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Introduction of Interrupted Secondary Structure in Supercoiled DNA as a Function of Superhelix Density: Consideration of Hairpin Structures in Superhelical DNA

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PM2 DNA was prepared with different superhelical densities (σ) in order to examine the relationship between supercoiling and the occurrence of a region(s) of unpaired bases in this DNA. A previous study showed that CH_3HgOH reacts with native superhelical PM2 DNA more rapidly than the nicked form II. This evaluation of binding, monitored through the change of sedimentation velocity, was repeated on PM2 DNA I with different superhelical densities. Early binding is detected by an increase in sedimentation velocity and occurs with molecules with σ' values between -0.025 and -0.037. The conversion of form I to form II with the single-strand-specific endonuclease from *Neurospora crassa* also occurs above a σ value of -0.025. This data strongly supports the view that supercoiling produces interrupted secondary structure. The question whether the interrupted regions remain single stranded in character or form small intrastrand hairpin regions is considered by examining which model best fits the CH_3HgOH-induced sedimentation velocity changes and the standard sedimentation velocity versus the superhelical density curve for the in vitro made DNAs. The hairpin model offers the most satisfactory explanations for all the results of this and previous studies.

DNAs which are covalently closed, circular, and supercoiled are widespread among living organisms and represent the nucleic acid or DNA intermediate of a large variety of viruses (1, 30). They include the DNA of PM2 bacteriophage; replicating and intracellular forms of ϕX, λ, and P22; simian virus 40 (SV40), polyoma, and papilloma viruses (1, 30); and very recently the proviral DNAs of the murine leukemic and avian sarcoma RNA viruses (13, 16). It is striking that a large fraction of viral superhelical DNAs have the capacity to integrate into their host cell chromosome (30). In order to explore structural features that might be related to biological function, we have previously examined the susceptibility of the secondary structure of supercoiled DNA to chemical probes. From the reactivity with formaldehyde (7) and methylmercury hydroxide (3), it was concluded that superhelical DNAs have regions of altered or interrupted secondary structure containing unpaired bases. A similar conclusion was reached by other investigators studying the action of the single-strand-specific endonucleases, *Aspergillus* S1 nuclease (2, 12, 20) and *Neurospora crassa* endonuclease (18). T4 gene 32 protein interacts specifically with SV40 DNA I at two sites (22, 29). Recently it was shown that gene 32 protein-binding sites map in any of eight sharply defined regions on PM2 DNA; however, only one to three sites per molecule can be detected (4).

In order to have a full understanding of the behavior of superhelical viral DNA and to understand possible structure-function relationships, we have examined the development of altered secondary structure as a function of supercoiling, using PM2 DNA. Two additional papers (6, 19) will be presented characterizing the reaction of superhelical SV40 DNA with a water-soluble carbodiimide that reacts specifically with unpaired thymine and guanine (21).

MATERIALS AND METHODS

Preparation of PM2 DNA (form I). PM2 DNA form I was prepared as described previously by Espejo, Canelo, and Sinsheimer (10) and Dean and Lebowitz (7). The DNA was dialyzed in 0.1 M NaCl-0.01 M $Na_2B_4O_7$ (pH 9.0), buffered, and stored frozen at -20 C.

Preparation of PM2 DNA (form II) as substrate for DNA ligase. PM2 DNA form I was converted to form II by an *Escherichia coli* tRNA-associated endonuclease I (14). The incubation mixture (60 μl) contained 83 mM glycine (pH 7.0), 5 mM $MgCl_2$,

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0.5 M NaCl, 0.2 μg of *E. coli* tRNA, 0.6 μg of PM2 DNA form I, and 40 μg of endonuclease I-tRNA complex. The reaction was incubated at 37 C. At 10-min intervals, aliquots were removed and centrifuged in the model E ultracentrifuge to monitor the conversion of form I to form II DNA. After determining the incubation time needed to put one nick in each of the form I DNA molecules, the reaction mixture was scaled up 200-fold to prepare substrate for polynucleotide ligase. The reaction was terminated by addition of EDTA to a final concentration of 0.033 M. The ligase substrate was incubated at 75 C for 15 min, to inactivate the endonuclease, and dialyzed for 24 h against four changes (50 volumes) of the following buffered solution: 0.01 M Tris-hydrochloride (pH 7.8), 2 mM $MgCl_2$, 1 mM EDTA, 100 μg of bovine serum albumin per ml, and 10 μM NAD^+ (24). The ligase substrate was stored frozen at −20 C.

Preparation of in vitro DNAs. Ligase substrate was incubated with DNA ligase at 30 C for 30 min in the presence of different concentrations of ethidium bromide. The enzymatically closed DNA was separated from form II DNA by centrifugation in an ethidium bromide-cesium chloride density gradient (27) for 48 h at 42,500 rpm and 5 C using an SW50L rotor in a Spinco model L2-65B preparative centrifuge. The ethidium bromide was removed from the DNA by isoamyl alcohol extraction followed by passage through a Dowex 50 column. The DNA was dialyzed in 0.1 M NaCl-0.1 M Tris-hydrochloride (pH 8.0) and stored frozen at −20 C. These DNAs are designated "purified" DNAs (85 to 90% in vitro form I, 10 to 15% form II DNA). "unpurified" DNAs (approximately 50% in vitro form I, 50% form II) refer to those DNAs that after ligase closure were extracted with isoamyl alcohol to remove ethidium bromide, dialyzed versus 0.01 M Tris-hydrochloride (pH 7.8)−2 mM $MgCl_2$-1 mM EDTA, and stored frozen at −20 C.

