Electrophoretic analysis of covalently closed SV40 DNA: Boltzmann distributions of DNA species.

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ABSTRACT

Covalently closed relaxed SV40 DNA [SV40(I')] generated by polynucleotide ligase closure of nicked circular SV40 DNA was analyzed by agarose gel electrophoresis. The DNA can be resolved into a series of bands differing in superhelical density whose intensities are approximately symmetrical about a central most intense band. Densitometric analysis of the gel pattern has revealed that the distribution of DNA species conforms to a Boltzmann distribution and has enabled us to derive an equation for the free energy of superhelix formation for SV40 DNA. We believe the observed bands reflect the time-averaged distribution of thermally induced fluctuations in DNA chain conformation in solution at the time of ligase catalyzed phosphodiester bond formation. Densitometric analysis of native supercoiled SV40 DNA, partially unwound in the presence of ethidium bromide, demonstrates that the separation between adjacent bands is approximately half that seen with SV40(I'). Agarose gel electrophoresis was also used to measure the change in average base rotation angle as a function of temperature by a procedure independent of ethidium dye binding.

INTRODUCTION

Agarose gel electrophoresis has proven to be a highly versatile tool for analysis of DNA structure. It has been used extensively for molecular weight determinations and for analysis of restriction enzyme-generated DNA fragments (1). Recently, Keller and Wendel (2) used agarose gels to follow the progressive relaxation of native supercoiled SV40 DNA [SV40(I)] after incubation with the DNA relaxation enzyme from KB cells. SV40(I) was converted from a rapidly migrating supercoiled molecule to a more slowly migrating covalently closed relaxed molecule through a series of discrete partially relaxed intermediates with superhelical densities ranging from zero to that of SV40(I). The fact that the partially relaxed intermediates migrated as discrete bands between SV40(I) and nicked circular SV40 DNA [SV40(II)] suggested that agarose gels are highly sensitive to the tertiary structure of covalently closed molecules. We have also observed similar multiple DNA species with electrophoretic mobilities intermediate between those of SV40-(II) and SV40(I). These species were apparent after electrophoresis of
relaxed covalently closed SV40 DNA [SV40(I')] generated either by ligase-catalyzed closure of SV40(II) or by complete relaxation of SV40(I) by the DNA relaxation enzyme (3) from CV-1 monkey cells. These results suggested that circular covalently closed DNA in its lowest thermodynamic energy state exists as a collection of species whose average superhelical density (4) is zero under reaction conditions, but which consists of a distribution of discrete molecular species in which both positively and negatively supercoiled molecules are represented. We have analyzed the set of covalently closed DNA bands obtained following electrophoresis of SV40(I') DNA generated by ligase closure of SV40(II), and have used the resulting data to derive an equation for the free energy of superhelix formation for SV40 DNA.

MATERIALS AND METHODS

I. DNA and Enzymes

SV40(I), labelled with [3H]-thymidine (2-6 x 10^6 cpm/µg) or sodium [14C] formate (1-3 x 10^3 cpm/µg) was purified from CV-1 cells infected with SV40 at 0.01 pfu/cell by Hirt lysis (5), deproteinization of the Hirt supernatant with chloroform:isoamyl alcohol (24:1), isopycnic banding in CsCl-ethidium bromide gradients (6), treatment with RNase A, and velocity sedimentation in 5-20% neutral sucrose gradients to remove oligomers. SV40(II) was prepared by limited digestion of SV40(I) (25 µg/ml) in 0.1 M Tris, pH 7.4, 10 mM MgCl2 for 20 minutes at 28°C with pancreatic DNase I (0.5 ng/ml, Sigma, electrophoretically pure). These conditions converted approximately 60% of SV40(I) to SV40(II). SV40(II) was purified by sedimentation through neutral sucrose gradients.

Ligation of SV40(II) (12-15 µg/ml) was performed according to Modrich and Lehman (7) using T4 polynucleotide ligase (Miles Laboratories) at 0.2 units/ml in the presence of 10 mM Mg2+ at 37°C. SV40(I') was isolated by sedimentation through neutral sucrose gradients containing 40 µg/ml ethidium bromide (Calbiochem), removal of the ethidium bromide from the pooled SV40- (I') fractions by passage through Dowex 50 in 1 M NaCl, and concentration by ethanol precipitation.

