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Chemical Modification of Simian Virus 40 DNA by Reaction with a Water-Soluble Carbodiimide

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Superhelical simian virus 40 (SV40) DNA I can be modified with *N*-cyclohexyl-*N'*-β-(4 methylmorpholinium)ethylcarbodiimide (CMC). The reaction produces an increase in the sedimentation velocity of DNA I from 21 to 22.5*S* and a decrease in its buoyant density in CsCl from 1.694 to 1.688. A comparable shift in buoyant density is observed in a saturated ethidium bromide-cesium chloride gradient where form II, which has been exposed to CMC, shows no shift. The CsCl-buoyant density data allows us to estimate that 108 mol of CMC are bound per mol of SV40 DNA I. In the subsequent paper an alternative procedure has been used to locate CMC sites, and the extent of the regions available to bind CMC have been measured.

In the previous paper, a model was presented for superhelical DNA in which sufficient supercoiling produces regions of interrupted secondary structure which are capable of forming intrastrand hairpins (18). In order to characterize these altered regions, it would be extremely useful to react unpaired bases in covalently closed structures with a reagent that forms a covalently linked product. This would allow the removal of excess reagent prior to enzymatic studies without the potential interference of the reagent with the respective enzyme during the reaction. The water-soluble *N*-cyclohexyl-*N'*-β'-(4 methylmorpholinium)ethylcarbodiimide (CMC) reacts with unpaired thymine and guanine (Fig. 1) under mild conditions and has been utilized extensively to study nucleic acids suspected of having regions of interrupted secondary structure, i.e., tRNA (5, 7, 12). This reagent has also been used to modify M13 and PM2 DNAs in order to analyze the sense of supercoiling and the angle of unwinding of ethidium bromide (13). Consequently, we have used CMC to probe the structure of simian virus 40 (SV40) DNA I. The results will be presented in two papers. First, we will characterize the effect of modification on sedimentation velocity ($s_{20,w}$) and the buoyant density in CsCl in the absence and presence of saturating ethidium bromide. The second paper (6) reports the mapping of the unpaired regions of SV40 DNA I by using ^{14}C-labeled CMC and endo R·*Hin* D restriction enzyme digestion products to locate the sites of reactivity for the superhelical molecule. These results will be discussed, and they are consistent with a hairpin model that has been previously proposed (18).

[1] This research was conducted while on sabbatical leave from Syracuse University, Syracuse, N.Y. 13210.

MATERIALS AND METHODS

Virus and cell culture. BSC-1 or Vero cells were grown in Eagle basal medium supplemented with 2 mM glutamine and 10% fetal calf serum. Confluent monolayers were infected with SV40 (plaque-purified, small plaque stain) at an input multiplicity of 50 to 100 PFU/cell. Full details for infection have been described previously (15).

Preparation of virus and radioactive SV40 DNA from virions. At 24 to 30 h after infection, either [^{14}C]thymidine (1 μCi/ml, 60 mCi/mmol) or [^{3}H]-thymidine (50 to 100 μCi/ml, 20 to 25 mCi/μmol) from New England Nuclear Corp., Boston, Mass., was added to the infected cells. Media and cells were collected from plastic petri dishes and harvested by centifugation for 3 h at 35,000 rpm in a type 42 rotor. The supernatant fluid was removed, 10.0 ml of phosphate-buffered saline was added to each pellet, and the pellet was dispersed. The cell-virus solution was sonicated for 30 s and brought to a final concentration of 1.3% in deoxycholate and 0.0125% in trypsin. The solution was incubated for 30 min at 37 C and then chilled to precipitate deoxycholate and clarified by centrifugation for 10 min at 10,000 rpm. The supernatant solution was layered over a CsCl cushion of ρ = 1.38. Virus was collected as a band below the interface after a 12-h centrifugation at 4 C at 25,000 rpm in an SW25 rotor. The virus was further purified by density gradient centrifugation using CsCl, ρ = 1.33, and centrifuging the virus for 16 h at 38,000 rpm in an SW39 rotor. The density of the viral band was 1.33. SV40 DNA was prepared from virions by treatment with 1% sarcosyl and heating at 50 C for 1 h. The detergent solution was brought to 1.564 in density using solid CsCl and to a final

