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Site-specific cleavage of supercoiled DNA by ascorbate/Cu(II)

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ABSTRACT

We have investigated ascorbate/Cu(II) cleavage of double-stranded DNA in the presence and absence of DNA negative torsion. We found that ascorbate/Cu(II) cleavage shows a site-specificity that is dependent on negative torsion and is influenced by the nature of the salt, ionic strength, and pH. This provides strong evidence for involvement of local DNA conformation in ascorbate/Cu(II) specific cleavage sites, that differs from the previous reports on cleavage of linear double-stranded DNA and secondary structures assumed by single-stranded DNA. The data indicate specific binding of Cu(II) ions to sites in the negatively supercoiled DNA. Fining mapping of the cleavage sites does not reveal any known DNA conformation, nor does it indicate any sequence identity among the sites cleaved. However, identification of a major site of cleavage of supercoiled DNA at physiological ionic strength, pH and temperature, along with fact that ascorbate and Cu(II) are normal cell constituents, suggests the torsion-dependent, site-specific interactions could have biological significance.

INTRODUCTION

Ascorbic acid is known to be selectively toxic to several malignant cell lines (1, 2) and cause virus inactivation (3, 4). Ascorbic acid has also been shown to increase the frequencies of mutagenesis, carcinogenesis and sister-chromatid exchanges (5, 6, 7). All these activities can be significantly enhanced in the presence of micromolor concentration of copper ions (1, 3). Chiou (2) demonstrated that mixing of ascorbic acid and Cu(II) very efficiently generated reactive oxygen species which had protein and DNA scission activities. Free hydroxyl radicals were identified to be responsible for initiating cleavage of DNA strands by abstracting hydrogen atoms from the deoxyriboses along the DNA backbone.

By using linearized, end-labeled DNA fragments from plasmid pBR322, Chiou et al (8) showed some sequence specificity of the DNA-scission activity of ascorbate in the presence of copper ions. Tracts of purines were more frequently cleaved than the rest of the sequences. However, explanation of the relatively higher sensitivity of purine tracts toward ascorbate/Cu(II) could be very difficult due to the complicated interactions between copper ions and DNA (9, 10). Copper ions are known to interact with both phosphates and the bases through coordination binding (9) which could be strongly affected by DNA sequences and conformation (11). One obvious question is whether the ascorbate/Cu(II) cleavage of DNA involves binding of copper ions to some specific sites on a DNA molecule which could account for the scission specificity. Recently, Kazakov et al (12) investigated ascorbate/Cu(II) scission of single-stranded DNA. An unique cleavage site was found in a 302 nucleotide DNA fragment. The high cleavage specifity was attributed to tight binding of copper ions and localized generation of hydroxyl radicals at a sequence which was shown

to adopt a stem-loop conformation Therefore, involvement of DNA secondary structure in the site-specific cleavage of DNA by ascorbate/Cu(II) was proposed.

Torsional stress associated with negatively supercoiled DNA may induce DNA conformational changes (13, 14), such as cruciform structure at an inverted repeat sequence (15). In order to correlate site-specific DNA cleavage by ascorbate/Cu(II) with altered DNA conformation, we investigated the DNA-scission specificity of double-stranded DNA in the presence or absence of negative torsion. In this paper, we report a negative torsion-dependent, site specific cleavage of DNA by ascorbate/Cu(II). The cleavage sites were identified by fining mapping. The ascorbate/Cu(II) cleavage of double stranded-DNA under torsional stress was also studied as functions of salt type, salt concentration, pH and presence or absence of the strong chelator EDTA, and compared with DNA cleavage by other hydroxyl radical generating systems.

MATERIALS AND METHODS

Plasmid and plasmid preparation

A 512 bp HinfI fragment of the J-C intron of mouse immunoglobulin κ-gene (16) was blunt-ended by Klenow fill-in and inserted into the SmaI site of pUC12-W1(15) to generate pUC12-W1/512. Supercoiled plasmid DNA was grown and prepared as described (17).

Ascorbate/Cu(II)cleavage of supercoiled plasmid DNA

1 μg DNA was incubated in a buffer containing 10 mM Hepes, 1 mM ascorbic acid (Sigma, sodium salt), 1 μM CuSO4, 0.5 mg/ml polyd(I) and 0.005% H_2O_2 (Sigma, 30% solution) at 4°C for 30 minutes. Though hydrogen peroxide can be generated when oxygen molecules are reduced by ascorbic acid (3, 12), low concentration of H_2O_2 was included as catalyst to accelerate the cleavage reaction. pH, salt type and salt concentration varied with the purpose of each experiment. Solutions of ascorbic acid and H_2O_2 were made fresh right before use. The mean number of single-strand scissions were limited to less than one per DNA molecule (only half of the supercoiled DNA molecules were converted to relaxed circles and no linearized molecules were generated in our experimental condition). To show site specific DNA scissions, the relaxed DNA molecules were linearized with either EcoRI or HindIII and followed with S1 nuclease digestion. S1 nuclease is from BRL and the reaction was carried out in buffer recommended by the vendor. Since S1 nuclease only cleaves the phosphodiester bonds opposite to the ones initially cleaved by ascorbate/Cu(II), the size of the double-stranded DNA fragments generated after S1 nuclease treatment reflect the distance between the sites of ascorbate/ Cu(II) scissions and EcoRI or HindIII restriction sites. Agarose gel electrophoresis was used to resolve the DNA fragments which allowed a low resolution mapping of the sites of DNA scissions.