II. Electrophoresis

Electrophoresis was performed in 0.7% agarose (Seakem) formed into 0.6 x 15 cm cylindrical gels or 24 x 13 x 0.4 cm slab gels. Buffer was circulated between the upper and lower buffer chambers in the slab gel apparatus so as to maintain constant pH. Electrophoresis buffer was 40 mM Tris, 5 mM sodium acetate, 1 mM EDTA adjusted to pH 8.2 with acetic acid (1).
Cylindrical gels were run at 100V (6.7 V/cm) for 3–4 hours and slab gels at 60V for 18 hours (2.6 V/cm) at room temperature (21±2°C). Gel temperatures were monitored by inserting a thermocouple into the gel. Temperatures generally increased less than 2° over the course of the run. Samples for electrophoresis (25–50 µl) were made 5% in sucrose for cylindrical gels and 4% in Ficoll 400 (Pharmacia) for slab gels. Ficoll gave better resolution and less trailing with slab gels than did sucrose.

III. Photography and Quantitation

Gels were stained for 45 minutes in freshly prepared electrophoresis buffer containing 1 µg/ml ethidium bromide (8). DNA was visualized by placing gels over a long-wave ultraviolet light source (C50 transilluminator, Ultraviolet Products). Where DNA was to be quantitated by densitometry, photographs were taken on Kodak Pan-X film using a Crown Graphic camera equipped with a UV filter (Ultraviolet Products) and a Kodak No. 9 Wratten gelatin filter. A Kodak photographic step tablet (No. 1A, O.D. range 0.05 to 3.05) was photographed with each slab gel to assess the linearity of the film response. Negatives were traced on a Joyce-Loebl microdensitometer. Reconstruction experiments showed that under the conditions used, peak height in the densitometer trace was linearly proportional to DNA concentration. Photographs not used for quantitation were taken with a Polaroid MP-4 camera using Polaroid Type 55 P/N film, a UV filter, and a Kodak No. 23 Wratten gelatin filter. DNA was also quantitated by slicing individual bands from slab gels and counting in a Nuclear Chicago Mark II scintillation counter after shaking gel slices for 24 hours at 37°C in ACS scintillator (Amersham/Searle).

RESULTS

I. Multiple DNA Species in SV40(I')

When a ligase reaction mixture containing SV40(II) and SV40(I') is subjected to electrophoresis on 0.7% agarose gels as described in Materials and Methods, the covalently closed relaxed SV40(I') product is resolved into a series of bands which migrate more rapidly than the covalently open SV40-(II) substrate (Figure 1). (In this system, covalently closed superhelical SV40(I) migrates farther than any SV40(I') band (see figure 1)). The distribution of bands is approximately symmetrical in intensity about a central most intense band. We believe this distribution reflects the distribution of thermally induced fluctuations in chain conformation among SV40(II) molecules in solution at the time of ligase-catalyzed conversion of SV40(II) to
Electrophoresis was performed on 0.7% agarose slab gels as described in Materials and Methods. Direction of electrophoresis is from left to right. The band at the extreme left represents SV40(II) as well as two or three SV40(I') components.

SV40(I'). In other words, the population of SV40(II) molecules in solution, having a mean distortion from the thermodynamic ground state of zero, exists as a distribution of molecules about that mean, some molecules having no distortion and others having positive or negative deviations in \( \beta \), the duplex winding number (4), at any given instant. Since an increasing amount of thermal energy input is required for increasing changes in \( \beta \), the greater the deviation in \( \beta \) from the mean, the fewer will be the molecules in that conformational state at any instant. When these covalently open SV40(II) molecules are converted to SV40(I') by ligase, deviations from the average chain conformation present in the SV40(II) molecules at the instant of ligation will be locked into it by covalent closure. Under appropriate conditions, these different conformational states, now no longer interconvertible, can be resolved on agarose gels.

Keller and Wendel (2) have observed similar bands with electrophoretic mobilities intermediate between those of SV40(I) and SV40(II) after incomplete reaction of SV40(I) with the relaxation enzyme from KB cells. They have suggested that the DNA in each band differs from the DNA in the bands immediately adjacent to it by one superhelical turn. We also assume that adjacent species differ by a single superhelical turn since any variation in the superhelicity of SV40(I') molecules under ligase reaction conditions must occur as integral numbers of turns.

