Oxidative and Nonoxidative Mechanisms of Site-Specific DNA Cleavage Induced by Copper-Containing Metallothioneins†

Shinji Oikawa,‡ Masaaki Kurasaki,§ Yutaka Kojima,§ and Shosuke Kawanishi*,‡

Department of Public Health, Faculty of Medicine, Kyoto University, Kyoto 606, Japan, and Department of Environmental Medicine and Informatics, Graduate School of Earth Environmental Science, Hokkaido University, Sapporo 060, Japan

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ABSTRACT: DNA cleavage induced by metallothionein (MT) containing copper was investigated by a DNA sequencing technique. Reconstituted Cd_7-MT showed no ability to cause DNA cleavage. Commercially available rabbit MT I caused DNA cleavage, suggesting that DNA cleavage is due to the metal contained in commercial MT. Cu_2Cd_5-MT and Cu_{12}-MT were prepared by the treatment of commercial rabbit MT I with $[Cu(CH_3CN)_4]ClO_4$. Cu_{12}-MT frequently induced an alteration of thymine residues, especially in the 5′-GTC-3′ sequence, and piperidine treatment led to chain cleavage at the thymine residues. The site specificity was similar to that obtained with Cu(I) plus H_2O_2. H_2O_2 enhanced DNA cleavage induced by Cu_{12}-MT. Catalase and a Cu(I)-specific chelating agent, bathocuproine, inhibited DNA cleavage. These results suggest that Cu(I) and H_2O_2 have important roles in the production of active species causing DNA cleavage. Commercial MT and Cu_2Cd_5-MT induced DNA cleavage much less than Cu_{12}-MT, but gave particularly specific DNA cleavage. Cu_2Cd_5-MT induced cleavage specifically at the central guanine residue of the 5′-GGT-3′ sequence. A similar cleavage pattern was obtained with commercial MT. No effect of piperidine treatment suggests that the DNA cleavage might not be due to base damage and/or liberation. The DNA cleavage was inhibited efficiently by EDTA, but not by bathocuproine and catalase. Experiments with DNA ligands, albumin, and denatured DNA suggest that commercial MT and Cu_2Cd_5-MT induce nonoxidative cleavage of the deoxyribose phosphate backbone through its DNA recognition. These two types of cleavage mechanisms are discussed in relation to the possible role of Cu-MT in carcinogenesis.

The biological significance of copper has recently attracted much interest in connection with carcinogenicity and mutagenicity (Agarwal *et al.*, 1989). Copper is an essential component of chromatin (Dijkwel & Wenink, 1986; Saucier *et al.*, 1991) and is known to accumulate preferentially in the heterochromatic regions (Bryan *et al.*, 1976). Copper sulfate ($CuSO_4$) showed clastogenic effects on the bone marrow chromosomes of mice *in vivo* (Agarwal *et al.*, 1990). Li *et al.* (1991) reported that copper accumulated in liver tissues of LEC rats that had spontaneously developed hepatocellular carcinomas, suggesting that the abnormal copper metabolism is involved in hepatic carcinogenesis in the LEC rats. The exposure of DNA to H_2O_2 in the presence of Cu(I) or Cu(II) is known to result in the induction of a variety of oxidative lesions, including DNA strand breaks and base modifications (Que *et al.*, 1980; Marshall *et al.*, 1981; Goldstein & Czapski, 1986; Yamamoto & Kawanishi, 1989, 1991; Kawanishi & Yamamoto, 1991; Ito *et al.*, 1992). Therefore, it is reasonable to consider the possibility of copper involvement in cellular DNA cleavage.