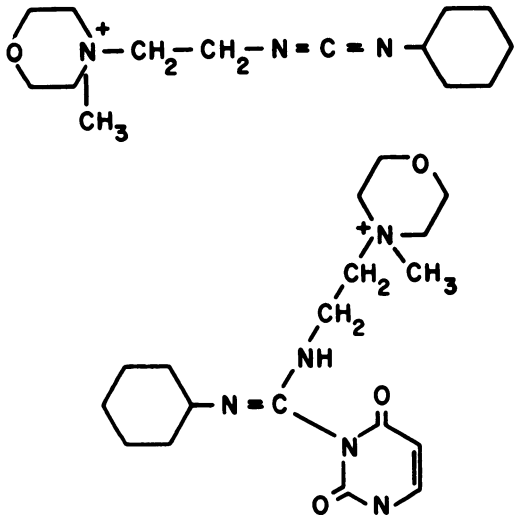

FIG. 1. *Structural formula of N-cyclohexyl-N'-β-(4, methylmorpholinium)ethylcarbodiimide (CMC) and the reaction product with uracil. A similar reaction at the imino site occurs for thymine and guanine.*

concentration of 330 μg of ethidium bromide per ml. This solution was centrifuged at 40,000 rpm in a 50Ti rotor for 48 h at 20 C, and DNA I and II were isolated. Ethidium bromide was removed by extraction with isopropanol, and the DNA solution was extensively dialyzed against the appropriate buffer.

Chemicals and carbodiimide reaction. All chemicals were reagent grade, and buffers were prepared in deionized water. The water-soluble CMC was purchased from Aldrich Chemical Co., Milwaukee, Wisc. The modification of SV40 DNA (50 to 100 μg/ml) was carried out at 37 C using a 500- to 700-fold excess mol of CMC per mol of DNA phosphate in 10 mM $Na_2B_4O_7$, 100 mM Na_2SO_4, pH 8.0. DNA concentrations between 50 to 100 μg/ml were utilized due to the slow rate of the reaction. The reaction was followed by sedimentation velocity changes. Reaction mixtures were exhaustively dialyzed against the appropriate buffer to remove excess CMC before the various centrifugation analyses described below.

Centrifugation. SV40 DNA was layered onto a 12-ml 5 to 30% sucrose gradient containing 10 mM NaCl, 10 mM Tris, and 1 mM EDTA, pH 7.5. Gradients were centrifuged for 16 h at 30,000 rpm or 18 h at 26,000 rpm at 10 C in a SV41 Ti rotor. Sedimentation is from right to left.

Partial specific volume measurement of CMC. The partial specific volume of CMC was determined using a Mettler-Paar DMAO2D precision density meter. The details of the use of this mechanical oscillation technique for partial specific volume measurements have been described by Kratky et al. (10). Ten measurements were made at 20 C; the average partial specific volume of CMC is 0.81387 ± 0.0007.

Equilibrium centrifugation and buoyant density analysis. Solutions for equilibrium centrifugation contained modified CMC SV40 DNA and marker SV40 DNA, 10 mM $Na_2B_4O_7$, pH 8.0, 330 μg of ethidium bromide and enough CsCl per milliliter to bring final density to 1.564 g/ml. These solutions (9 ml/tube) were centrifuged in the Spinco 50 fixed-angle rotor at 36,000 rpm for 48 h at 30 C. In order to evaluate the density shift in CsCl (initial $\rho = 1.70$) due to CMC modification, a density gradient was evaluated after centrifugation in an SW40 rotor. The amount of CMC bound was evaluated using the equation of Bauer and Vinograd (2) $\nu' = \theta(\bar{\upsilon}_3 + \Gamma') - (1 + \Gamma')/(1 - \bar{\upsilon}_4 \Gamma')$, (1) where ν' = grams of reagent bound per gram of DNA; θ is the buoyant density of the modified DNA; $\bar{\upsilon}_3 = 0.479$, the partial specific volume of anhydrous DNA; Γ' = the net hydration of the modified DNA, and $\bar{\upsilon}_4 = 0.814$, the partial specific volume of CMC, which was determined as described above. Since the density shift for CMC modification is extremely small, Γ' can be considered equivalent to the hydration for native SV40 DNA, which is 0.272 g of water per g of DNA (2). The moles of CMC per mole of nucleotide was obtained by multiplying ν' by 430/423, the respective molecular weights of a Cs nucleotide and CMC.

RESULTS

(i) Sedimentation velocity analysis. Studies with probes of the structure of superhelical DNA have shown that the initial reactivity produces an increase in sedimentation velocity (4, 8, 13, 18). Consequently, changes in sedimentation rate were used to examine whether the water-soluble carbodiimide, CMC, reacts with SV40 DNA I.

