and the type of gel used. If there were sufficient ^{32}P counts in a sample, the sample was divided in two. In this case, half of the sample would later undergo acid depurination (A>G) and the other half would undergo neutral depurination (G>A). In samples in which there were fewer ^{32}P counts, the entire sample was used for the neutral depurination reaction.

NaAc was added to each tube to a final concentration of 0.3 M, after which 3 volumes of 95% EtOH were added. After mixing and chilling to -20°C (or to -70°C), precipitates were collected by centrifugation in an Eppendorf centrifuge (12,000 x g, 4°C, 15-30 minutes).

Depurination and Hydrolysis

G>A:

Dried precipitates were resuspended in 20 µl of 20 mM NaPi pH 7.0, 1 mM EDTA. Depurination proceeded at 90°C for 15 minutes after which the samples were placed on ice. When cooled, 1 N NaOH was added to an approximate

final concentration of 0.1 N (i.e., 2 µl 1 N NaOH/20 µl sample) and the mixtures were taken up in silanized drawn melting point capillaries (Kontes Glass Co.). The capillaries were sealed using a small flame and were then submerged in a 90°C bath for 30 minutes for the alkaline hydrolysis step. (Dermicel First Aid Tape [Johnson and Johnson] is excellent for labeling multiple capillaries to be submerged in 90°C H_2O. When wrapped around a capillary and then stuck to itself, this tape does not come off at elevated temperatures.)

A>G:

Alternatively, dried precipitates were resuspended in 20 µl of double-distilled water, after which they were placed on ice in the cold-room to permit thorough chilling. An ice-cold solution of 0.5 N HCl was added to a final concentration of 0.1 N (i.e., 5 µl 0.5 N HCl/20 µl sample). The sample was vortexed and replaced on ice. Incubation was at 0°C for a total of 2 hours, and each sample was vortexed in the cold every 15 minutes. Depurination was terminated by the addition of 250 µl of an ice-cold solution of 0.3 M NaAc, 10 mM Hepes pH 7.5, 1 mM EDTA followed by the addition of 3 volumes of ice-cold 95% EtOH. After at least 30 minutes at -20°C, precipitates were collected by centrifugation. The resulting pellets were washed with 1 ml of ice-cold 95% EtOH, after which they were dried and resuspended in 20 µl of 0.1 N NaOH, 0.1 mM EDTA. The mixtures were taken up in silanized melting point capillaries (Kontes Glass Co.) and were subjected to the conditions for alkaline hydrolysis at depurinated sites described for the neutral depurination reactions (i.e., submersion in 90°C H_2O for 30 minutes).

Following alkaline hydrolysis, the capillaries were removed from the 90°C bath and placed at room temperature to cool. The contents of the capillaries

were transferred to 1.5 ml tubes. One of the two following things was then done: (1) An equal volume of a solution of 10 M urea (Schwarz/Mann, Ultra Pure), 0.05% BPB, 0.05% XCFF was added. (2) 100-150 µl of a solution of 0.3 M NaAc, 10 mM Hepes pH 7.5, 1 mM EDTA was added. Occasionally, additional carrier was added at this point (5-10 µg of a solution of tRNA in H_2O). 5 volumes of ice-cold 95% EtOH were then added and the mixtures were placed at -20°C or -70°C for at least one hour, after which the precipitates were collected by centrifugation at 4°C at 12,000 x g in an Eppendorf centrifuge for 15-30 minutes. The pellets were dried and resuspended in 5-25 µl of a solution of 7 M urea, 5 mM Tris borate pH 8.3, 0.1 mM EDTA, 0.05% BPB, 0.05% XCFF.

All resuspended samples were heated at 90°C for 15 seconds-1 minute just prior to loading on the sequencing gels.

Sequencing Gels

Sequencing gels were cast between glass plates 0.25 inches thick. The dimensions of the gels were 0.15 cm (thickness) x 33 cm (width) x 40 cm (length). Spacer bars and slot-formers were made of plexiglass. Acrylamide (AA), Bis (N,N'-methylene-bis-acrylamide), APS (ammonium persulfate), and Temed (N,N,N',N'-tetramethylethylenediamine) were purchased from Bio-Rad Laboratories. Urea (ultra pure) was purchased from Schwarz/Mann. 10% polyacrylamide gels were cast using a ratio of Bis/total acrylamide of 1:20 (w/w) and contained 7 M urea, 2 x TBE (100 mM Tris borate, pH 8.3, 2 mM EDTA). 20% polyacrylamide gels were cast using a ratio of Bis/total acrylamide of 1:30 (w/w) and contained 7 M urea, 1 x TBE (50 mM Tris borate, pH 8.3, 1 mM EDTA).