EDTA-Fe(II) cleavage of supercoiled DNA

The hydroxyl radical cutting reaction was carried out as described by Tullius and Dombroski (18). Different aliquots of 100 μM Fe(II) per 200μM EDTA were added to a solution containing 10 mM Hepes, 1 μg DNA, 0.5 mg/ml polyd(I) and H_2O_2 of a final concentration of 0.005%, and the mixture was incubated at 4°C for 30 minutes. pH and ionic strength of the solution vary with experiments. After EDTA-Fe(II) treatment, the DNA was processed as described above.

High resolution mapping of DNA scissions

The DNA treated with ascorbate plus copper ions were cut on both sides of the scissions with proper restriction enzymes. The restriction products were resolved on agarose gels. After ethidium bromide staining, the bands of DNA fragments containing scissions were cut out and DNA was isolated by electroelution. One of the strand of a DNA fragment

was selectively labeled either with ^{32}P-γ-dATP (ICN, >3000 Ci/mmole) by T4 kinase (BRL) or with ^{32}P-α-dNTP (ICN, >3000 Ci/mmole) by DNA polymerase (BRL, Klenow). The labeled DNA fragment was denatured by adding one volume of 98% deionized formamide, 10 mM EDTA, 0.2% bromophenol blue and 0.2% xylene cyanol) and heating at 95°C for three minutes, and followed by sequencing gel electrophopresis and autoradiography (19).

RESULTS

Ascorbate/Cu(II) cleavage of supercoiled DNA in different conditions

Figure 1A shows a map of plasmid pUC12-W1/512. Restriction sites which were used for later mapping of DNA scissions are indicated. Figure 1B outlines the strategy of detecting site-specific cleavage of supercoiled plasmid DNA by ascorbate and copper ions. Torsion-induced local alterations of DNA conformation may quickly return to duplex when torsion is released by a single nicking event. If the ascorbate/Cu(II) system recognizes an altered DNA conformation as a highly prefered target, the target will no longer exist after the first nicking event and the subsequent nicking of the relaxed DNA will be more or less random. Therefore, we selected conditions which generated less than one nick per DNA molecule as described in Materials and Methods. The nicked DNA was then digested to completion with either EcoRI or HindIII to generate linear DNA molecules about half

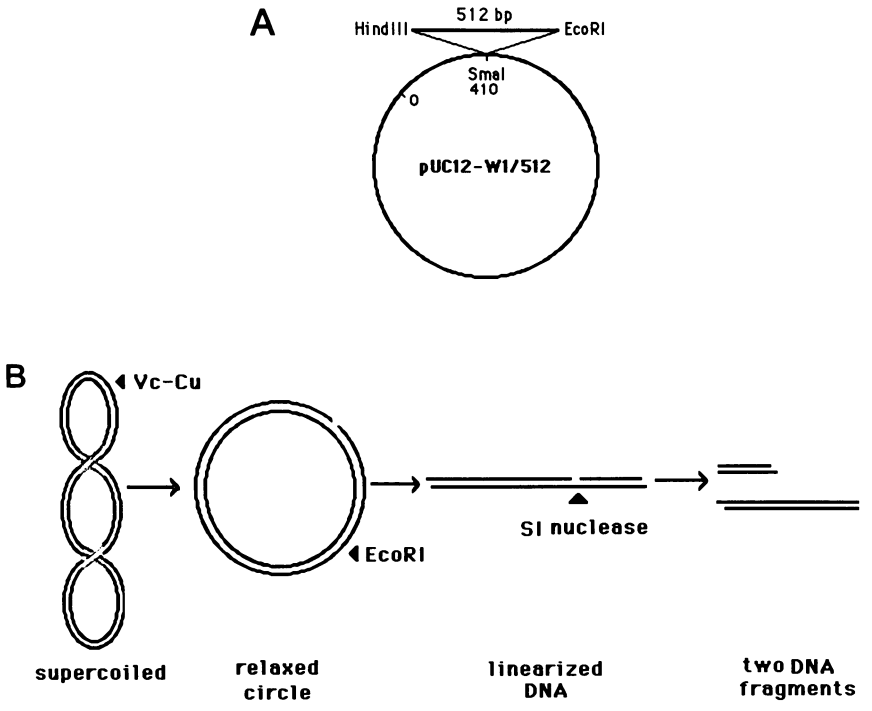

Figure 1. A: *Construction and map of pUC12-W1/512.* An enhancer-containing 512 bp HinfI fragment of the J-C intron of mouse immunoglobulin κ-gene was blunt-ended and inserted into the SmaI site of pUC12-W1. B: *Strategy of detecting site-specific cleavage of supercoiled DNA by ascorbate/Cu(II).* Vc = ascorbic acid;