When tritium SV40 DNA was incubated with a 500- to 750-fold excess of reagent (moles of CMC per moles of nucleotide) at 37 C for 18 to 24 h and examined by sucrose gradient velocity analysis, we consistently observed that the sedimentation velocity of DNA I increases from 21 to 22.5S (Fig. 2). However, there is no change in the $s_{20,w}$ of SV40 DNA II. This result suggests that the superhelical form is far more reactive than the open circle form. However, since we have no measure of the relative structural sensitivities of DNA I and DNA II to CMC modification we cannot conclude that the reaction is highly preferential or specific for the superhelical DNA I based solely on $s_{20,w}$ criteria. Consequently, we examined the change in buoyant density in two different experiments.

(ii) Buoyant density analysis in cesium chloride and cesium chloride-ethidium bromide gradients. Separated DNA I and DNA II were reacted with CMC under the conditions employed above; however, this time respective reactivities were monitored by examining whether a buoyant shift was produced by the modification. It would be expected from the measured density of CMC of 1.229 that the buoyant density would be lowered by CMC binding. The first analysis examined the

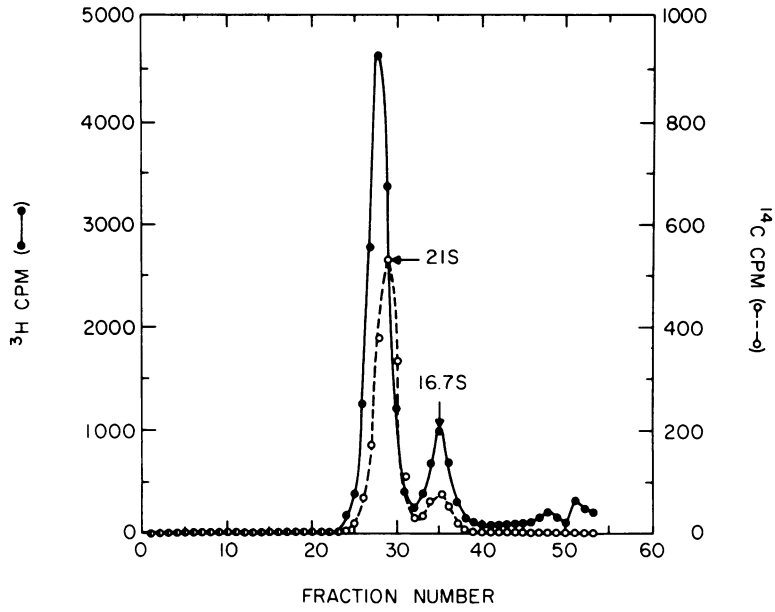

FIG. 2. *^{3}H-labeled SV40 DNA (●) was reacted with CMC as described in the text. The reaction product was then analyzed by velocity sedimentation in a neutral sucrose gradient as described in Materials and Methods. ^{14}C-labeled DNAs I and II (○) were added as sedimentation markers. Sedimentation is from right to left.*

change of buoyant density in a cesium chloride gradient in order to detect the buoyant shift. Figure 3 shows that CMC-reacted SV40 DNA I shifts in buoyant density from 1.694 to 1.688.

In order to detect the possible conversion of DNA I to DNA II as well as the extent of reaction of DNA II alone, the CMC reaction was analyzed in saturation ethidium bromide-cesium chloride gradients. This approach allows us to detect the presence of DNA I and DNA II simultaneously. There is a comparable shift of DNA I (Fig. 4A) to a lower buoyant density value relative to the shift seen in the cesium chloride gradient (Fig. 3). It can be seen that some conversion of DNA I to DNA II has occurred. In this situation the converted product remains modified, as indicated by the density shift relative to the marker. Figure 4B examines the CMC modification of DNA II. Although the marker DNA II is low, there is no indication that DNA II, which has been treated with CMC, reacts with the reagent to the extent observed for DNA I. These results suggest that DNA I contains structural features in which bases are accessible to a bulky, water-soluble carbodiimide (Fig. 1).

(iii) Binding analysis. Using the analysis and equation developed by Bauer and Vinograd (2), which was presented in the Materials and Methods section, we can estimate the amount of CMC bound from the buoyant density measurements and the partial specific volume of the reagent. The binding ratio (moles of CMC per moles of nucleotide) was found to be 0.01. Since SV40 DNA contains approximately 10,800 nucleotides (3), we estimate 108 CMC molecules reacted with SV40 DNA I. Since each CMC reacts with a thymine or guanine, this represents 108 base pairs, which is 2% of the total number of base pairs. This is in excellent agreement with the measurement binding using radioactive ^{14}C-labeled carbodiimide (6).