DNA ligase. DNA ligase was isolated from *E. coli* 1100 (an endonuclease-minus strain) and purified on DEAE-cellulose according to the procedure of Olivera and Lehman (23).

***E. coli* tRNA-endonuclease I complex.** A tRNA-associated endonuclease I was isolated from *E. coli* JC411 according to the procedure of Goebel and Helinski (14). The enzyme makes a single cut in one strand of circular duplex DNA and produces 5'-phosphoryl and 3'-hydroxyl termini which can be closed by polynucleotide ligase. The enzyme from a cleared Brij lysate was used in all experiments and was stable for several months when stored in 50% (vol/vol) glycerol at −15 C.

***N. crassa* endonuclease.** The single-strand-specific endonuclease was a generous gift of K. Bartok. The incubation mixture (40 μl) contained 0.1 M Tris-hydrochloride (pH 8.0), 0.01 M $MgCl_2$, 0.1 M NaCl, 0.9 μg of DNA, and 0.06 to 0.14 U of enzyme. After incubation for 10 min at 37 C, the reaction was terminated with 5 μl of 0.1 M EDTA, pH 7.7.

Chemicals. Ethidium bromide was a gift of Boots Pure Drug Co., Ltd., Nottingham, England. Dowex AG 50W-X4 and DEAE-cellulose (Cellex D) were purchased from Bio-Rad Laboratories, Richmond, Calif. Optical grade cesium chloride and cesium sulfate were obtained from Harshaw Chemical Co., Cleveland, Ohio. Methylmercury hydroxide (panogen concentrate) was donated by NOR-AM agricultural products, Woodstock, Ill.

Analytical ultracentrifugation. All band sedimentation experimentation was performed at 20 C in a Beckman model E ultracentrifuge equipped with a photoelectric scanner and multiplexer. Type I, double-sector 12-mm band centerpieces were used (Beckman Instruments Inc., Fullerton, Calif.). Sedimentation coefficients were calculated from a least-squares analysis of log distance (centimeters) versus time (minutes) using an IBM 360-50 computer. Sedimentation values were corrected to standard conditions for sodium DNA ($s^0_{20,w}$) according to the method of Bruner and Vinograd (5). The densities of the sedimentation solvents were determined from refractive index readings taken on a Zeiss refractometer.

(i) The detection of the binding of CH_3HgOH. The detection of the binding of CH_3HgOH to DNA was performed by band sedimentation velocity as described above, as well as in a previous study (3). In order to apply the Bruner and Vinograd (5) empirical method for correcting sedimentation coefficients in cesium salts, it is necessary to know the buoyant density change for each CH_3HgOH concentration, plotted as pM, the -log [CH_3HgOH]. This procedure also requires a test for the linearity of the uncorrected S value times the sedimentation-solvent viscosity relative to H_2O (η_{rel}) versus the density of the sedimentation solvent (5). In the case of a bound metal ligand to DNA, we would also have to independently measure the binding as a function of solvent density in order to correct for ionic effects on binding. This approach requires a vast amount of data and has not been attempted in other situations where it is also required (31). Since the amount of in vitro made superhelical DNA was low, we could not use this fully rigorous empirical approach. However, the linearity of the uncorrected $s \times \eta_{rel}$ for PM2 DNA versus Cs_2SO_4 density was established (T. A. Beerman, unpublished data). Furthermore, in a previous study (3) it was shown that the correction for the measured buoyant density shift with CH_3HgOH decreased the respective $s'_{20,w}$ (Bruner and Vinograd [5], correction assuming no change in buoyant density) values in proportion to the amount of CH_3HgOH bound and that the buoyant density transition followed the initial sedimentation velocity transition very closely (3). Hence, the buoyant density correction does not change the character of the observed $s'_{20,w}$ transition, and relative comparisons of binding are valid using $s'_{20,w}$ versus pM data for each superhelical DNA (Fig. 3).

(ii) Binding experiments with CH_3HgOH. Binding experiments with CH_3HgOH were performed at 36,000 rpm in 0.65 M Cs_2SO_4-0.005 M $Na_2B_4O_7$ (pH 9.0), density 1.187 g/ml. The concentration of CH_3HgOH was determined by atomic absorption spectrophotometry as described previously (3). Calculated amounts of stock solution of CH_3HgOH (1.5×10^{-3} M) were added to the sedimentation solvent with a Manostat digital pipette.