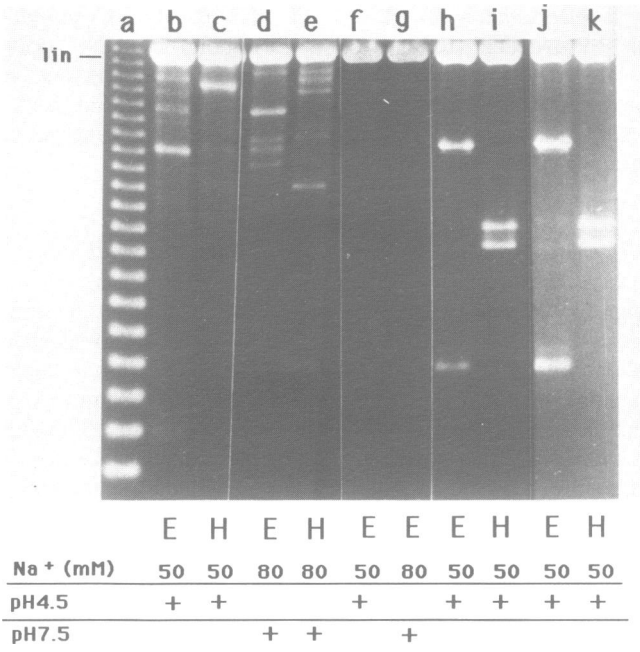

Figure 2. A: *Vc/Cu(II) cleavage of supercoiled and linearized plasmid DNA*. Cleavage reactions were carried out as described in Materials and Methods and the pH and NaCl concentration of each reaction are listed below lanes. The restriction enzyme (E = EcoRI, H = HindIII) used to linearize DNA is shown at the bottom of each lane. Lane a: 123 bp ladder as size marker. Lanes b to e: supercoiled DNA was treated with Vc/Cu(II) followed by restriction cleavage and S1 nuclease digestion. Lanes f and g: supercoiled DNA was linearized with EcoRI before Vc/Cu(II) treatment and S1 nuclease digestion. Lanes h to k: supercoiled DNA was first digested with either S1 nuclease (lanes h and i) or Mungbean nuclease (lanes j and k) followed with EcoRI or HindIII restriction. Lin = linearized plasmid.

of which had a single nick on one of the two strands. To visualize the sites of nicking, the linear DNA was treated with S1 nuclease which cleaves the phosphodiester bond opposite the nick on the complementary strand to generate two DNA fragments. DNA fragments produced after S1 nuclease treatment were resolved on agarose gels. If the initial nicking by ascorbate/Cu(II) was at specific sites, discrete bands should be observed on agarose gels.

Figure 2 shows the ascorbate/Cu(II) cleavage of both supercoiled and linearized DNA. A comparison of the cleavage of the same plasmid by two DNA single-strand specific S1 and Mungbean endonucleases is also presented. Reaction conditions and the restriction enzymes used to linearize nicked DNA are listed below the lanes. pH 4.5 was initially chosen for comparison with the cleavage patterns of the same DNA by S1 and Mungbean nucleases, both of which function at pH 4.5 and are often used to probe DNA conformational changes(13, 14). Also, at pH4.5 two inverted repeat sequences in pUC12-W1/512 are known to assume cruciform structures (15). A near physiological pH7.5 was also chosen. Titration of ascorbate and Cu(II) concentrations was carried out leading to the optimized reaction condition as described in Materials and Methods. Higher concentrations of ascorbate and Cu(II) ions result in higher frequency of non-specific cleavage. Under the two conditions described above, ascorbate/Cu(II) nicks the plasmid at one major site and three minor

sites when the plasmid was supercoiled (lanes b, c, d and e). Although the cleavage pattern is different under different reaction conditions, the results clearly demonstrate that ascorbate/Cu(II) introduces the first nick into a supercoiled plasmid DNA molecule at very specific sites. Many types of non-B-DNA are induced and stablized by DNA negative torsion (20). If any of these form the target of ascorbate/Cu(II), the targets should not be present in the linear DNA of the same plasmid. To test the possibility, supercoiled pUC12-W1/512 DNA was first completely linearized by restriction enzyme EcoRI and followed by ascorbate/Cu(II) treatment and S1 nuclease digestion. As shown in lanes f and g, no site-specific cleavage of the linear double-stranded DNA was observed. The strict torsion-dependence of site-specific ascorbate/Cu(II) cleavage of double-stranded DNA provides strong evidence that local DNA conformational changes are involved. To examine whether any of the ascorbate/Cu(II) cleavage sites coincide with the S1 and mungbean nuclease cleavage sites in pUC12-W1/512, supercoiled plasmid was first digested with either S1 nuclease (lanes h and i) or Mungbean nuclease (lanes j and k) at 4°C for 30 minutes and followed by EcoRI or HindIII restriction. Two sites in the plasmid are recognized by both S1 and Mungbean nucleases. They are correspondent to the two palindromic sequences but not to any site of ascorbate/Cu(II) cleavage. It can be concluded that cruciforms do not form the highly prefered targets of ascorbate/Cu(II) cleavage in double-stranded DNA as opposed to single-stranded DNA (12) and the secondary structures attacked by ascorbate/Cu(II) do not possess any apparent single-strand features.

Distinct patterns of ascorbate/Cu(II) cleavage of supercoiled DNA in different conditions (Figure 2) strongly indicate influence of either ionic strength or pH on the sites of cleavage. Therefore, the ascorbate/Cu(II) cleavage of supercoiled DNA was further tested as a function of these parameters. Figure 3A shows the salt concentration dependence of ascorbate/Cu(II) cleavage of supercoiled pUC12-W1/512. The salt concentration titration was carried out at pH 4.5 which is about the pH of Hepes solution without adding any Na^+. The cleavage at the site labeled c is first detected when NaCl concentration is increased to 20 mM, reaches and remains at maximum between 40 and 80 mM, and becomes weak at 160 mM. The sites labeled 'a' and 'b' are detected at higher Na^+ concentration (40 mM) and seem to become stronger with the increase of Na^+ concentration. Site 'b' is favored in the presence of 1 mM to 16 mM $MgCl_2$ which is consistent with the favorable cleavage at site 'b' at high concentrations of Na^+, since divalent Mg^{2+} is known to be much more efficient in stablizing DNA structures than the monovalent Na^+(11). Figure 3B shows the pH dependence of ascorbate/Cu(II) cleavage of supercoiled pUC12-W1/512. The Hepes buffer was first adjusted to pH7.5 with NaOH and the Na^+ concentration was adjusted to 80 mM. HCl was then added to obtain buffers with different pH without changing sodium concentration. A dramatic effect of pH on the cleavage pattern is observed. Site 'a' forms the strongest cleavage site at pH from 5.0 to 6.0 and completely disappears when pH is increased to 6.5. Site 'c' exists from pH 4.5 through 6.5 and completely disappears at pH 7.0. Site 'g' is first detected at pH 6.5 and becomes stronger at pH7.0 and 7.5. At pH7.5 a strong band 'd' appears at the same position as site 'b'. Since site 'b' is favored at pH5.0 and 6.0 and becomes weaker at pH 6.5 and 7.0, site 'd' may be different from site 'b', which can not be resolved by the low resolution gel electrophoresis. Two other new sites 'e' and 'f' appear at pH7.5.

