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Osmium tetroxide: a new probe for site-specific distortions in supercoiled DNAs

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ABSTRACT

Supercoiled plasmids Col E1 and cDm 506 (a Col E1 derivative carrying the *D. melanogaster* histone gene repeat) were treated with OsO_4 in presence of pyridine and the reaction products were analyzed using different approaches. Gel electrophoresis showed that OsO_4 binding to supercoiled DNA induced its relaxation without nicking. The amount of osmium bound to DNA (as determined electrochemically) increased with the extent of DNA relaxation. As a result of osmium modification of supercoiled cDm 506, a single denaturation "bubble" was observed in the electron microscope. Mapping of the osmium binding site by S1 nuclease cleavage followed by restriction enzyme digestion has revealed one major site in the intergenic spacer between the H1 and H3 histone genes of *D. melanogaster*. This site differs from the site cleaved by S1 nuclease in supercoiled DNA in the absence of osmium.

INTRODUCTION

Osmium tetroxide in the presence of pyridine and other ligands binds through addition across the 5,6 double bond of the pyrimidine rings in single-stranded nucleic acids (1-4). Recently we have shown (5,6) that under stuitable conditions osmium binds also to distorted regions in the double-helical DNA, e.g. in the vicinity of single-strand interruptions and thymine dimers. The amount of osmium bound to DNA can be determined with high sensitivity by modern electrochemical methods (7,8) such as differential pulse polarography (6), and stripping voltammetry (9).

It has been shown by many authors that supercoiled DNAs are sensitive to single-strand specific (region-specific) nucleases (10-18) and react with some chemical agents such as formaldehyde, carbodiimide and methyl-mercury hydroxide (12-14) more readily than do relaxed circles or linear molecules. These properties of supercoiled DNAs have been explained by some authors (10,12,14,15) by the presence of cruciform structures at specific sites induced by superhelicity of the DNA molecule.

In this paper we have attempt to use osmium tetroxide as a structural

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probe of supercoiled DNAs, and we have found that osmium binds to specific sites which can be recognized by nuclease S1 and visualized as a denaturation "bubble" in the electron microscope.

MATERIALS AND METHODS

Plasmid isolations were performed as previously described (19,20). Nuclease S1 was isolated from Takadiesterase (21) or purchased from Worthington. Osmium tetroxide from Fisher Scientific Co. was kindly donated by Professor H.M. Sobell, Sephadex G 50 medium was purchased from Pharmacia Fine Chemicals. Other chemicals were of analytical grade.

DNA modification with osmium tetroxide

A typical reaction mixture contained supercoiled Col E1 DNA at a concentration of 60 μg/ml (cDm 506 DNA at a concentration of 20 μg/ml) in 7.5 mM NaCl, 0.75 mM sodium citrate, pH 7.0 (SSC/20) or in 5 mM Tris buffer with 0.5 mM EDTA pH 7.9 (TE buffer), 1×10^{-3} M OsO_4 and 4 % (v/v) pyridine. The reaction mixture was incubated for 21 h at 26° C, and the reaction was terminated by passing the mixture through the Sephadex G 50 medium column or by ethanol precipitation. If the product was to be used for electrochemical measurements dialysis was carried out against SSC/20 for 20 hours at 4°C. In some experiments the reaction mixture was directly applied to the gel and electrophoresed; the results of these experiments showed no difference as compared with those where the product was purified by ethanol precipitation and/or dialysis.

S1 nuclease digestion was performed under conditions used by Lilley (13). Electrophoresis was carried out in 1 percent agarose slab gels. Following electrophoresis the gels were stained with ethidium bromide and photographed.

For determination of osmium content in Col E1 DNA the differential pulse stripping voltammetry (9) was used. Measurements were performed with a Model PAR 174 A Polarographic Analyzer under conditions previously described (6,9). The samples for electron microscopy were spread by the formamide spreading technique (22) and the photographs were taken with a Zeiss E. M. 109.