Several lines of evidence indicate that metallothionein (MT)[1] may play a role in various carcinogenic processes. Direct evidence indicates that certain tumor types have highly concentrated MT (Cherian *et al.*, 1994). Mammalian MT has 61 or 62 amino acids, including 20 cysteine residues (Kojima *et al.*, 1976; Kägi & Kojima, 1987). This protein is extremely inducible by copper, as well as by cadmium (Hamer, 1986). Cd-MT contains seven Cd ions, three bound to the amino-terminal β-domain of residues 1−30 and four bound to the carboxyl-terminal α-domain of residues 31−61 (Otvos & Armitage, 1980). Cu-MT contains twelve Cu ions, six bound in the β-domain and six in the α-domain (Nielson *et al.*, 1985). MT functions in copper homeostasis and in the detoxification of nonessential metal ions (Kägi & Kojima, 1987; Hamer, 1986). MT is also a potent hydroxyl free radical (•OH) scavenger, and its antioxidant properties *in vivo* have been suggested by the observation that MT protected cellular hydrogen peroxide (H_2O_2)-induced DNA cleavage (Chubatsu & Meneghini, 1993). On the other hand, Müller *et al.* (1991) reported that Cd,Zn-MT induced strand breaks to isolated DNA. Sakurai *et al.* (1994) also reported that an unusual accumulation of copper induced Cu-MT in the liver of LEC rats. However, at present, it remains to be clarified whether or not the role of MT is to protect from or enhance carcinogenesis.

In this study, we prepared several kinds of Cu-reconstituted MT, which contained different amounts of copper ions, and examined the site specificity of Cu-MT-induced DNA cleavage by using ^{32}P 5′-end-labeled DNA fragments of defined sequence.

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* Author to whom correspondence should be addressed.
‡ Kyoto University.
§ Hokkaido University.

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[1] Abbreviations: MT, metallothionein; DTPA, diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid.

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EXPERIMENTAL PROCEDURES

Materials

[γ-^{32}P]dATP (222 TBq/mmol) was purchased from Du Pont-New England Nuclear. Restriction enzymes (*Ava*I, *Xba*I, *Pst*I, and *Bam*HI) and T_4 polynucleotide kinase were purchased from New England Biolabs. $CuCl_2$, other metals, and ethanol were from Nacalai Tesque, Inc. (Kyoto, Japan). DTPA, EDTA, and bathocuproinedisulfonic acid were from Dojin Chemicals Co. (Kumamoto, Japan). Rabbit liver MT I, Lys-Cys-Thr-Cys-Cys-Ala (56−61 residues of MT), berenil (diminazene aceturate), and catalase (45 000 units/mg from bovine liver) were from Sigma Chemical Co. Chromomycin A_3 was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Chelex 100 was from Bio-Rad Laboratories.

Methods

Preparation of ^{32}P 5′-End-Labeled DNA Fragments. DNA fragments were prepared from plasmid pbcNI, which carries a 6.6 kb *Bam*HI chromosomal DNA segment containing the human c-Ha-*ras*-1 protooncogene (Yamamoto *et al.*, 1989). Singly labeled 261 bp fragment (*Ava*I* 1645−*Xba*I 1905), 341 bp fragment (*Xba*I 1906−*Ava*I* 2246), 98 bp fragment (*Ava*I* 2247−*Pst*I 2344), and 337 bp fragment (*Pst*I 2345−*Ava*I* 2681) were obtained according to the method described previously (Yamamoto *et al.*, 1989). The asterisk indicates ^{32}P labeling, and nucleotide numbering starts with the *Bam*HI site (Capon *et al.*, 1983).

Preparation of Cd_7-Reconstituted MT. Apo-MT was prepared by acidification of commercially available MT I in 0.1 N HCl, followed by filtration on a Centricon-3 (Amicon, Stonehouse, UK) equilibrated with 0.01 N HCl. The apo-MT was reconstituted with 10-fold Cd(II) ions per mole of protein. The unbound metals were removed with Chelex 100. Protein concentrations were quantified by amino acid analysis following hydrolysis *in vacuo* with 6 N HCl. The concentration of cysteine was determined as cysteic acid after performic acid oxidation. The concentration of cadmium was measured with Hitachi flame atomic absorption spectrophotometer Model 180-30.

Preparation of Cu-Reconstituted MT. $[Cu(CH_3CN)_4]ClO_4$ as a Cu(I) donor was synthesized by the method of Hemmerich and Sigwart (1963). The 0.5−1.0 mM commercial MT I in 10 mM Tris/5 mM HCl was incubated with 1−20 mol equiv of Cu(I) using $[Cu(CH_3CN)_4]ClO_4$ dissolved in acetonitrile at room temperature for 20 min. The mole equivalents indicates 1 mol of metal ions per mole of protein or per 20 mol of SH groups. After incubation, an aliquot of Chelex 100 was added to each incubation mixture to remove the unbound metals. After centrifugation, the absorption spectra, metal concentrations, and amino acid compositions were determined.