Fine mapping of ascorbate/Cu(II) cleavage sites in pUC12-W1/512

Fine mapping of sites on a DNA molecule which are hypersensitive to endonucleases (21) or chemical reagents (21, 22) are frequently used to derive the local DNA conformation

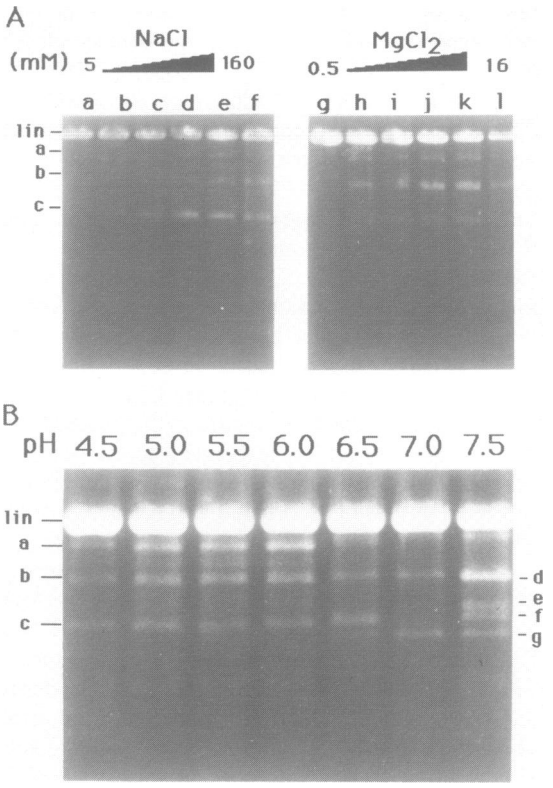

Figure 3. A: *Salt type and salt concentration dependence of Vc/Cu(II) cleavage of supercoiled plasmid DNA.* NaCl concentration (lanes a to f): 5, 10, 20, 40, 80, and 160 mM; and $MgCl_2$ concentration (lanes g to l): 0.5, 1, 2, 4, 8, and 16 mM. pH is 4.5 for all reactions. EcoRI was used to linearize DNA after Vc/Cu(II) treatment. B: *pH dependence of Vc/Cu(II) cleavage of supercoiled plasmid DNA.* Vc/Cu(II) cleavage reaction was done in the presence of 80 mM NaCl, and the pH of each reaction is listed above each lane. EcoRI was used to linearize DNA after Vc/Cu(II) treatment.

which may account for the hypersensitivity. A low resolution map of ascorbate/Cu(II) cleavage sites (see Figure 4A) was derived from the results of Figure 2. For mapping the major cleavage site at pH 7.5, after ascorbate/Cu(II) nicking of the supercoiled plasmid, the DNA was digested with HinfI and a 396 bp restriction fragment containing the cleavage site was isolated by agarose gel eletrophoresis and electroelution. For mapping the major site at pH4.5 and the three minor sites at pH7.5, a 602 NarI-DraI fragment was isolated and purified. Figure 4B shows the strategy of mapping the nicks on each strand of the restriction fragments. After selectively labeling one strand, the DNA was denatured and resolved on a denaturing polyacylamide gel. Figure 5 is the autoradiograph of the gel. One major band is observed in lane 9 in the 396 bp HinfI fragment. In lane 10 where the labeled DNA was digested with S1 nuclease, no new band is observed which shows that either ascorbate/Cu(II) attacks only one strand of the DNA or it attacks both strands at the same position. In lane 6 there is one major cleavage site in the 602 DraI-NarI fragment

where the supercoiled plasmid was treated with ascorbate/Cu(II) at pH4.5, and in lane 7 there are three cleavage sites where the plasmid was treated at pH 7.5. The fainter bands likely represent a background of random cleavage sites. No band is present in lanes where DNA has not been treated with ascorbate/Cu(II). The positions of all bands match well with the low resolution mapping. No correspondent sites are present on the complementary strand (data not shown). Asymmetric cleavage of DNA by endonucleases and chemical reagents are often observed (22, 23, 24) which reflects the asymmetry of local DNA conformation. Figure 4 also shows the nucleotide sequences around the bases which are hypersensitive to ascorbate/Cu(II).