RESULTS

Osmium tetroxide induces relaxation of superhelical DNAs without nicking

Circular DNAs of plasmids Col E1 and cDm 506, a Col E1 derivative carrying the D. melanogaster histone gene repeat, were treated with OsO_4 in

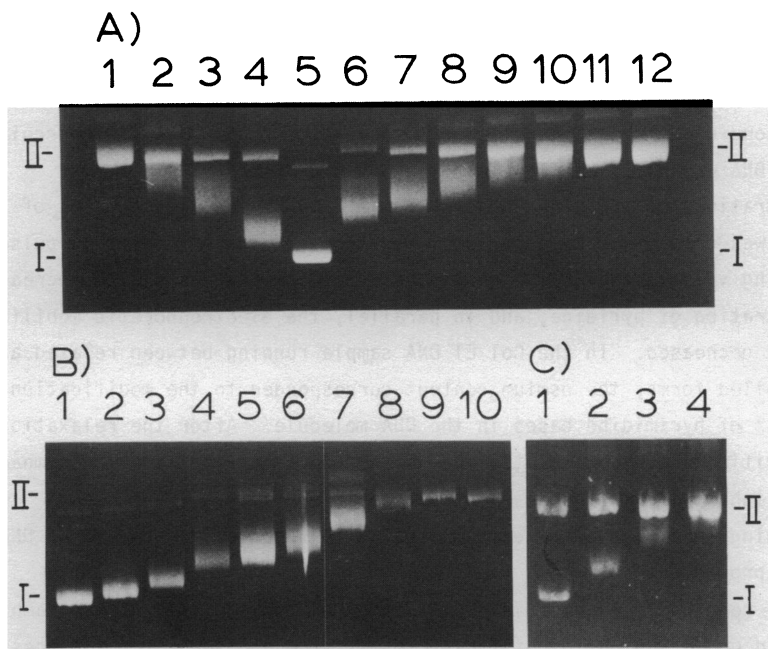

Fig. 1 Conditions for plasmid cDm 506 (A,B) and Col El (C) OsO_4 modification (A) 1-4 Dependence on OsO_4 concentrations (4% pyridine, 26°C, 21 hours):1, 1×10^{-3} M; 2, 5×10^{-4} M; 3, 2×10^{-4} M; 4, 1×10^{-4} M. (A) 6-12 Dependence on pyridine concentrations (5×10^{-4} M OsO_4, 26°C, 21 hours); 6, 1%; 7, 2%; 8, 3%; 9, 4%; 10, 5%; 11, 6%; 12, 7%. Lane 5, unmodified supercoiled DNA. (B) Time course (1×10^{-3} M OsO_4, 4% pyridine, 26°C): 1, 0 min; 2, 1 min; 3, 2 min; 4, 4 min; 5, 8 min; 6, 16 min; 7, 60 min; 8, 120 min; 9, 240 min. Lane 10, unmodified relaxed DNA. (C) Dependence on temperature (1×10^{-3} M OsO_4, 2% pyridine, 21 hours): 2, 4°C; 3, 26°C; 4, 37°C. Lane 1, unmodified DNA. Each well contained 1 µg (A,B) or 0.6 µg (C) of DNA incubated in 50 µl (A,B) or 20 µl (C) of the reaction mixture under the indicated conditions. The positions of supercoiled and nicked forms of plasmid DNA are indicated by I, and II, respectively.

the presence of pyridine and their electrophoretic mobility was measured. This treament (1×10^{-3} M OsO_4, 4% pyridine, 21 h at 26°C) resulted in the shift of all supercoiled DNA into the relaxed form, with little or no change in the mobility of the nicked relaxed form (data not shown). The change in the mobility of the supercoiled DNAs does not appear to be due to a simple chain scission, since osmium-relaxed Col El DNA did not denature as a result of heating to denaturation temperature, while its nicked form was denatured under the same conditions. Denaturation was followed by gel electrophoresis and differential pulse polarography (8) (data not shown).

By changing the OsO_4 and pyridine concentrations (Fig. 1 A), the reaction time (Fig. 1 B) and the temperature (Fig. 1 C) the mobility of the reaction product can be made to vary almost continuously from that corresponding to the supercoiled DNA to that of relaxed DNA. Supercoiled Col E1 DNA was modified (1×10^{-3} M OsO_4, 21 h at 26°C) at various concentrations of pyridine ranging from 0.2 to 5%, and the content of osmium was determined in each sample by means of the differential pulse stripping voltammetry (9). The amount of osmium increased with increasing concentration of pyridine, and in parallel, the electrophoretic mobility of the DNA decreased. In the Col E1 DNA sample running between relaxed and supercoiled forms, the osmium content corresponded to the modification of about 4% of pyrimidine bases in the DNA molecule. After the relaxation of the modified DNA was completed the uptake of osmium continued, accompanied by a slight increase in the electrophoretic mobility. Experiments concerning the kinetics of osmium binding to supercoiled and linear DNAs are in progress.