Analyses of DNA Cleavage Induced by Commercial MT, Cu_2Cd_5-MT, Cu_8Cd_2-MT, and Cu_{12}-MT. The standard reaction mixture in a microtube (1.5 mL Eppendorf) contained 0.5 mM MT, 1 μM per base of sonicated calf thymus DNA, and ^{32}P 5′-end-labeled DNA fragment in 200 μL of 10 mM sodium phosphate buffer (pH 7.9) containing 5 μM DTPA. After incubation at 37 °C for 60 min, the DNA fragments were heated at 90 °C in 1 M piperidine where indicated and treated as previously described (Yamamoto *et al.*, 1989). The treated DNA fragments were electrophoresed on an 8% polyacrylamide/8 M urea gel, and the autoradiogram was obtained by exposing X-ray film to the gel.

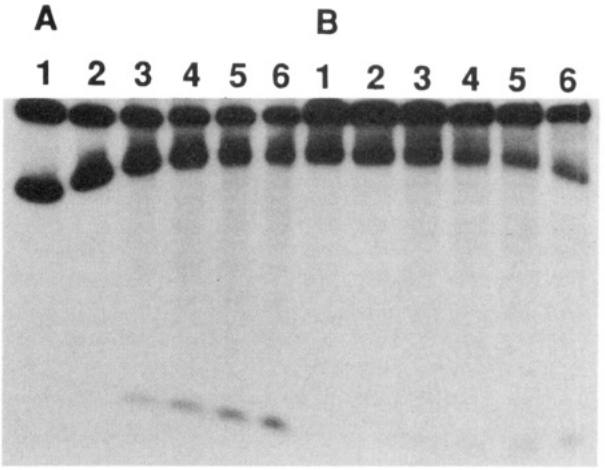

FIGURE 1: Autoradiogram of ^{32}P-labeled DNA fragments incubated with commercial MT. The reaction mixture contained the ^{32}P 5′-end-labeled 337 bp fragment (*Pst*I 2345−*Ava*I* 2681), 0.5 mM commercial MT, 1 μM per base of sonicated calf thymus DNA, and 5 μM DTPA in 200 μL of 10 mM sodium phosphate buffer at pH 7.9. After incubation at 37 °C for the indicated durations, followed by nothing (A) or the piperidine treatment (B), the DNA fragments were analyzed by the method described in Experimental Procedures. (A) Lane 1, control, 120 min; lane 2, 0 min; lane 3, 30 min; lane 4, 60 min; lane 5, 90 min; lane 6, 120 min. (B) Lane 1, control, 120 min; lane 2, 0 min; lane 3, 30 min; lane 4, 60 min; lane 5, 90 min; lane 6, 120 min.

The preferred cleavage sites were determined by direct comparison of the positions of the oligonucleotides with those produced by the chemical reactions of the Maxam−Gilbert procedure (Maxam & Gilbert, 1980), using a DNA-sequencing system (LKB 2010 Macrophor). A laser densitometer (LKB 2222 UltroScan XL) was used for the measurement of the relative amounts of oligonucleotides from treated DNA fragments.

RESULTS

Metal Analyses of Commercial and Reconstituted MT. Metal analysis of commercial MT gave average values of 0.3 mol of Cu, 5.4 mol of Cd, 1.7 mol of Zn, and 0.0003 mol of Fe per mole of protein. Cd_7-MT contained seven Cd atoms per protein and only a negligible amount of Cu and Zn.

Cu_2Cd_5-MT, Cu_8Cd_2-MT, and Cu_{12}-MT, which contain two Cu ions plus five Cd ions, eight Cu ions plus two Cd ions, and twelve Cu ions alone, respectively, were prepared by the treatment of commercial rabbit MT I with $[Cu(CH_3CN)_4]ClO_4$. The metal content of Cu_2Cd_5-MT was found to be 2.2 mol of Cu and 4.7 mol of Cd per mole of protein. The metal content of Cu_8Cd_2-MT was found to be 8.3 mol of Cu and 1.9 mol of Cd per mole of protein. In Cu_{12}-MT, 12.2 mol of Cu was present per molecule with only negligible traces of Cd and Zn.