Possible mechanisms of the site-specific cleavage of supercoiled DNA by ascorbate/Cu(II)

Mixing Cu(II) ions and ascorbic acid generates hydroxyl radical (·OH) by a known mechanism (3). Hydroxyl radical initiates cleavage of DNA strands by abstracting a hydrogen atom from the deoxyriboses along the DNA backbone (25). Due to its small size, hydroxyl radical can readily diffuse to each base so that it cleaves DNA molecules nearly equally at each position. However, if a DNA sequence assumes an unusual conformation, the relative reactivity of each base within the sequence toward hydroxyl radical may be different (18). The strict torsion-dependence suggests one possible mechanism of the site-specific cleavage of DNA by ascorbate/Cu(II), i.e. local DNA conformational changes render some bases much more reactive toward hydroxyl radical than those within normal B-DNA duplex. To test this possibility, we investigated the cleavage of supercoiled pUC12-W1/512 by EDTA-Fe(II). Reacting with H_2O_2, the EDTA-Fe(II) complex generates diffusable hydroxyl radical and the complex is known not to bind to DNA (25). Due to lack of sequence-specificity of DNA cleavage, EDTA-Fe(II) is often used for foot-printing of binding sites of proteins and antitumor drugs on DNA (26). The reaction condition is similar to that for ascorbate/Cu(II) reaction except that ascorbate is replaced with EDTA-Fe(II) of different concentrations. As shown in Figure 6A, only uniform smears of DNA are seen on the agarose gel at all concentrations of EDTA-Fe(II). The result ruled out the conformation-oriented hypersensitivity toward diffusable hydroxyl radical.

Chemical studies have shown that copper ions interact with both bases and phosphates of DNA through coordination binding. DNA conformation is suggested to play a great role in determination of binding specificity by governing the number of binding sites and spatial arrangement of these sites (11). Cu(II) binding to DNA may be determined by DNA conformation. Direct interaction of ascorbate with the bound Cu(II) ions and localized generation of hydroxyl radicals may provide another possible mechanism to explain the site-specific cleavage of supercoiled DNA by the complex. To test whether binding of Cu(II) ions to DNA is required for the site-specific cleavage, the strong metal-ion chelator EDTA was added at different concentrations to DNA before and after addition of Cu(II) ions, and the effect on the site-specific cleavage was compared. Figure 6B shows that the site specific cleavage was completely inhibited when 1 μM EDTA was added before Cu(II), while 1 μM EDTA only slightly inhibited the site-specific cleavage when the DNA had been preincubated with Cu(II) on ice for 30 minutes. At 5 μM concentration, EDTA completely abolished the site-specific cleavage, whether it was added before or after the addition of Cu(II) ions. The inhibitory effect presumably is due to the effective competition of EDTA with DNA for Cu(II) ions. The higher concentration of EDTA required to inhibit the site-specific cleavage after preincubation of DNA with Cu(II) ions indicates that binding of copper ions to DNA is necessary for the site-specific cleavage of DNA.

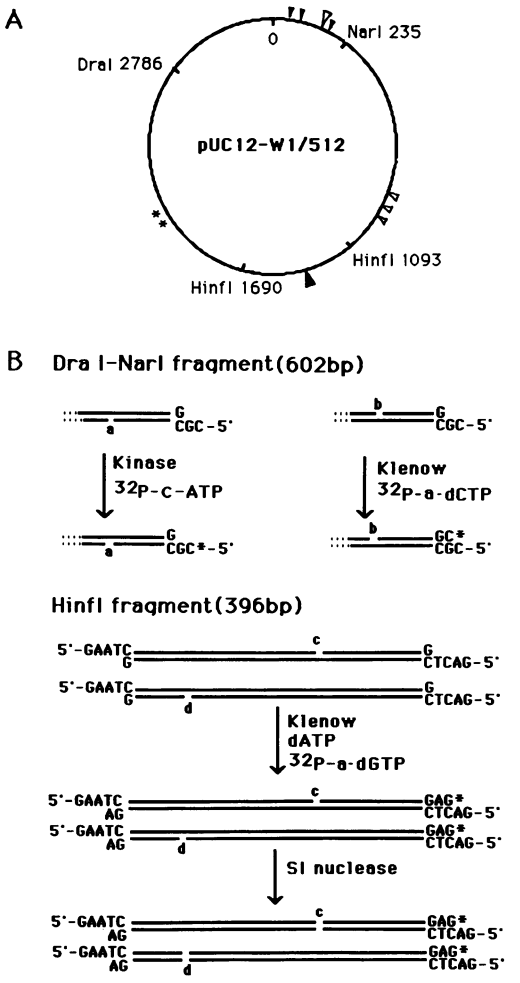

Figure 4. A: *Low-resolution map of Vc/Cu(II) cleavage sites in pUC12-W1/512.*
▼: Cleavage sites at pH 7.5 and 80 mM NaCl; ▽: cleavage sites at pH4.5 and 50 mM NaCl or LiCl. *: the two palindromic sequences which form cruciforms sensitive to S1 and Mungbean nucleases.
B: *Outline of strategy of mapping cleavage sites on each strand of duplex DNA.* Hypothetical nicks are shown to explain the strategy. For the 602 bp DraI-NarI fragment, the 5′ end of lower strand was labeled by T4 kinase with ^{32}P-γ-dATP for mapping nick a, and the 3′ end of upper strand was labeled by Klenow-fill-in with ^{32}P-α-dCTP for mapping nick b. For the 396bp HinfI fragment, the 3′ end of the upper strand containing nick c was selectively labeled by Klenow-fill-in with ^{32}P-α-dGTP by adding cold dATP and ^{32}P-α-dGTP taking advantage of the difference between the two HinfI recognition sequences; and for detecting nick d in the lower strand of the 396 bp fragment, the above labeled DNA was further digested with S1 nuclease which will nick the upper strand at sites opposite the cleavages in the lower strand. All the labeled DNA is then resolved on denaturing sequencing gels.