Agarose gels do not discriminate well between DNA molecules having similar numbers of superhelical turns when the number of superhelical turns per molecule (\( \tau \), the superhelical winding number) is either very close to
zero (2) or when it is quite large. Under normal conditions in standard electrophoresis buffer, native SV40(I) migrates as a single band. If, however, ethidium bromide is included in the gel and buffer at a concentration of 15 ng/ml, it effects a partial unwinding of the DNA. Under these conditions, native SV40(I) is also resolved into a series of bands (Figure 2).

Conversely, under certain conditions, such as high Mg\(^{++}\) concentration in the gels, SV40(I') migrates such that the set of covalently closed bands normally seen has an electrophoretic mobility the same as that of SV40(II).

If the SV40(I') derived from SV40(II) were sealed with ligase and electrophoresed under temperature and ionic conditions identical to those in the ligase reaction, the mean superhelical density of the SV40(I') during electrophoresis should be zero and some of the molecules (those which have zero superhelical turns under the conditions of electrophoresis) should co-migrate with SV40(II). However, the ionic conditions of ligation and electrophoresis are not identical in these experiments. Consequently, as is apparent in Figure 1, the mean superhelical density of the SV40(I') population is not zero under the conditions of electrophoresis. Since the ionic strength of the electrophoresis buffer is less than that of the ligase reaction mixture, and since a decrease in ionic strength of the solvent results in the introduction of positive superhelical turns in covalently closed DNA molecules (9), it would be expected that the SV40(I') bands observed would contain positive superhelical turns. Figure 3 demonstrates that this is the case. When SV40(I') is electrophoresed in the presence of increasing quantities of ethidium bromide, the migration of the SV40(I') bands increases uniformly relative to that of SV40(II). If the SV40(I') DNA were negatively supercoiled under electrophoresis conditions, it would be expected that the
mobility of the SV40(I') bands would first decrease and then increase as positive superhelical turns were introduced by the ethidium bromide (4). In a related study (10), we have shown that this effect does occur with negatively supercoiled SV40(I).

II. The Free Energy of Superhelix Formation

DNA molecules in thermal equilibrium with the surroundings may be considered to be distributed over a number of states. The number of molecules in the \( n \)th energy state \( (N_n) \) relative to the number in the state with the lowest energy \( (N_0) \) is given by the Boltzmann equation

\[
\frac{N_n}{N_0} = g_n \exp\left(-\frac{E_n}{RT}\right)
\]

where \( g_n \) is a degeneracy factor which in this case is equal to unity. \( E_n \) is the energy of the \( n \)th state and \( R \) and \( T \) have their usual meanings. Bauer and Vinograd (11) have shown that the free energy of superhelix formation is proportional to the square of the superhelical winding number. A plot of \(-\ln N_n/N_0\) versus \( \tau^2 \) should therefore be a line from whose slope the free energy of superhelix formation for SV40 DNA can be calculated.

In order to determine \( N_\circ \) and \( N_1 \), SV40(I') species resolved on vertical slab gels were photographed. The amount of DNA in each band was then deter-
determined by tracing the negative with a Joyce-Loebl microdensitometer. A typical trace is shown in figure 4. Background levels were evaluated by tracing on either side of the sample channel and averaging. For purposes of comparison, individual bands of radioactive $[^3H]$ SV40(I') DNA were also sliced from the gels and counted. The distributions of DNA species determined by these two different methods agreed closely with one another.

Figure 4: Densitometric Profile of SV40(I').

Electrophoresis and densitometry were performed as described. This tracing demonstrates the loss of resolution in the vicinity of SV40(II). (The extreme left hand peak is comprised of SV40(II) and two or three SV40(I') components). The base line was established by tracing each side of the sample channel (lower two traces) and averaging .