The continuous changes in the electrophoretic mobility of DNA samples modified under various conditions (Fig. 1) strongly support our suggestion that nicking cannot be the reason for the observed DNA relaxation. We therefore suggest that osmium binding might stabilize denatured regions in supercoiled DNAs (10-18). Osmium binding results in the formation of permanently unpaired bases (E. Paleček, unpublished) and should thus produce a coupled loss of duplex and superhelical turns. It can be expected that such a process will be assisted by the high free energy of the supercoiled (underwound) molecule, until the free energy is substantially lowered and the molecule becomes partially or fully relaxed. If this is correct osmium binding to supercoiled DNA should result in the formation of many small denatured sites or one relatively large single-stranded DNA region.

Modification of superhelical DNA results in a single denaturation "bubble" visible in the electron microscope

In order to obtain some more information about the structural changes brought about by osmium modification of supercoiled DNA, cDm 506 DNA was examined in the electron microscope. The osmium-modified DNA displays a single denaturation "bubble" and looks less supercoiled than the unmodified control (Fig. 2 a,b). The size of this "bubble" appears to be similar or slightly larger than that observed with the same DNA after treatment with the *E. coli* single-strand binding protein (19). Similar treatment of the

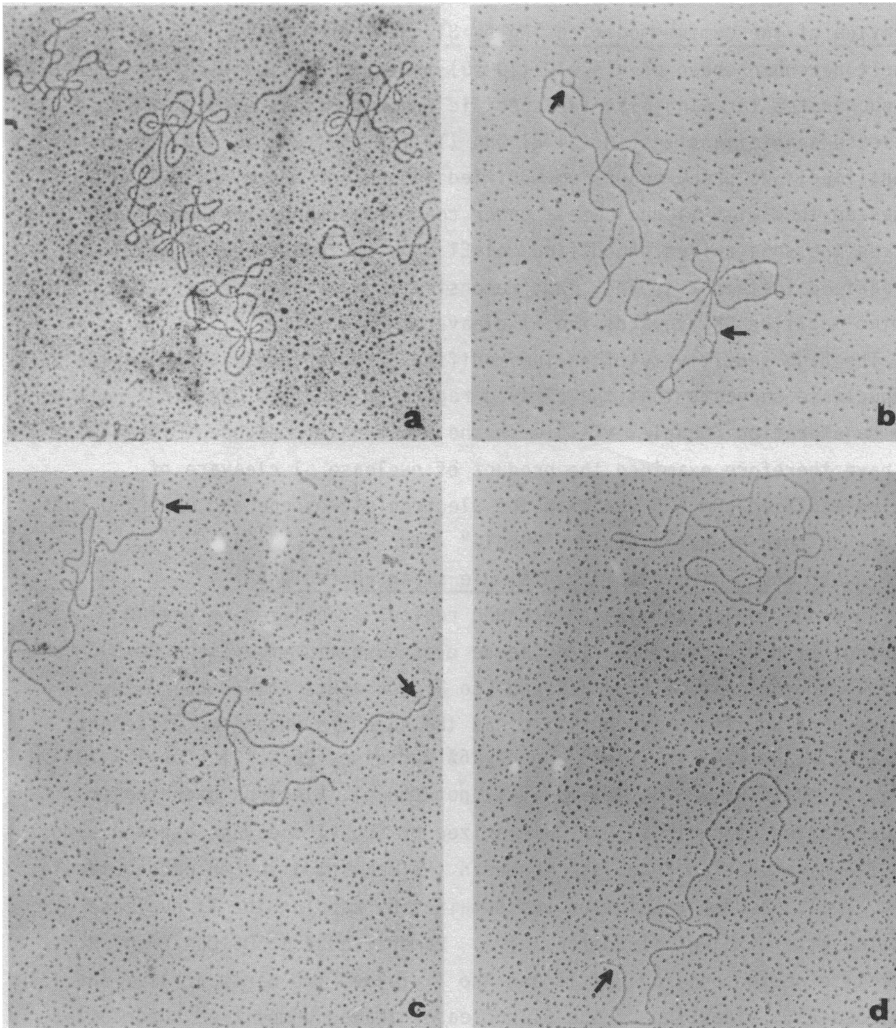

Fig. 2 Electron micrographs (35000x) of the osmium-modified plasmid cDm 506 DNA and of its fragments. a, Osmium-free supercoiled DNA; b, osmium-modified DNA; c, osmium-modified DNA after Eco RI cleavage; d, osmium-modified DNA after S1 cleavage. 1 µg of DNA was treated under the same conditions as Fig. 1 A, lane 6, and precipitated with ethanol (in presence of Na acetate 200 mM pH 7.0). The pellet was resuspended in 15 µl of TE buffer. Aliquots of 5 µl were taken, and the medium adjusted for Eco RI (10 units, 60 minutes, 37°C) digestions into 15 µl. The reactions were stopped by addition of 15 µl of phenol and the DNA was purified by phenol and chloroform extractions before spreading. The samples were spread by the formamide spreading technique. All the photographs were taken with a Zeiss E.M. 109.

relaxed circular DNA did not yield a denaturation "bubble".

Cleavage of the osmium-modified DNA by S1 nuclease