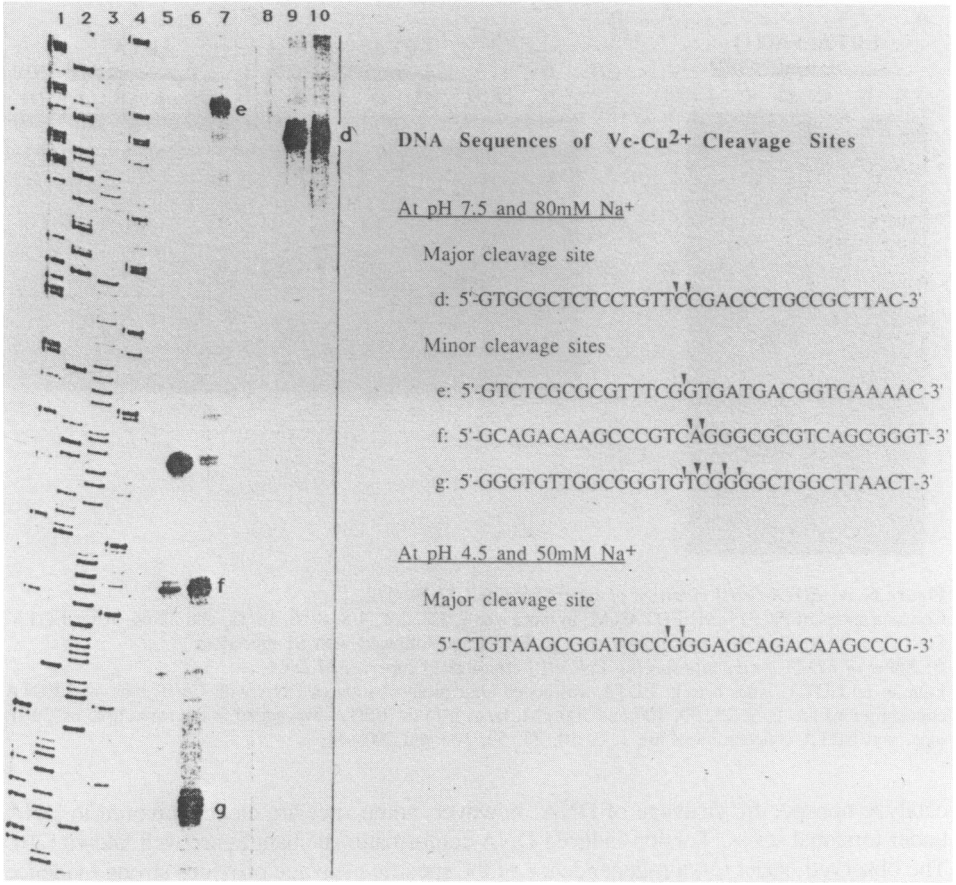

Figure 5. Sequencing gel resolution of Vc/Cu(II) cleavage sites and sequences around the cleavage sites. Lanes 1 to 4 are the four sequencing reactions (G, A, T and C) of a DNA of known sequence used as size markers (which has been calibrated against Maxam and Gilbert sequencing reactions of the fragments containing the Vc/Cu(II) cleavage sites).

Lanes 5, 6 and 7 are the 602 bp DraI-NarI fragment labeled by Klenow-fill-in (the upper strand shown in figure 4B. Lane 5: plasmid without being treated with Vc/Cu(II); lane 6: plamid treated with Vc/Cu(II) at pH4.5 and 50 mM NaCl; and lane 7: plasmid treated with Vc/Cu(II) at pH7.5 and 80 mM NaCl. Lanes 8 to 10 are the 396 bp HinfI fragment labeled by Klenow-fill-in (the upper strand shown in figure 4B). Lane 8: plasmid without being treated with Vc/Cu(II); lane 9: plasmid treated with Vc/Cu(II) at pH7.5 and 80 mM NaCl; lane 10: the same labeled DNA as in lane 9 except digested with S1 nuclease after end-labeling. Bands d, e, f, and g are the same as those labeled with the same letters in Figure 3.

DNA sequences around each cleavage site are also presented.

DISCUSSION

In this paper, we report a negative torsion dependent, site-specific cleavage of double-stranded DNA by ascorbate/Cu(II). The site-specific cleavage occurs only when the DNA is under negative torsional stress. As indicated by Figure 5, Vc/Cu(II) does appear to