(iii) Superhelix densities. Superhelix densities (σ, the superhelix density = τ/β^0 where τ equals the

number of supercoils and β^0 is numerically equal to 0.1 times the number of base pairs in the DNA or the number of superhelical turns per ten base pairs) were determined by sedimentation velocity-dye titration at 34,000 rpm in 2.83 M CsCl-0.01 M Tris-hydrochloride (pH 8.0), density 1.35 g/ml, using the dye-binding data previously obtained for this solution (15). The appropriate amounts of a stock solution of ethidium bromide (175 μg/ml) were added to the sedimentation solvent with a Manostat digital pipette. The ethidium bromide concentration was determined on a Cary 15 spectrophotometer using a reciprocal extinction coefficient of 81.6 μg/ml per absorbance unit at 487 nm (15).

(iv) Standard sedimentation velocity versus superhelix density curves. To establish $s^0_{20,w}$ versus superhelix density curves, samples of DNA were centrifuged at 34,000 rpm in 2.83 M CsCl-0.01 M Tris-hydrochloride (pH 8.0), density 1.35 g/ml (31).

The analytical centrifuge was also used to determined the extent of conversion of form I to form II DNA by endonuclease. (i) The activity of *E. coli* tRNA-endonuclease I complex was measured by centrifuging aliquots of the incubation mixture at 30,000 rpm and 20 C in 1.5 M CsCl-0.05 M sodium phosphate (pH 12.8), density 1.2 g/ml. (ii) The activity of *N. crassa* endonuclease was assayed by centrifugation of the reaction mixtures at 34,000 rpm and 20 C in 1.5 M CsCl-0.4 M Tris-hydrochloride (pH 8.0), density 1.2 g/ml.

RESULTS

An ethidium bromide titration of the respective in vitro ligase-closed DNAs is shown in Fig. 1. The drug needed to achieve the minimum for each sample was converted to superhelix density, $-\sigma$ (1). Superhelical density values reported in this study are based on a 12° unwinding angle for ethidium bromide.

Change in $s^0_{20,w}$ as superhelix density increases. The sedimentation velocity coefficients of a family of in vitro, ligase-closed DNAs are plotted as a function of superhelix density in Fig. 2. As the superhelix density increases from -0.009 to -0.025 the sedimentation coefficient rises from 25.3 to 28.6s. Then there is a drop in $s^0_{20,w}$ to 26.8, even though the superhelix density continues to increase from -0.025 to -0.043. At superhelix densities greater than -0.043 the $s^0_{20,w}$ again rises.

Binding of CH_3HgOH depends on the extent of supercoiling. Since previous studies had suggested that supercoiling created a region(s) of unpaired bases (2-4, 7, 12, 17, 18, 22), it was of interest to assess the binding of methylmercury hydroxide (a measure of altered secondary structure) as a function of superhelix density. In prior studies of the reaction of methylmercuric hydroxide with PM2 DNA (3) we had already shown (i) that both the $s'_{20,w}$ and the buoyant density of superhelical PM2 DNA increase as a function of methylmercury hydroxide concentration, whereas the nicked form does not. (ii) As the CH_3HgOH concentration approaches the region where normal duplex structure is induced to denature, we observe a dip in $s'_{20,w}$ associated with loss of supercoils, and we can measure this decrease (3). (iii) A buoyant density binding analysis allowed us to estimate that 3.7% of the bases contain methylmercury in the first phase of the transition (3). Based on these findings, we have used changes in sedimentation rate to assess the reactivity of methylmercuric hydroxide with PM2 DNA of varying superhelicity. An examination of the effects of CH_3HgOH on the $s^0_{20,w}$, corrected for the buoyant density change, reveals that the predominant effect is on the frictional coefficient of the DNA (3). The increase in molecular weight due to mercurial binding is small in the pM region studied, and we can qualitatively discuss $s'_{20,w}$ in terms of structural transitions in the DNA (Fig. 3).

An in vitro DNA with a superhelix density equal to that of the native molecule has the same affinity for CH_3HgOH as described for the native supercoiled DNA (3) (Fig. 3B). However, there is no early binding of CH_3HgOH (pM 4.62 to 3.82) to in vitro DNAs with a superhelical density of -0.018 (Fig. 3F) or -0.025 (Fig. 3E). At a superhelix density between -0.025 and -0.037 we begin to see an early reaction with CH_3HgOH; the $s'_{20,w}$ increases from 27.2s to 29.8s (Fig. 3D). We conclude that DNAs with superhelix densities greater than -0.037 bind progressively more CH_3HgOH, as reflected by the initial increase in $s'_{20,w}$ (Fig. 3A-D), whereas DNA with a superhelix density of -0.025 or below does not produce a region(s) containing sufficient unpaired bases to be detected by reaction with CH_3HgOH.

Cutting with *N. crassa* endonuclease depends on superhelix density. Another, and perhaps more sensitive, way to detect disordered secondary structure in superhelical DNA is to determine susceptibility to a single-strand-specific endonuclease. Recent studies in other laboratories (see Introduction) have shown that native superhelical DNAs have enough "single-strand-like" character to be susceptible to attack by single-strand-specific endonucleases.