A plot of $-\ln N_i/N_0$ as a function of $\tau^2$ is shown in figure 5. It should be noted that the most intense band does not necessarily represent the precise center of the distribution. Pulleyblank et al. (12) have defined the quantity $\epsilon$ as the difference between the duplex winding number $\beta$ of a covalently open DNA and the value of $\beta$ for a covalently closed DNA with $\tau=0$ under identical environmental conditions. The quantity $\epsilon$ (-0.5 < $\epsilon$ < 0.5) must be added to the integer number of superhelical turns in a given species in the distribution to obtain the true number of superhelical turns by which the molecules in that species differ from the lowest thermodynamic energy state. The central most intense band is therefore displaced from the true mean by the value of $\epsilon$. $\epsilon$ was determined from the densitometer trace shown in Figure 4 by a curve-fitting procedure, and was found to have a value of 0.36 for the DNA shown.

The data shown in Figure 5 gives an equation for the free energy of superhelix formation for SV40 DNA

$$E_\epsilon/RT = 0.163 \tau^2$$

(2)
Figure 5: Plot of $-\ln \frac{N_f}{N_0}$ vs. $\tau^2$ for SV40(I').

Values of $-\ln \frac{N_f}{N_0}$ were obtained from tracings as described. The assumption has been made for this plot that adjacent bands in the densitometer trace differ by a single superhelical turn. The line was fitted to the data points by a least squares procedure. Coefficients of determination were routinely greater than 0.96.

Mass determinations by scintillation counting gave comparable results. Six independent determinations gave values of $0.163 \pm 10\%$ for the coefficient of $\tau^2$.

As shown in Figure 2, SV40(I) can also be resolved into a series of bands in which the DNA is symmetrically distributed when it is subjected to electrophoresis on gels containing 15 ng/ml ethidium bromide. In contrast to SV40(I'), a greater number of species are observed at equivalent DNA concentrations. Data obtained from densitometric analysis of the distribution of the SV40(I) species shown in Figure 2 resulted in a different coefficient in the equation for the free energy of superhelix formation

$$E_f/RT = 0.046 \tau^2$$

(3)

It is of interest to note that the coefficient in this equation is only about one-fourth of the value obtained for SV40(I'). Comparison of the
SV40(I) with the SV40(I') band distribution also indicates that there are twice as many bands per cm of gel when the two populations are electrophoresed an equivalent distance. If the previous assumption that adjacent bands in the SV40(I') distribution differ by one superhelical turn is valid, this result implies that adjacent bands in the SV40(I) distribution differ by approximately one-half of one superhelical turn. Since the coefficient in equation 2 will decrease as the square of the difference in \( \tau \) between adjacent bands, separation of the SV40(I) bands by one-half of one superhelical turn would predict a coefficient in equation 2 for SV40(I) one-quarter of that determined for SV40(I'), and that is what is observed. (Of course, any number of superhelical turn differences between adjacent bands \( \Delta \tau \) in which \( \Delta \tau (I')/\Delta \tau (I) = 2 \) would also lead to a fourfold difference in the value of the coefficient in equation 2.) Further analysis of the SV40(I') distribution indicates that it may be considered to be composed of two separate distributions, each identical to the SV40(I') distribution. That is, if every other band in the SV40(I) distribution is analyzed in the same manner as for SV40(I'), two distributions (bands \( n, n+2, n+4, \ldots \) and bands \( n+1, n+3, n+5, \ldots \) ) identical to that seen for SV40(I') and having coefficients in equation 2 approximately the same as is found for SV40(I'). A model for how these two distributions are generated and how they are related to each other will be presented in a subsequent paper (13).

III. Migration of SV40(I') as a Function of Temperature of Covalent Closure

Wang (9) has shown that the average base rotation angle for DNA varies as a function of temperature and ionic strength. It follows that if nicked circular DNA is in thermal equilibrium with its environment, variation in the temperature at which the ligase reaction is performed should result in a concomitant variation in the average base rotation angle of the covalently closed ligase product, and therefore in a change in \( \tau \) with temperature of sealing.