Recipes for 300 ml of acrylamide solution are as follows:

10%: 100 ml 30% (w/v) acrylamide ([28.5 gm AA + 1.5 gm Bis]/100 ml); 126 gm urea; 30 ml 20 x TBE (1.0 M Tris borate, pH 8.3, 20 mM EDTA); dd H_2O to slightly <300 ml; 1.92 ml 10% (w/v) APS; dd H_2O to 300 ml; 60 µl Temed.

20%: 100 ml 60% (w/v) acrylamide ([58 gm AA + 2 gm Bis]/100 ml); 126 gm urea; 15 ml 20 x TBE; dd H_2O to slightly <300 ml; 1.92 ml 10% (w/v) APS; dd H_2O to 300 ml; 30 µl Temed.

The first four components were mixed and the urea was dissolved by stirring at 37°C, after which the solution was cooled to room temperature. After cooling, the APS was added and the volume of the mixture was adjusted to 300 ml by the addition of more dd H_2O. The solution was Millipore-filtered to remove particulate material, following which it was degassed. Immediately before pouring the gel, the appropriate amount of Temed was added. Both of the recipes above result in a polymerization time of roughly 30 minutes at room temperature.

Gels were poured with the aid of a funnel whose tip was held against the inside surface of the larger glass plate. Slot-formers were inserted directly into the solution after pouring. Following polymerization, the gels were permitted to stand at room temperature for at least 12 hours. This "aging" resulted in the formation of more even slots.

After removing the slot-formers, the gels were assembled in the buffer tanks. At this point, residual acrylamide as well as particulate material (including walls of polyacrylamide formed due to small spaces between the slot-former and the glass plates) were removed from the sample wells by aspirating with a drawn Pasteur pipette. Failure to clean the slots usually resulted in uneven slots and/or great difficulty in loading the samples.

For 10% gels, the electrophoresis buffer was 2 x TBE. 10% gels were pre-electrophoresesd at 600 V for at least one hour and then at 800 V for at least one hour prior to loading the samples. After loading the samples, electrophoresis was at 800 V, usually until the XCFF was approximately 3-4 cm from the bottom of the gel (∿8-8.5 hours). Under these electrophoresis conditions, the XCFF comigrates with fragments of roughly 35 nucleotides in length (A. Maxam, personal communication).

For 20% gels, the electrophoresis buffer was 1 x TBE. 20% gels were pre-electrophoresed at 800 V for at least one hour and then at 1000 V for at least one hour prior to loading the samples. After loading the samples, electrophoresis was at 1000 V usually until the BPB was 0-1 cm from the bottom of the gel (∿9-9.5 hours). Under these electrophoresis conditions, the BPB comigrates with fragments of roughly 10-13 nucleotides in length (A. Maxam, personal communication).

Just prior to loading the samples, the sample wells were flushed to remove urea which had leached out of the top of the gel into the wells. Samples (heated at 90°C just prior to loading) were applied in volumes of anywhere from 5-25 µl. To later facilitate comparisons of purine patterns in terms of intensities of bands, care was taken (when at all possible) to apply the same number of ^{32}P counts of corresponding samples from naked DNA and from virus in adjacent slots.

Following electrophoresis, the shorter of the two gel plates was removed and the gel, still on the larger plate, was wrapped in Saran wrap. Scotch "Magic Tape" was placed on the Saran wrap over areas of the gel not containing sample, and the tape was marked with ^{14}C ink (anywhere from 5-50 µCi ^{14}C/ml of ink)

to label the positions of the slots, the BPB, the XCFF, and the bottom and top of the gel. After the ink dried, 1-2 sheets of film (14" x 17", Kodak No-Screen film or Kodak XR-5 film) were placed on the covered gel and the entire assembly (in an exposure holder) was sandwiched between two aluminum plates (each 0.5 inches thick) which were then bolted together. Exposure was at -20°C for anywhere from roughly 1-30 days, depending upon the number of ^{32}P counts which had been applied to the slots of the gel.

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