DISCUSSION

In previous studies on the kinetics of the CMC reaction with various polynucleotides, it was demonstrated that secondary structure plays a major role (5, 7, 12). The reactivity of CMC is very fast with single-stranded DNA and extremely slow with native DNA (12). Consequently, the enhanced reaction of SV40 DNA I and CMC would argue for the existence of unpaired bases in superhelical DNA (4, 8, 18). However, an alternate proposal is that the enhanced reactivity is a result of the high free energy of DNA I, which assists any process that removes superhelical turns (3, 16, 17). Any reaction that produces a coupled loss of duplex and superhelical turns would lower the free energy. If this situation were true for the CMC reaction, we would anticipate a significant loss of supercoils. The extent of the reaction in terms of base pairs is 2%. This unwinding would cause a loss of 50% of the superhelical

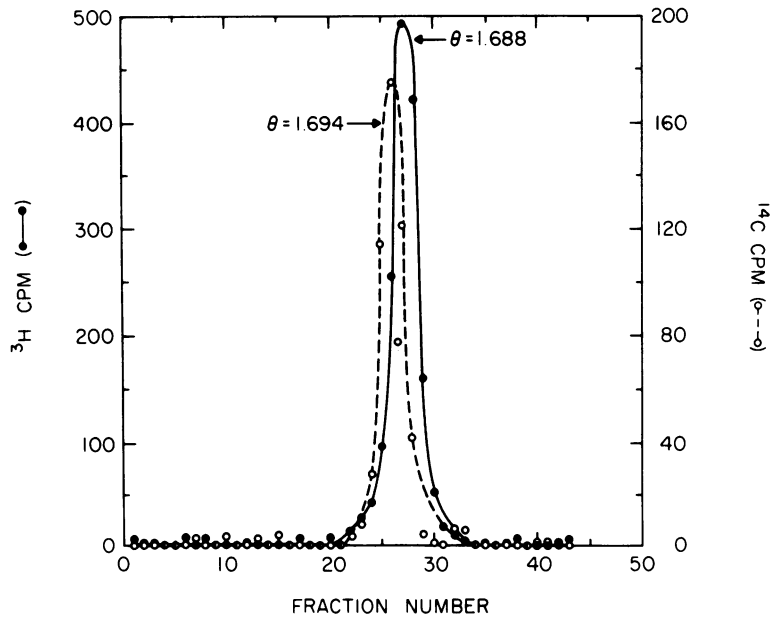

FIG. 3. *Isopycnic centrifugation of ^{3}H-labeled CMC-reacted SV40 DNA (●) in CsCl. ^{14}C-labeled SV40 DNA (O) was added as a marker.*

turns, using a superhelix density of 0.039 for SV40 DNA I (9), and postulating that the secondary structure was intact. This is a conservative estimate, since it is highly likely that CMC would disrupt adjacent base pairs due to the distortion of the helix caused by the large size of this reagent.

The relationship between the relative buoyant separation and superhelix density in cesium chloride-ethidium bromide density was determined by Gray et al. (9). Their results predict an increased separation between DNA I and DNA II of 1.15 relative to untreated SV40 DNA, due to a 50% reduction in superhelix density estimated above. The loss of superhelical turns by chemical modification would require less drug to remove these remaining turns and, consequently, an increased buoyant density for DNA I should result. We have seen that this is not the case, since CMC-modified DNA I shifts toward a lower buoyant density (Fig. 4A). This shift is close to the decrease observed in the CsCl gradient (Fig. 3). CMC lowers the buoyant density as expected from its partial specific volume, and this has been confirmed independently (13). Hence, it appears from the dye-density gradient that superhelical turns are not lost upon reaction. Recently new reports have been published regarding the angle of unwinding of ethidium and, consequently, the superhelix density. Three reports (11, 13, 17) suggest that the angle should be increased to 26°, whereas another (14) claims that a 17° angle is correct. This latter value was determined using linear DNA (14), whereas the other studies used circular DNA (11, 13, 17). Computer modeling supports the lower value because the most favorable conformation for drug intercalation gives a helical unwinding of 18° (1). It was suggested in this study that circular DNA may contain different conformations, and this might explain the observed difference in estimated unwinding angles. If the 26° unwinding angle is accepted, SV40 DNA I would have a superhelix density of −0.085. This would reduce the effect of CMC modification and cause the removal of only 25% of the supercoils. Even under these conditions, it isn't possible to reconcile the lower buoyant density of modified SV40 DNA I and the above significant loss of supercoils occurring at the same time, since the latter should cancel the change produced by CMC if we apply the analysis according to Gray et al. (9). This view is further supported by the fact that the initial reaction of PM2 DNA with CMC produces no loss of superhelical turns, as measured by sedimentation velocity-dye titration (A. Chaudhuri, G. Kitos, and J. Lebowitz, unpublished data), and that M13 DNA has been reported to show an apparent increase in superhelical content similar to that seen for the HCHO and CH_3HgOH (4, 8, 18). The similarity of the effects produced by CMC, HCHO, and CH_3HgOH point to structural features of superhelical DNA that are a result of supercoiling. It is highly unlikely,