N. crassa endonuclease also preferentially attacks native supercoiled PM2 DNA as compared to nicked circular form II DNA. To determine the effect of supercoiling, a number of in vitro DNAs of different superhelical content were incubated with *N. crassa* endonuclease. Conditions were chosen where there was no conversion of the nicked circular form II to form III (a linear duplex). The results are presented in Fig. 4. In one set of experiments, EDTA was

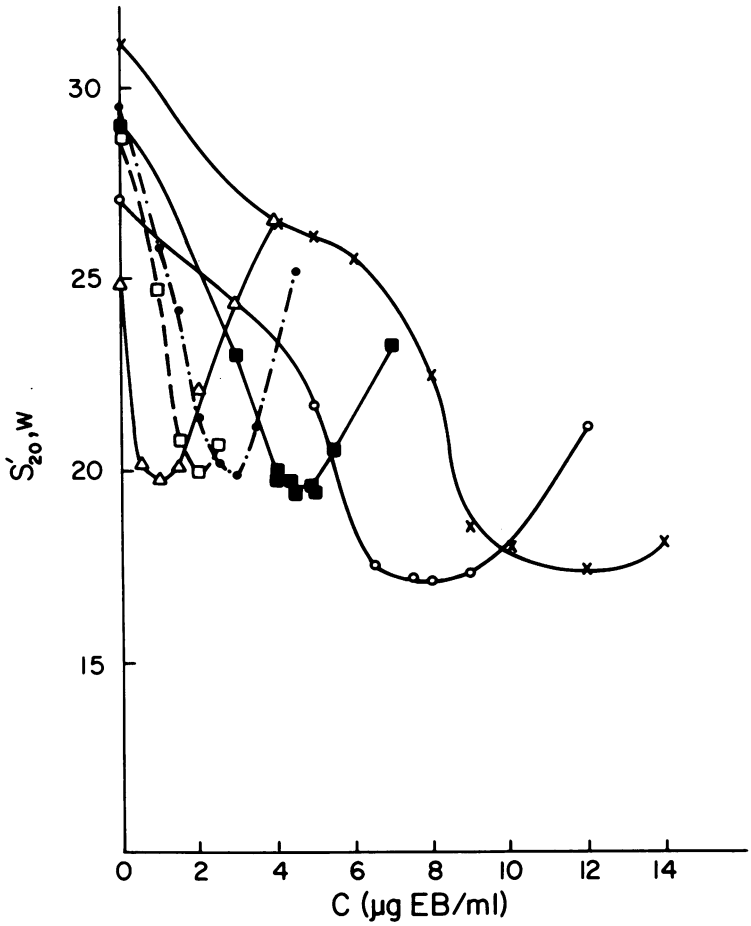

FIG. 1. *Sedimentation velocity-ethidium bromide (EB) titrations of six closed circular PM2 DNAs in 2.83 M CsCl-0.01 M Tris-hydrochloride (pH 8.0) at 20 C in a Beckman model E ultracentrifuge. Standard sedimentation coefficient $s'_{20,w}$ uncorrected for the buoyant effect of bound ethidium versus free drug concentration. Superhelix densities: (△——△) −0.009; (□——□) −0.018; (●——●) −0.025; (■——■) −0.037; (○——○) −0.053 native PM2; and (X——X) −0.069.*

present because the DNA samples were so small it would have been difficult to remove the EDTA. The enzyme is inhibited approximately 50% by 6.25×10^{-4} EDTA as shown in the inset. Approximately 60 to 70% of form I is converted to form II by 1.5 U of enzyme per ml. In contrast, less than 5% nicking of φX RF I is seen with this low concentration of enzyme (18). The fact that PM2 DNA has a greater superhelix density (−0.053) than φX RF I (−0.036) (8) supports our model, which predicts it would be more susceptible to attack by the enzyme. The two curves in Fig. 4 demonstrate how the enzyme activity depends on the extent of supercoiling. Although there is a quantitative difference in the amount of enzyme activity in the presence and absence of EDTA, the qualitative results are the same. The closed circular duplex DNA of PM2 is not subject to attack by the single-strand-specific endonuclease until the DNA molecule has a superhelix density of −0.029. As more supercoils are put into the molecule, the DNA is more readily converted to form II by the enzyme. An in vitro DNA molecule with more superhelical turns than the native molecule is more susceptible to attack by the enzyme than is the native DNA. It is clear that the extent of supercoiling determines the susceptibility of closed circular duplex DNA to *N. crassa* endonuclease. The correlation of the appearance of nicking activity and the binding of CH_3HgOH at σ values between −0.025 and −0.037 is excellent.

DISCUSSION

This study was undertaken to explore the behavior of a number of features of superhelical DNA as a function of superhelix density. The

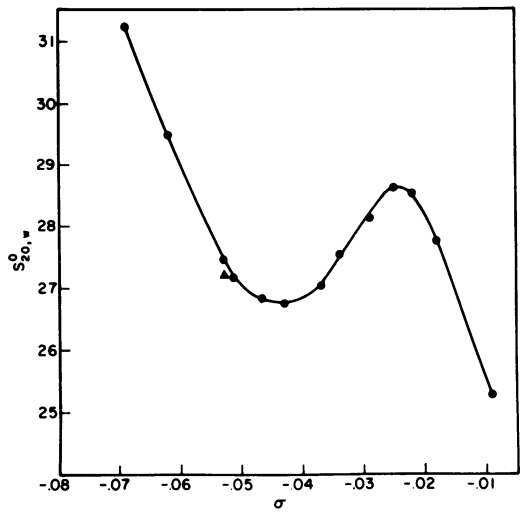

FIG. 2. *Sedimentation velocity coefficients ($s^0_{20,w}$) as a function of superhelix density, σ. In vitro DNA samples (0.7 μg) were centrifuged at 34,000 rpm in 2.83 M CsCl-0.01 M Tris-hydrochloride (pH 8.0) at 20 C in a Beckman model E ultracentrifuge. Symbols: (●) In vitro, ligase-closed, supercoiled DNAs; (▲) native PM2 DNA form I.*