It has been shown previously (10-13) that supercoiled circles may act as substrates for single-strand-specific endonucleases. Recently S1 nuclease sensitive sites in Col E1 DNA (13,18) and cDm 506 DNA (19) were identified. We prepared osmium-modified samples of these two DNAs and digested them with S1 nuclease. Under conditions where the nuclease only nicked the supercoiled unmodified Col E1 DNA, the modified DNA was linearized (data not shown), thus demonstrating a higher sensitivity of the osmium-modified DNA towards the S1 cleavage.

The high sensitivity of osmium-modified circular duplex DNA towards the S1 nuclease suggests that the cleaved region in modified DNA might well be the denaturation "bubble" visible in the electron microscope (Fig. 2 b). We have therefore examined the product of nuclease S1 cleavage of osmium-modified cDm 506 DNA with the electron microscope and observed cuts on one or both branches of the "bubble" (Fig. 2 d).

Mapping of the osmium-stabilized single-stranded regions

In order to locate single-stranded regions stabilized by osmium binding, we restricted the S1 nuclease cleaved osmium-modified cDm 506 DNA. Gel electrophoresis of Hind III and Eco RI fragments showed one major osmium binding site. Its position was the same one under two different reaction conditions (5×10^{-4} M OsO_4, 6% pyridine, 26°C, 21 h or 1×10^{-3} M OsO_4, 4% pyridine, 26°C, 1.5 h) (data not shown). Electron microscopic visualization of modified DNA, linearized by Eco RI cleavage, showed almost all the denaturation "bubbles" to be in the same position on each molecule (Fig. 2 c). Better resolution was obtained by mapping the S1 nuclease cutting site with Bgl II and/or Xho I. Unmodified cDm 506 DNA (Fig. 3, lane 1) digested only by Bgl II and Xho I yielded two bands at 11.7 and 1.1 kb (lane 2). Digestion of the S1 nuclease cleaved unmodified supercoiled DNA with the same restriction enzymes (lane 3) produced two new bands of 10.5 and 1.2 kb (the latter overlapped with the 1.1 kb band), both derived apparently from the 11.7 kb fragment (lane 2). There was still some remaining 11.7 kb band (lane 3) probably generated from the relaxed DNA which was not cleaved by S1 nuclease. These data are in agreement with the previously mapped S1-sensitive site in the supercoiled cDm 506 DNA (19).

Cleavage of the osmium-modified DNA (lane 5) by S1 nuclease produced a linear 12.8 kb fragment (lane 6) which had the same mobility as the unmodified linearized DNA. The subsequent incubation with Xho I yielded a