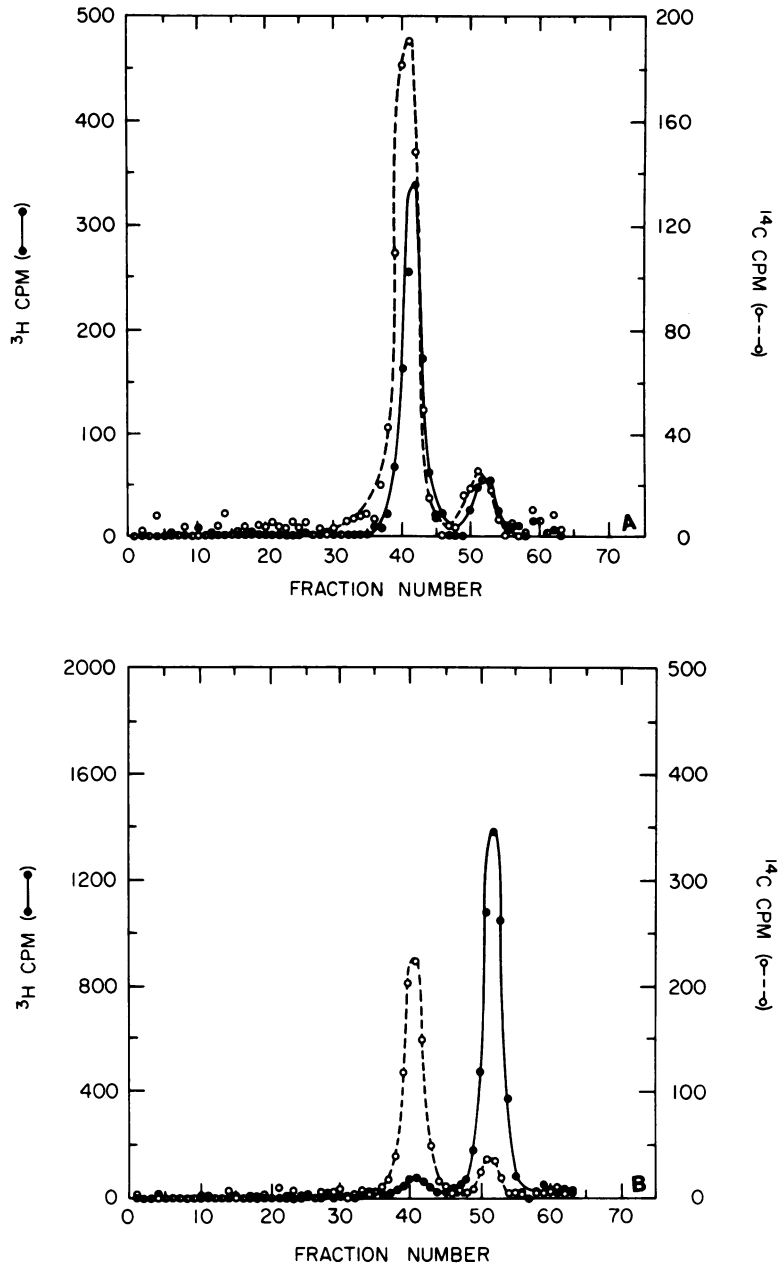

FIG. 4. *(A) The fractionation of ^{3}H-labeled CMC-reacted SV40 DNA (●) by isopycnic cesium chloride-ethidium bromide centrifugation. ^{14}C-labeled SV40 DNA (○) was added as a marker. ^{3}H-labeled CMC form II (○) created upon reaction of pure form I. (B) The fractionation of ^{3}H-labeled form II (●) in cesium chloride-ethidium bromide after exposure to CMC under reaction conditions identical to these causing modification of DNA I.*

from all the considerations raised above, that superhelical DNA can be considered to be an intact duplex. We interpret the results of this study to be consistent with the view that supercoiling produces regions of unpared bases. The cumulative data support a model, proposed in the previous paper (18), that these interrupted regions can form hairpin structures. The sedimentation velocity increase is a result of the disruption of these hairpin sites which produces coil regions that increase the flexibility of the DNA. It is interesting to note that the reaction

of PM2 DNA with CMC produces a much larger percentage of change in sedimentation velocity than SV40 DNA (14; A. Chaudhuri et al., unpublished data). This is consistent with the much larger superhelical density for PM2 DNA and the production of more hairpin regions.

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