results show that superhelical PM2 DNA does not bind CH_3HgOH by sedimentation velocity criteria (initial increase) until the molecule has achieved a superhelix density between −0.025 and −0.037. Molecules with superhelix densities greater than −0.037 have an increasingly higher affinity for CH_3HgOH. Furthermore, closed circular DNA is not cleaved by *N. crassa* endonuclease, an enzyme specific for disordered DNA, unless it has a superhelix density of −0.029.

The results strongly suggest that a mechanism exists for the production of interrupted secondary structure as a function of superhelical turns. We have not attempted to explore the structural details leading to this altered secondary structure. However, it is worthwhile to consider what is the nature of the interrupted secondary structure. Is it simply regions of single-strand DNA which exist as random coils, or does this structure fold and produce hairpins? One of these alternative models should help explain the following observations: (i) the increase in $s'_{20,w}$ upon the initial reactivity of CH_3HgOH (a similar effect is observed with HCHO [7], the reaction of a water-soluble carbodiimide [19, 25] and the production of photodimers by UV irradiation [8]); (ii) the behavior of $s^0_{20,w}$ versus $-\sigma$ (Fig. 2); (iii) the analysis of superhelical content of modified DNAs published in recent years (3, 7).

It is very striking that all the above perturbations of superhelical DNA create comparable effects in sedimentation velocity, yet they are vastly different probes of the secondary structure of DNA. For example, both CH_3HgOH (3) and HCHO (7) produce a $\Delta s'_{20,w} = +3$ in PM2 DNA I for an estimated 4% reactivity. Similar changes occur in the other respective cases cited above. Also of considerable importance is the fact that none of the above probes causes a loss of superhelical turns upon the initial reaction with form I (see below). This suggests to us that initial reaction occurs at a common site or sites for all of the above agents and, consequently, a similar effect is responsible for the initial perturbation.

It is impossible to account for the velocity changes being produced by mass changes, since the reagents used are very diverse in molecular weight. In the case of UV irradiation, no mass change is possible. Therefore, we conclude that chemical modification at the interrupted or altered secondary structure is predominantly a frictional change. However, it is difficult to see how reaction at preexisting single-strand coil regions would decrease the frictional coefficient significantly. Consequently, the simplest conceptional model is the disruption of hairpin regions that are partially hydrogen bonded. These localized "helix-coil" transitions could be induced by all of the reagents discussed above. We interpret the effect produced by UV irradiation in the following way. The efficiency of introducing photoproducts into folded, denatured DNA is greater than native DNA (28), and we envision that there is a preferential disruption of hairpin regions by pyrimidine dimers and cytosine hydrates. This would produce behavior similar to that observed for chemical disruption of hairpin sites. The results of the initial modification of PM2 DNA I by a water-soluble carbodiimide parallels the behavior produced by UV irradiation (25; A. Chaudhuri et al., manuscript in preparation).

Consequently, the change in frictional coefficient produced by chemical modification is due to increased flexibility caused by the production of "coil" sites which would readily account for the observed increase in $s'_{20,w}$. This is illustrated in Fig. 5. Chemical reaction with reagents specific, or preferential, for unpaired bases would be expected to occur at loops of the hairpin (Fig. 5B). We view the disruption of the hairpins by CH_3HgOH as the cause of the first transition observed in $s'_{20,w}$ (Fig. 3). This is due to the generation of points of increase flexibility in the DNA as the rigidity of the intrastrand helical regions undergoes a "helix-to-coil transition". The second phase of the reaction would be the propagation of the denaturation into neighboring regions of duplex structure. This unwinding of duplex turns would be coupled to