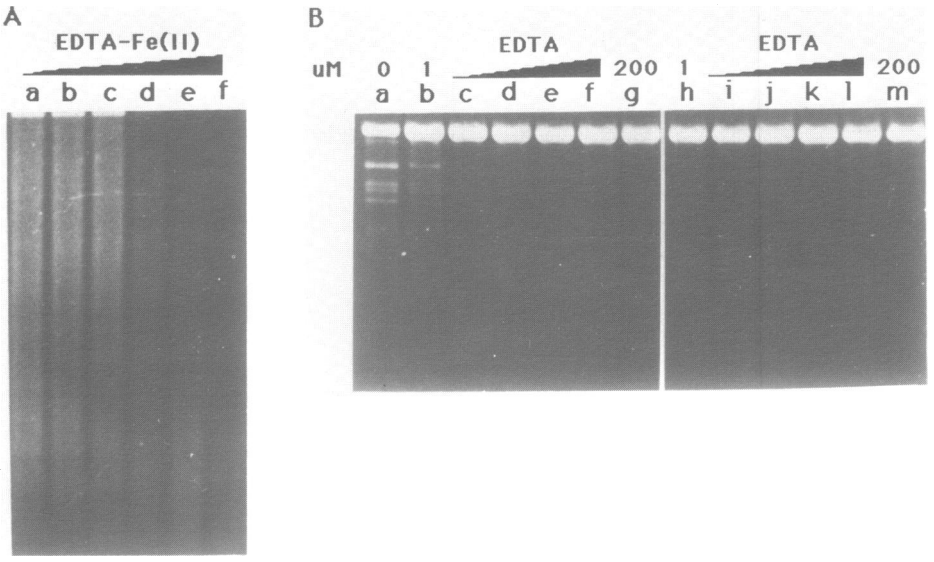

Figure 6. A: *EDTA-Fe(II) cleavage of supercoiled pUC12-W1/512.*
Concentrations of Fe(II) [μM]/EDTA[μM] in lanes a to f: 1/2, 2/4, 4/8, 8/16, 16/32, and 32/64. After EDTA-Fe(II) treatment, the DNA was linearized with EcoRI and followed with S1 digestion.
B: *Effect of EDTA on the site-specific Vc/Cu(II) cleavage of supercoiled DNA.*
Lane a: no EDTA; lanes b to g: EDTA was added after preincubation of DNA with Cu(II) ions, and EDTA concentrations are 1, 5, 25, 50, 100 and 200 μM; lanes h to m: EDTA was added at the same time as Cu(II) ions, and EDTA concentrations are 1, 5, 10, 25, 50, 100 and 200 μM.

catalyze nonspecific cleavage of DNA; however, some sites are clearly favored in DNA under torsional stress. Torsion-induced DNA conformational changes are well known (20). The observed, strict torsion-dependence of the specific cleavage provides strong evidence that local DNA conformational change may determine the site-specificity.

Further evidence of involvement of DNA secondary structures in determining the site-specificity is from the dramatic influences of salt and pH on the specificity of cleavage. Salt type, ionic strength and pH are well known to affect DNA secondary structures (11). For example, cruciformation at different palindromic sequences may show entirely different salt dependance (27), and sequences of alternating purines and pyrimidines in a linear DNA molecule assume left-handed helix only in the presence of certain types of ions and at certain concentrations (28). At low pH protonation on N-7 of guanine may cause rotation of guanine out of the helix (29), and the most dramatic influence of pH on DNA secondary structure is exemplified by H-form DNA adopted by homopurine-homopyrimidine tract at acidic pH in superhelical DNA (23, 24).

Requirement of specific binding of Cu(II) ions to the cleavage sites was suggested by the higher concentration of EDTA needed to inhibit the site-specific cleavage when the DNA was preincubated with Cu(II) ions. Binding of Cu(II) ions to DNA was also concluded by Kazakof et al (12) for the site-specific cleavage of single-stranded DNA by ascorbate/Cu(II). Direct interaction of ascorbic molecules with the bound Cu(II) ions and localized generation of nondiffusable hydroxyl radical is a likely mechanism which can

explain the high specificity of DNA cleavage. Localized generation of nondiffusable hydroxyl radical has been suggested to account for the site-specific cleavage of DNA by several reducing agents which bind to DNA (12, 30). Our observation of the highly site-specific cleavage of one strand of double-stranded DNA by ascorbate/Cu(II) confirms that hydroxyl radical is formed at the sites of Cu(II) binding and is not freely diffusable. Stereochemically determined specific interaction between ascorbate molecules and Cu(II) ions tightly bound to DNA is possible, but has not been tested. An important question can be asked whether copper ions which bind to DNA and determine the site-specific cleavage by ascorbate/Cu(II) are also accessible to other reducing agents of copper-dependent redox reactions.

Since the DNA at the cleavage sites does not show any sequence identity, no rule can be derived to predict which sequence can form a prefered target of ascorbate/Cu(II), nor could we derive any known property of the local DNA conformation. Furthermore, the hypersensitive bases do not agree with the observation of Chiou et al (8) who found that in a linear DNA the bases most susceptible to ascorbate/Cu(II) were GT and GC and the sequences least susceptible were pyrimidine clusters. For instance, the major site in pUC12-W1/512 at pH7.5 is located in a highly pyrimidine-rich region of the strand. The discrepancy is probably due to the existence of two levels of specificity of ascorbate/Cu(II) cleavage of double-stranded DNA: sequence-directed specificity and conformation-directed specificity. The latter is dependent on the presence of torsional stress or in single-stranded DNA where intrastrand base-pairing can happen. More sequence data are needed to unravel the sequence and conformation requirements of ascorbate/Cu(II) hypersensitive sites. Nevertheless, we have demonstrated torsion-dependent site-specific cleavage of double-stranded DNA by ascorbate/Cu(II).

The site-specific cleavage of supercoiled DNA by ascorbate/Cu(II) could be of biological significance for the following reasons: i) ascorbic acid and Cu(II) are normal cellular constituents; ii) DNA in nature is negatively supercoiled; and iii) a major site-specific cleavage happens at physiological pH, ionic strength and temperature.