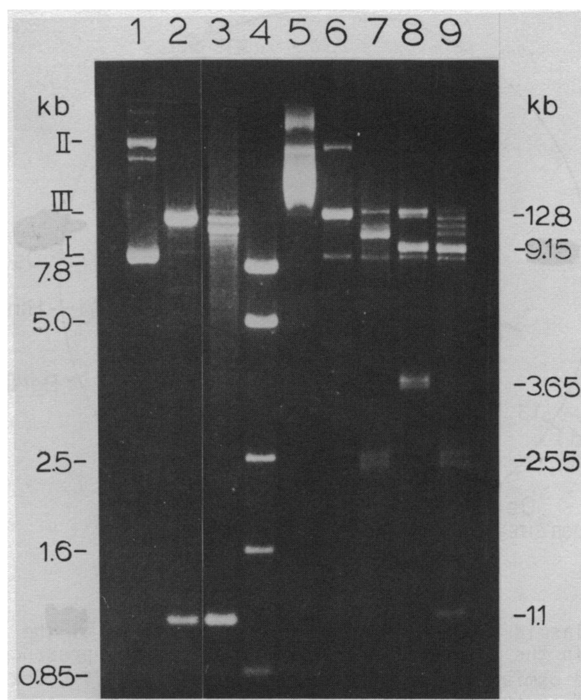

Fig. 3 Mapping of the osmium-stabilized simple strand regions in the plasmid cDm 506. The lanes were loaded with DNA (1-4) or OsO_4 modified DNA (5-9) that had been treated with the following enzymes: 1, none; 2, Xho I and Bgl II; 3, Xho I and Bgl II after S1; 4, molecular weight markers (Hind III products of cDm 506: 7.8 and 5.0 kb; Hae III products of ds M13:2.5, 1.6 and 0.85 kb); 5, no enzyme (overloaded); 6, S1; 7, Xho I after S1; 8, Bgl II after S1; 9, Xho I and Bgl II after S1. Each well contained 1 μg of DNA incubated with 1×10^{-3} M OsO_4, 2° pyridine at 26°C for 90 min. The reaction was stopped by ethanol precipitation and the DNA was resuspended in 10 μl of TE buffer, and 10 μl of 60 mM sodium acetate buffer (pH 4.6)/100 mM sodium chloride/2 mM zinc chloride, plus 0.1 unit of S1 endonuclease were added. S1 digestion proceeded for 30 min at 37°C. The reaction was terminated by phenol, the DNA was purified by phenol and chloroform extraction, precipitated by ethanol and resuspended in suitable buffers for the various restriction enzymes (10 units, 37°C, 1 hour). The position of linear form of plasmid DNA is indicated by III, other symbols as Fig. 1.

10.25 kb and paired 2.6 - 2.5 kb fragments (lane 7). Incubation with Bgl II (subsequent to S1 digestion) yielded 9.15 kb and a pair of 3.7 - 3.6 kb fragments (lane 8). When nuclease S1 was followed by digestion with both restriction enzymes, fragments of 9.15 kb, 2.6 - 2.5 kb and 1.1 kb appeared (lane 9). As shown in Fig. 4, the S1-generated fragments unambiguously

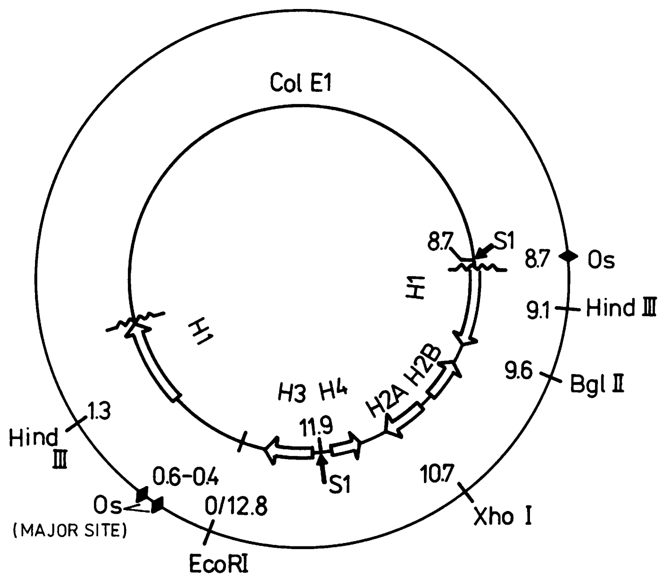

Fig. 4 Map of plasmid cDm 506 DNA showing the positions of the nuclease S1 cleavage sites in the absence (inner circle) and in the presence of covalently bound osmium (outer circle), major site at 0.4-0.6 kb, and minor site at 8.7 kb from the Eco RI site. The positions of the five histone genes and of restriction sites used in the mapping are also shown.

locate the major S1 nuclease-sensitive site lying in the spacer between histone genes H1 and H3. A small fraction of the molecules were also cleaved at another minor site originating the 8.2 kb fragment in lanes 6 to 9. This minor S1 nuclease site is near or at the minor site obtained by the S1 nuclease on unmodified supercoiled DNA (19).