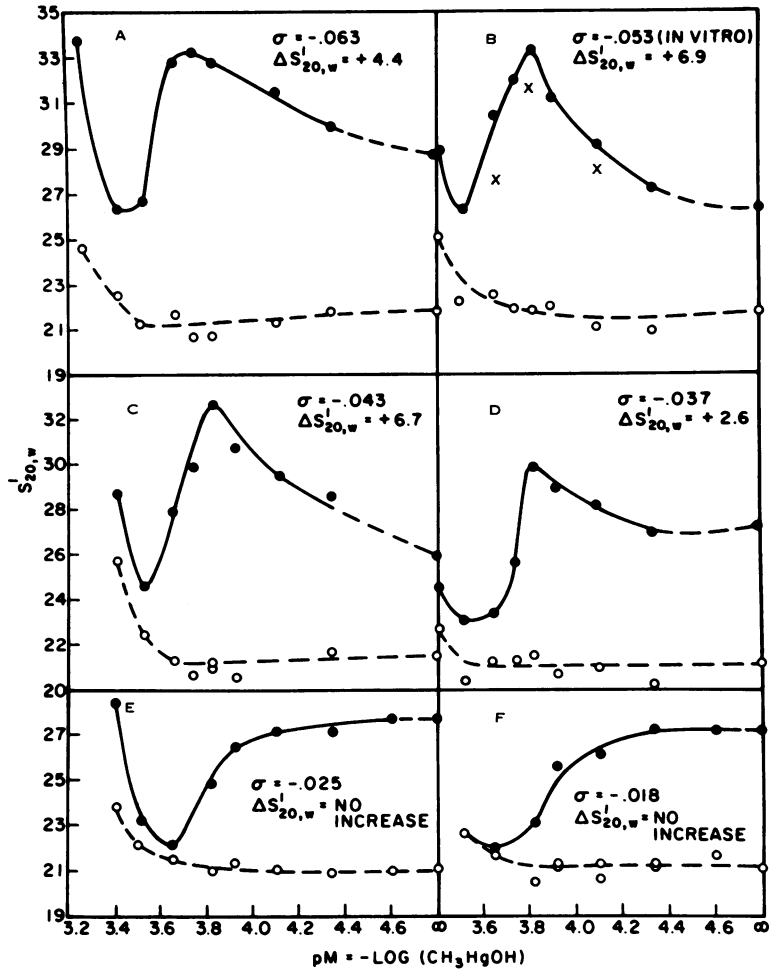

FIG. 3. *Changes in sedimentation velocity ($s'_{20,w}$) as a result of binding CH_3HgOH to in vitro DNAs. Experiments were performed at 36,000 rpm in a Beckman model E ultracentrifuge at 20 C. The sedimentation solvent was 0.65 M Cs_2SO_4-0.005 M $Na_2B_4O_7$ (pH 9.0). Each frame (A-F) specifies the superhelix density (σ) and the respective change in $s'_{20,w}$ from the initial $s^0_{20,w}$ at pM ∞ to the $s'_{20,w}$ at the local maximum. The $s'_{20,w}$ values are uncorrected for the buoyant effect of bound CH_3HgOH, except for the X points shown in (B). The $s^0_{20,w}$ (X) values were obtained using the buoyant density data of Beerman and Lebowitz (3) and illustrate the displacement caused by correcting for the increase in buoyant density due to bound CH_3HgOH. Symbols: (●——●) In vitro, ligase-closed, supercoiled DNAs; (○——○) nicked circular PM2 DNA (form II).*

a loss of superhelical turns. At this point denatured coil regions are generated along with the loss of supercoils, and we finally observe the dip region seen in Fig. 3.

Superhelical molecules without hairpins would be expected to react with CH_3HgOH at intact duplex sites (A-T rich regions) which undergo transient opening. This would produce a coupled unwinding of duplex and superhelical turns. We believe this is the case for the lower superhelical-density molecules, $\sigma = -0.018$ and -0.025 (Fig. 3E,F). With the introduction of a superhelix density sufficient to produce hairpins, we would expect reactivity preferentially at these sites. This situation calls for the prediction that all molecules containing regions of unpaired bases would increase their $s'_{20,w}$ value well before undergoing a loss of superhelical turns as unwinding proceeds into intact duplex sites. It is interesting to examine the superhelical molecules containing σ values of -0.037, -0.043, and -0.053 in greater detail. Note that the maximum $s_{20,w}$ value of Fig. 3A-C exceeds any $s_{20,w}$ value of Fig. 2, including the $s_{20,w}$ value for the -0.063 molecule. Modified superhelical molecules generate much greater flexibility than the corresponding untreated molecules of Fig. 1.