A series of ligase reactions was performed at 0, 4, 16, 23, and 37°C and analyzed on cylindrical 0.7% agarose gels in standard electrophoresis buffer at 23°C. The resulting gel patterns are shown in figure 6. As the temperature differential between ligase reaction conditions and electrophoresis conditions increases, the set of SV40(I') species migrates with a greater mobility relative to the unreacted SV40(II). Densitometric analysis of the resulting gel patterns has allowed us to estimate the temperature dependence of the change in base rotation angle. The magnitude of the change was found to be \(-1.3 \times 10^{-2} \) degree/base pair/°C. This value agrees well with the value...
Figure 6: Migration of SV40(I') as a function of Ligation Temperature. Ligase reactions were performed as described and applied to cylindrical 0.7% agarose gels. Direction of migration is from top to bottom. From left to right, ligase reaction temperatures were 0°, 4°, 16°, 23°, and 37°C.

of \(-1.1 \times 10^{-2}\) degree/base pair/°C determined by Wang (9) when his results are re-calculated with a value for the angle of duplex unwinding due to ethidium binding of 26° (14) rather than 12°.

DISCUSSION

The overall conformational state of a closed circular DNA molecule may be described by the equation (4)

\[ \tau = \alpha - \beta \]  

(4)

where \(\tau\) is the number of superhelical turns, \(\alpha\), the topological winding number, is defined as the number of turns one strand makes about the other when the molecule is constrained to lie in a plane, and \(\beta\), the duplex winding number, is defined as the number of Watson-Crick turns in the molecule. In the covalently open SV40(II) molecule, free rotation is permitted about the phosphodiester bond opposite the nick, resulting in changes in the duplex winding number \(\beta\) with time. When ligase seals the nick, a value of the topological winding number \(\alpha\) is established in the molecule at that time and can no longer be altered. Since the chemical forces of Watson-Crick base pairing restore \(\beta\) to its mean value under reaction conditions, the time-averaged thermal fluctuations in chain conformation in SV40(II) are reflected as alterations in the superhelical winding number \(\tau\) of the covalently closed product.

Agarose gel electrophoresis has allowed us to observe and analyze discrete species of covalently closed DNA formed by ligase-catalyzed covalent closure of SV40(II). The distribution of SV40(I') species may be attributed
to thermal fluctuations in chain conformation. The species generated by ligase closure of SV40(II) must differ from each other by integral numbers of turns. Ligation places the 5' phosphoryl and 3' hydroxyl groups into close proximity. Because this position can be restored most simply by a complete 360° rotation of the phosphoryl and hydroxyl groups with respect to each other, we assume that adjacent species observable after electrophoresis of SV40(I') differ by a single superhelical turn.

The lowest free energy state of a population of DNA molecules under ligase reaction conditions will be the state shared by the largest number of molecules. Deviations from this state will occur at a frequency which is related to the free energy change involved. This relationship is described by the Boltzmann equation and has allowed us to calculate in a straightforward manner the free energy of superhelix formation for SV40 DNA. The fact that \(-\ln \frac{N_f}{N_i}\) is a linear function of \(\tau^2\) for both SV40(I') and SV40(I) confirms that each of these populations exists in a Boltzmann distribution.

Bauer and Vinograd (11) have reported a value of approximately 84 kcal/mole for the free energy of superhelix formation for native SV40(I) DNA. This value was determined from binding isotherms for ethidium bromide binding to SV40(I) in 5.8 M CsCl (11). If Bauer and Vinograd's value of \(\tau = -13\), based on an unwinding angle of 12° for ethidium (15) is corrected to -28 for an unwinding angle of 26° (14) and substituted into equation 2, a value of 80 kcal/mole is found. This is in excellent agreement with the value previously reported by Bauer and Vinograd.

The observation that the distribution of \(\tau\) is different for SV40(I) and SV40(I') is quite significant. It implies that there are constraints on SV40 DNA in the cell which do not apply to that same DNA in vitro. In vivo, SV40 DNA has bound to it a number of proteins, such as histones and relaxation enzymes, which are known to alter DNA conformation. The specific ways in which these proteins could act to generate the distribution seen for SV40(I) and why their absence prevents the same distribution from being seen for SV40(I') will be discussed in a subsequent paper (13).

The experiments which allowed us to calculate base rotation angle variation as a function of temperature were designed to avoid ambiguities introduced as a result of uncertainties (14,15) in estimating the unwinding angle due to ethidium binding. The technique we have employed has enabled us to observe directly the shift in band position as a function of the temperature of the ligation and thus to measure the change in base rotation angle in the absence of ethidium binding.
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