Cleavage of ^{32}P-Labeled DNA Fragments Induced by Commercial MT, Cu_8Cd_2-MT, and Cu_{12}-MT. The extent of DNA cleavage induced by MT was estimated by a gel electrophoretic analysis. Figure 1 shows that DNA cleavage is induced by commercial MT. Oligonucleotides were clearly detected on the autoradiogram as a result of commercial MT-induced DNA cleavage. The DNA cleavage increased with time. Even without piperidine treatment, oligonucleotides were formed (Figure 1A, lanes 3−6),

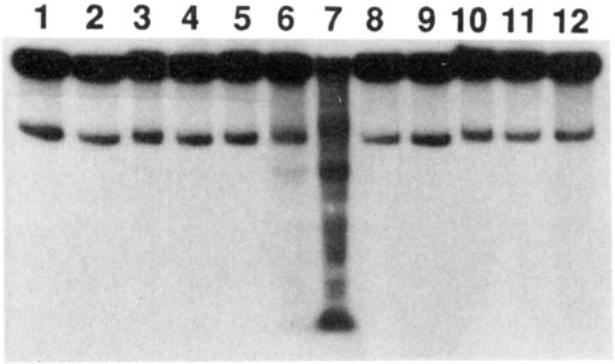

FIGURE 2: Comparison of DNA cleavage by Cu_{12}-MT with that by Cu_8Cd_2-MT. The reaction mixture contained the ^{32}P 5′-end-labeled 337 bp fragment, indicated concentrations of Cu-MT, 1 μM per base of sonicated calf thymus DNA, and 5 μM DTPA in 200 μL of 10 mM sodium phosphate buffer at pH 7.9. After incubation at 37 °C for 60 min, followed by the piperidine treatment, the DNA fragments were analyzed by the method described in Experimental Procedures. Lane 1, control; lane 2, 83 μM commercial MT; lane 3, 8.3 nM Cu_{12}-MT; lane 4, 83 nM Cu_{12}-MT; lane 5, 0.83 μM Cu_{12}-MT; lane 6, 8.3 μM Cu_{12}-MT; lane 7, 83 μM Cu_{12}-MT; lane 8, 8.3 nM Cu_8Cd_2-MT; lane 9, 83 nM Cu_8Cd_2-MT; lane 10, 0.83 μM Cu_8Cd_2-MT; lane 11, 8.3 μM Cu_8Cd_2-MT; lane 12, 83 μM Cu_8Cd_2-MT.

indicating cleavage of the deoxyribose phosphate backbone. The increased amount of oligonucleotides was not observed with piperidine treatment (Figure 1B, lanes 3−6), suggesting that base damage and/or liberation were not induced by commercial MT.

In order to clarify whether double helix is required for the DNA cleavage, an experiment with denatured DNA was performed. When double-stranded DNA fragments were denatured by heating at 90 °C for 10 min, oligonucleotide formation was decreased (data not shown). The secondary structure of DNA plays a role in DNA cleavage by commercial MT.

Figure 2 shows that more than 83 μM Cu_{12}-MT (1 mM per Cu) caused strong DNA cleavage (lane 7). Cleavage without piperidine treatment (data not shown) suggests breakage of the deoxyribose phosphate backbone by active species. The increased amount of oligonucleotides with piperidine treatment suggests that base damage and/or liberation were also induced by Cu_{12}-MT. On the other hand, 83 μM Cu_8Cd_2-MT (0.69 mM per Cu) caused slight DNA cleavage (Figure 2, lane 12). Cu_2Cd_5-MT, as well as Cu_8Cd_2-MT, also induced DNA cleavage much less than Cu_{12}-MT (data not shown). No or little DNA cleavage was observed with Cd_7-reconstituted MT (data not shown). These results indicate that Cd_7-MT has no ability to cleave DNA and that DNA cleavage is due to the metal contained in commercial MT.