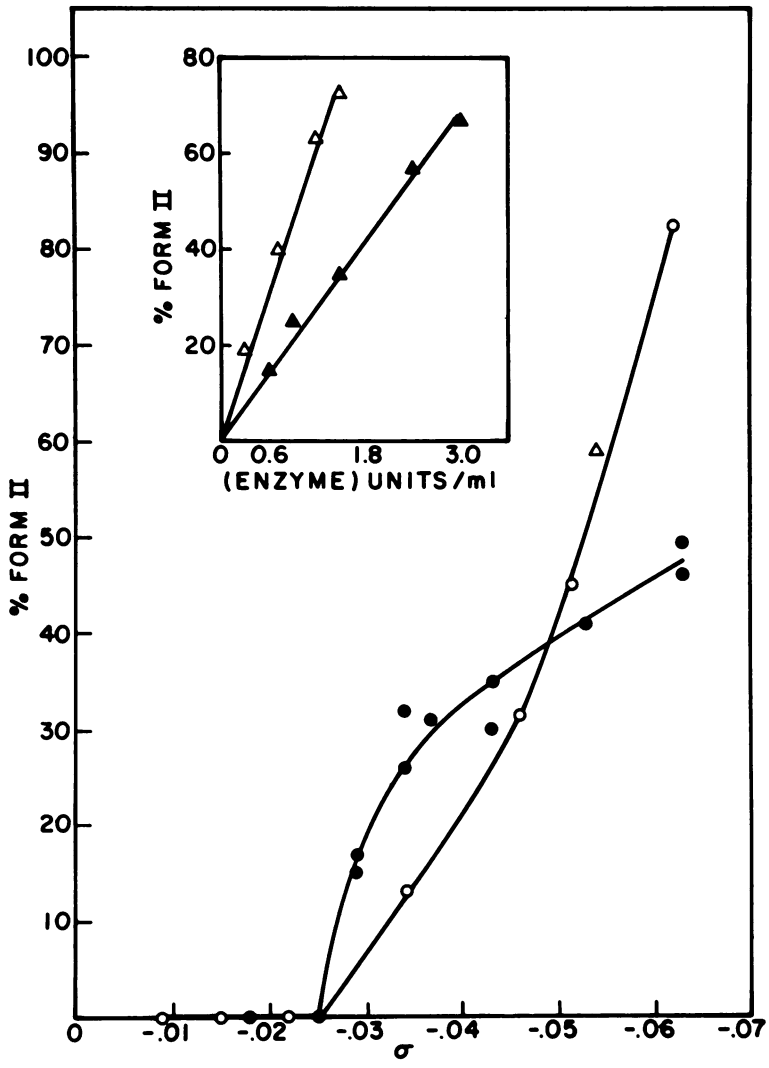

FIG. 4. *Activity of N. crassa endonuclease on in vitro, ligase-closed DNAs of different superhelix densities. The inset is the activity of the endonuclease on PM2 DNA form I as a function of enzyme concentration. Enzyme activity was measured as the amount of form I DNA converted to form II DNA after a 10-min incubation at 37 C. Symbols: (▲) Native PM2 DNA form I in the presence of 6.25 × 10⁻⁴ M EDTA; (△) native PM2 DNA form I (no EDTA); (●) "unpurified" in vitro DNAs incubated with 3 U of enzyme per ml in the presence of 6.25 × 10⁻⁴ M EDTA; (○) "purified" in vitro DNAs incubated with 1.5 U of enzyme per ml in the absence of EDTA.*

The model that supercoiling eventually produces hairpin regions requires us to consider their role in explaining Fig. 2, the behavior of $s^0_{20,w}$ versus $-\sigma$. A similar curve was obtained by Upholt et al. (31) for SV40 DNA, with the local maximum and minimum in good agreement with our results. They proposed that the first phase of Fig. 2 from σ equal to zero to the local maximum represents a reduction of hydrodynamic volume or an increase in compactness due to the introduction of superhelical turns. In addition, molecules at the local maximum ($\sigma = -0.024$) are coil-like in behavior with a capacity to have end-to-end contacts. The second phase of the $s^0_{20,w}$ versus $-\sigma$ transition, from the local maximum to the local minimum, represents a conversion from a "coil" to a "rod," due to the enhanced stiffening produced by additional supercoils. We extend these interpretations first offered by Upholt et al. for SV40 DNA (31) by considering the effects of the introduction of hairpin-like structures. If these structures occur simultaneously with the development of rodlike characteristics it would ap-

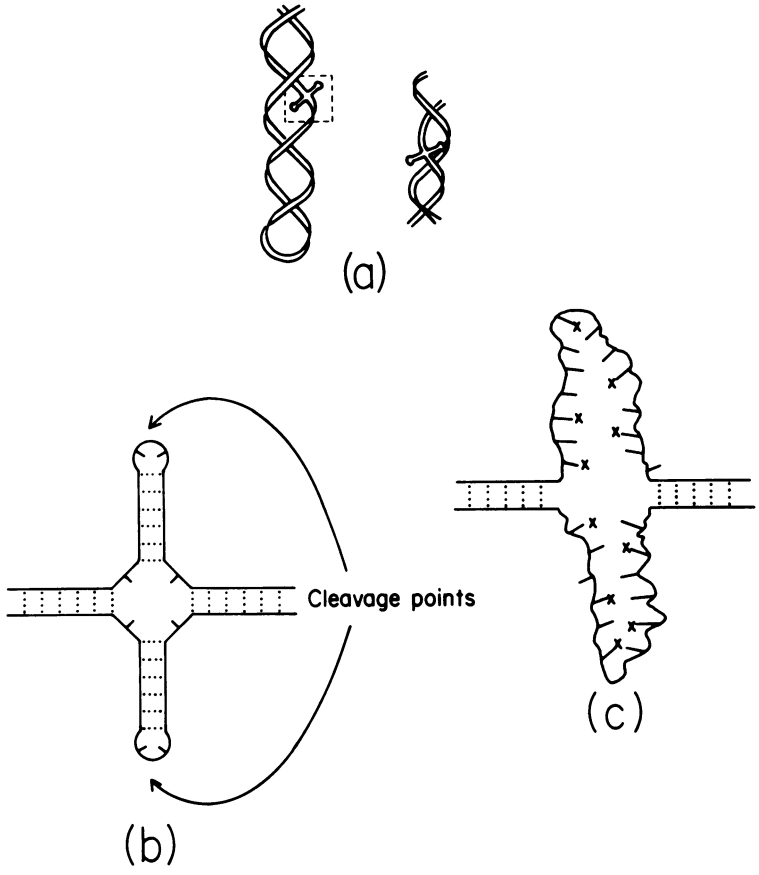

FIG. 5. *(a) Illustration of exaggerated hairpin regions in superhelical DNA. (b) Illustration of the sites for endonuclease cleavage and unpaired bases in a hairpin region. The figure represents only one of many possible types of hairpins which could be imagined with different degrees of base-pairing. (c) The figure represents the disruption of the hairpin by the binding of CH_3HgOH (X).*

pear that the sedimentation behavior would be a function of both structural features. We interpret the sedimentation behavior as a competition between the rodlike character of the DNA due to the stiffening produced by supercoiling versus the introduction of hairpin-like structures.