Metal ions are known to participate in stablization of numerous biological structures (31). Some metal ions, among which is Cu(II) have been found to be directly bound to specific sites of nucleic acid part of chromatin (11, 32) and thought to serve as linkers between nucleic acid and proteins. Even though DNA conformation has been suggested to determine the site-specificity of metal-ion binding to DNA, little is known about the structural properties of these binding sites due to lack of proper probes. Our current studies of ascorbate/Cu(II) cleavage of supercoiled DNA could provide a powerful probe for analyzing the sites of DNA where Cu(II) ions bind specifically. So far we do not have sufficient data to determine the structural properties of these sites, but the sequence data and resistance to S1 and Mungbean nucleases of these sites suggest that it may be a structure which has not been previously identified. Non-B DNA conformation is thought to play a role in specific interactions between DNA and regulatory proteins (20). Copper ions or other metal ions may recognize and stablize regions of certain non-B DNA and may also be involved in determining the specificity of protein-DNA interactions. Possible correlation between the cleavage sites and function of nucleotide sequences can not be concluded without a more extensive examination of positions of the cleavage sites present in other sequences of different functions.

The antivirus and antitumor activities of ascorbic acid have been attributed to extensive damage of DNA according to in vitro experiments (1, 2, 3). However, the situation in

vivo is much more complicated. Numerous species of hydroxyl radical scavengers within cells may quickly remove the hydroxyl radical before it can diffuse to DNA. A more likely mechanism is the highly site-specific cleavage of DNA which happens when ascorbic molecule interacts with Cu(II) ions bound to DNA to generate nondiffusable hydroxyl radical. Such a mechanism will be independent of intracellular concentrantion of hydroxyl radical and will not be affected by the presence of radical scavengers.

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REFERENCES

1. Bram, S., Frossard, P., Guichard, M., Jasmin, C., Augery, Y., Sinoussi-Barre, F. and Wray, W. (1980) Nature 284, 629–631.
2. Chiou, S.-H. (1983) J. Biochem. 94, 1259–1267.
3. Morgan, A.R., Cone, R.L. and Elgert, T.M. (1976) Nucl. Acids Res. 3, 1139–1149.
4. Wong, K., Morgan, A.R. and Paranchych, W. (1974) Can. J. Biochem. 52, 950–958.
5. Shamberger, R.J.(1984) Mutation Res. 133, 135–159.
6. Scarpa, M., Stevanto, R., Viglino, P. and Rigo, A. (1983) J. Biol. Chem. 259, 6695–6697.
7. Weitberg, A.B. (1987) Mutation Res. 191, 53–56.
8. Chiou, S.-H., Chang, W.-H., Jou, Y.-S., Chung, H.-M.M. and Lo, T.-B. (1985) J. Biochem. 98, 1723–1726.
9. Zimmer, C.H., Luck, G., Fritzsche, H. and Triebel, H. (1971) Biopolymers 10, 441–463.
10. Eichhorn, G.L. and Clark, P. (1965) Proc. Natl. Acad. Sci. USA 47, 778–782.
11. Sissoeff, I., Grisvard, J. and Guille, E. (1976) Prog. Biophys. Molec. Biol. 31, 165–199.
12. Kazakov, S.A., Astashkina, T.G., Mamaev, S.V. and Vlassov, V.V.(1988) Nature 335, 186–189.
13. Lilley , D.M.J. (1980) Proc. Natl. Acad. Sci. USA 77, 6468–6472.
14. Wang, J.C., Peck, L.J. and Becherer, K.(1982) Cold Spring Harbor Symp. Quant. Biol. 47,85–91.
15. Wang, Y. and Sauerbier, W. (1987) Gene 60, 303–306.
16. Foster, J., Stafford, J. and Queen, C. (1985) Nature 315, 423–425.
17. Wang, Y. and Sauerbier, W. (1987) Biochem. Biophys. Res. Comm. 146, 1503–1530.
18. Burkhorf, A.M. and Tullius, T.D. (1987) Cell 48, 935–943.
19. Maxam, A. and Gilbert, W. (1977) Proc. Natl. Acad. Sci. USA 560–564.
20. Wells, R.D. (1988) J. Biol. Chem. 263, 1095–1098.
21. Wohlrab, F., McLean, M.J. and Wells, R.D. (1987) J. Biol. Chem. 262, 6407–6416.
22. Kohwi-Shigematsu, T. and Kohwi, Y. (1985) Cell 43, 199–216.
23. Lee, J.S., Johnson, D.A. and Morgan, A.R. (1979) Nucl. Acids Res. 6, 3073–3091.
24. Lyamichev, V.I., Mirkin, S.M. and Frank-Kamenetskii, M.O. (1986) J. Biomol. Struct. and Dyn. 3, 667–669.
25. Tullius, T.D. and Dombroski, B.A. (1985) Science 230, 679–681.
26. Tullius, T.D. and Dombroski, B.A. (1986) Proc. Natl. Acad. Sci. USA 83, 5469–5473.
27. Sullivan, K.M. and Lilley, D.M.J. (1987) Cell 47, 817–827.
28. Elisson, M.J., Kelleher, R.J., Wang, H.-J., Habener, J.F. and Rich, A. (1985) Proc. Natl. Acad. Sci. USA 82, 8320–8324.
29. Courtois, Y., Fromageot, P. and Guschlbauer, W. (1968) Eur. J. Biochem. 6, 493–501.
30. Kuwabara, M., Yoon, C., Goyne, T., Thederahn, T. and Sigman, D.S. (1986) Biochemistry 25, 7401–7408.
31. Frieden, E. (1968) Sci. Am. 218, 102–114.
32. Lishanskaya, A.T. and Mosevitsky, M.I. (1975) Biochem. Biophys. Res. Cumm. 62, 822–829.