DISCUSSION

Experimental evidence obtained in recent year (10-19) clearly demonstrates that the introduction of superhelical turns into the DNA molecule results in local changes in its secondary structure. The altered regions can be characterized as more open compared to the rest of the molecule. Mapping of the sites of cleavage of some supercoiled DNAs by single-strand specific endonucleases has shown (13-18) that these sites are aligned with inverted repeat regions in the nucleotide sequence, and in Col E1, pBR322, and pVH51 DNAs (14,18) S1 nuclease cleavage at the loop of the inverted repeat was demonstrated. In the recombinant plasmid containing adenovirus DNA sequences the cleavage site at the stem of the hairpin

region where TATA box is located was recently identified (17). Moreover, it has been suggested that S1 nuclease also cleaves open regions at the junction between right-handed and left-handed segments in the supercoiled DNA molecule (24).

Distorted regions with exposed bases at specific sites of the DNA molecule represent potential recognition sites with important biological functions. Since, until recently, single-strand specific nucleases have been the only probes applied for identification of site-specific distortions in the supercoiled DNA molecules, development of other probes is desirable.

Osmium binding as a new probe for distorted regions in superhelical DNAs

It has been shown in this paper that OsO_4: pyridine binds to supercoiled DNAs (causing their relaxation without nicking) and forms single-stranded regions at specific sites (Figs. 2, 4). These sites are sensitive to S1 nuclease and thus can be easily located and mapped by restriction nucleases (Fig. 3). Moreover, as a result of osmium binding to supercoiled DNA a single denaturation "bubble" is observed in the electron microscope (Fig. 2). This "bubble" remains stable (without fixation) even after digestion by restriction nucleases yielding thus a possibility of binding site mapping without gel electrophoresis and nuclease S1 cleavage.

In addition to the single-strand specific nucleases, osmium may thus represent another probe for local distortions in DNA structure. As compared to nucleases this chemical probe has some advantages: (a) it can be used over a wider range of conditions including those under which certain purine-pyrimidine sequences are expected to undergo transitions to Z and other DNA forms (25-28), (b) it is potentially useful as a probe for DNA structure *in situ* (6), (c) individual osmium atoms may be visualized in the electron microscope by the technique introduced by Beer (1-4, 29). Osmium tetroxide does not cleave DNA strands and its interaction with the distorted regions is probably much simpler than that of complex nuclease molecules.

Specific sites of osmium binding in superhelical cDm 506 DNA

We have demonstrated that OsO_4: pyridine binds to one major site in the supercoiled cDm 506 DNA located in the intergenic spacer between H1 and H3 histone genes of *D. melanogaster* (Fig. 4). This site is cleaved by S1 nuclease and differs from that mapped by S1 nuclease in the absence of osmium by more than one kb. Thus, structural features recognized by OsO_4 are different from those recognized by S1 nuclease. It cannot be excluded

that this difference is due to different environmental conditions during the process of recognition of a specific site by the nuclease and the chemical agent. S1 nuclease digestion was performed at pH 4.6 in the presence of 1×10^{-3} M Zn^{++}, i.e. under conditions where protonation of bases may be not negligible, and where Zn^{++} might exert some effect on the DNA structure (1,2), while osmium binding took place close to the neutral pH (the subsequent S1 digestion was performed at a relaxed DNA molecule in which the S1 cutting site was determined by the preceding chemical reaction). Before the influence of the environmental conditions on the specificity of osmium binding is tested it appears at least equally probable that the sites mapped by osmium and nuclease S1 (in the absence of osmium) represent different distortions (either permanent or transient) existing in supercoiled cDm 506 DNA (regardless of pH between 4.6 and 7.9) which are recognized by different agents. A support for the latter explanation might be seen in the fact that the sites of binding of E. coli single-strand binding protein on the same DNA differed both from the sites of S1 nuclease cutting and osmium binding on the supercoiled DNA (19). This finding implies that there might be a large number of locally distorted regions in cDm 506 DNA.

When this work was completed two papers appeared (30,31) showing that bromoacetaldehyde selectively modifies supercoiled DNAs in the region sensitive to S1 nuclease cleavage. The reaction was performed at pH 4.5. Bromoacetaldehyde forms adducts with adenine and cytosine residues at nitrogen atoms involved in the Watson-Crick hydrogen bonding in contrast to the OsO_4 reaction which occurs outside the hydrogen bonding system at the 5,6 double bond of the pyrimidine ring. The results obtained with these two chemical agents may thus complement each other and give a more detailed picture of the complex DNA structure.

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