The local minimum in our view is the point where flexibility due to creation of disrupted secondary structure balances the stiffening produced by superhelical turns. Finally, $s^0_{20,w}$ increases because flexibility from hairpin joints outweighs further rodlike character. It is possible that as $-\sigma$ increases PM2 molecules would produce interrupted secondary structure not capable of intrastrand pairing. Such molecules would have flexibility points at the single-strand sites that would not undergo a local helix-to-coil transition. The production of significant single-strand coil sites could explain the steep rise in $s^0_{20,w}$ beyond the local minimum of Fig. 2 and could account for the branching seen in electron micrographs (31). It is interesting to note that the $\sigma = -0.063$ molecule (Fig. 3A) does not show a $\Delta s'_{20,w}$ increase as large as $\sigma = -0.053$ or -0.043 (Fig. 3C,D) molecules. In the latter cases the starting $s^0_{20,w}$ values are close (local minimum region), whereas the former is considerably higher. If this increase in the initial $s^0_{20,w}$ represents more fully unpaired sites, then we would not anticipate a significant frictional change at these sites for the binding of CH_3HgOH. This could explain the leveling-off process for $\Delta s'_{20,w}$ (Fig. 3) as $-\sigma$ increases beyond the local minimum.

An alternative model must be considered in regard to the data of this study, as well as previous studies, on the effects of probes for unpaired bases in superhelical DNA (3, 7). It could be argued that the superhelical molecule contains intact base-pairs that are under strain due to the torsional forces caused by supercoiling (1). This could result in regions that are open transiently with a much higher probabil-

ity than one would expect for normal duplex DNA. Such a situation would lead to the enhanced binding of chemical probes with the coupled loss of superhelical turns, which would lower the free energy of the molecule. Recent ^{3}H-exchange data do not support rapid transient opening of the supercoiled DNA (17). Since superhelical turns are coupled to duplex turns the corresponding loss of both is a necessary condition for the intact model.

There is no question that superhelical DNA is at a higher free energy relative to the open form. However, we believe that an intact model cannot account for the observations described in this and previous studies (3, 7). For example, a coupled unwinding of PM2 DNA by CH_3HgOH should have revealed a decrease in the superhelical content. It has been shown that the initial reaction with HCHO(7) and CH_3HgOH (3) does not produce this expected result. In fact the initial reaction appears to produce an "apparent" increase in supercoiling when measured by the dye-velocity titration (Fig. 1). This unexpected behavior has been repeated with another intercalating drug, hycanthone (A. Chaudhuri and J. Lebowitz, unpublished data). A similar effect is also observed when UV photoproducts are introduced into PM2 DNA at low dose levels (M. Woodworth-Gutai, J. Lebowitz, A. C. Kato, and D. T. Denhardt, manuscript in preparation). A report that a carbodiimide (6, 19) also produces this effect on M13 DNA I has appeared recently (24).

This persistently observed apparent increase in supercoiling, which is a reflection of more dye or drug needed to titrate initially modified DNA to a minimum $s'_{20,w}$ value (presumably zero superhelical content), is a puzzling result (3, 7). However, it may be a consequence of the procedure used to evaluate the superhelical content. An analysis of UV-treated PM2 DNA shows an apparent increase in ethidium bromide needed to reach the minimum $s'_{20,w}$ value in the titration but no change by the dye buoyant density procedure (15) using saturating amounts of propidium diiodide (M. Woodworth-Gutai et al., manuscript in preparation). This suggests that the hydrodynamic behavior of reacted DNA influences the minimum $s'_{20,w}$ value in the velocity-dye titration. In addition, it has been shown recently that superhelical DNA exists as a Boltzman distribution of superhelical molecules differing by one turn (9, 11, 26). Consequently, the interpretation of the behavior of superhelical DNA should take into account the above considerations for a possible explanation of the apparent increases in dye needed to reach the minimum $s'_{20,w}$ value in the titration. Although we do not have a complete interpretation of this behavior, the fact that the buoyant density analysis reveals no change in σ (6, 19) supports the interpretation that initial reactivity does not cause a coupled loss of duplex and superhelical turns. The inability to detect this coupled unwinding due to chemical modification requires that unpaired bases exist that are not linked topologically to supercoils. This view is strongly supported by the results in this study. We have extended our interpretation to a consideration of hairpin structures as the most satisfactory model of the hydrodynamic data ($s'_{20,w}$ versus σ) and the $s'_{20,w}$ behavior upon chemical modification.

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