Effects of Scavengers, Chelating Agents, and DNA Ligands on DNA Cleavage Induced by Cu_{12}-MT and Commercial MT. Figure 3 shows the effects of •OH scavenger and catalase on Cu_{12}-MT-induced DNA cleavage. DNA cleavage was inhibited by catalase (Figure 3, lane 4), whereas it was not inhibited by the •OH scavenger, ethanol (Figure 3, lane 3). H_2O_2 enhanced Cu_{12}-MT-induced DNA cleavage (Figure 3, lane 5). The addition of bathocuproine, a Cu(I)-specific chelating agent, inhibited Cu_{12}-MT-induced DNA cleavage (Figure 4B). These results suggest that Cu(I) and H_2O_2 have important roles in DNA cleavage.

On the other hand, commercial MT-induced DNA cleavage was inhibited efficiently by EDTA (Figure 4A, lane 4),

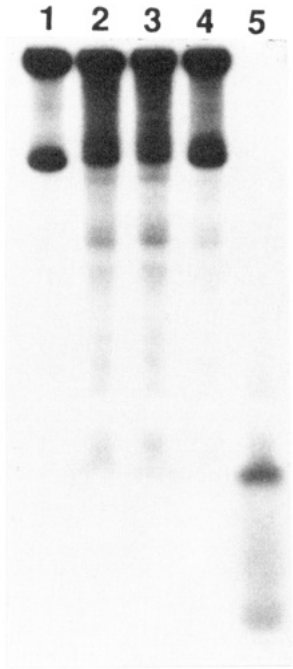

FIGURE 3: Effects of scavengers and H_2O_2 on DNA cleavage induced by Cu_{12}-MT. The reaction mixture contained the ^{32}P 5′-end-labeled 337 bp fragment, 42 μM Cu_{12}-MT (0.5 mM Cu), 1 μM per base of sonicated calf thymus DNA, and 5 μM DTPA in 200 μL of 10 mM sodium phosphate buffer at pH 7.9. After incubation at 37 °C for 60 min, followed by the piperidine treatment, the DNA fragments were analyzed by the method described in Experimental Procedures. Scavenger or H_2O_2 was added to the reaction mixture where indicated. Lane 1, control; lane 2, Cu_{12}-MT; lane 3, 0.8 M ethanol was added; lane 4, 30 units of catalase were added; lane 5, 200 μM H_2O_2 was added.

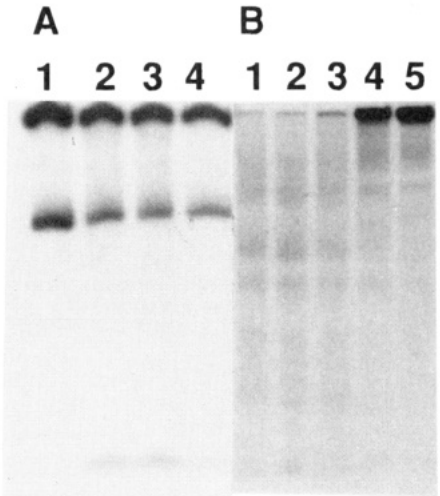

FIGURE 4: Effects of chelating agents on DNA cleavage induced by commercial MT and Cu_{12}-MT. The reaction mixture contained the ^{32}P 5′-end-labeled 337 bp fragment, 0.5 mM commercial MT (0.15 mM Cu) (A) or 42 μM Cu_{12}-MT (0.5 mM Cu) (B), 1 μM per base of sonicated calf thymus DNA, and 5 μM DTPA in 200 μL of 10 mM sodium phosphate buffer at pH 7.9. After incubation at 37 °C for 60 min, followed by the piperidine treatment, the DNA fragments were analyzed by the method described in Experimental Procedures. Chelating agent was added to the reaction mixture where indicated. (A) Lane 1, control; lane 2, commercial MT; lane 3, commercial MT + 0.15 mM bathocuproine; lane 4, commercial MT + 0.15 mM EDTA. (B) Lane 1, Cu_{12}-MT; lane 2, Cu_{12}-MT + 10 μM bathocuproine; lane 3, Cu_{12}-MT + 20 μM bathocuproine; lane 4, Cu_{12}-MT + 0.1 mM bathocurpoine; lane 5, Cu_{12}-MT + 0.5 mM bathocurproine.

but not by bathocuproine (Figure 4A, lane 3). The DNA cleavage was not inhibited by catalase (data not shown). In

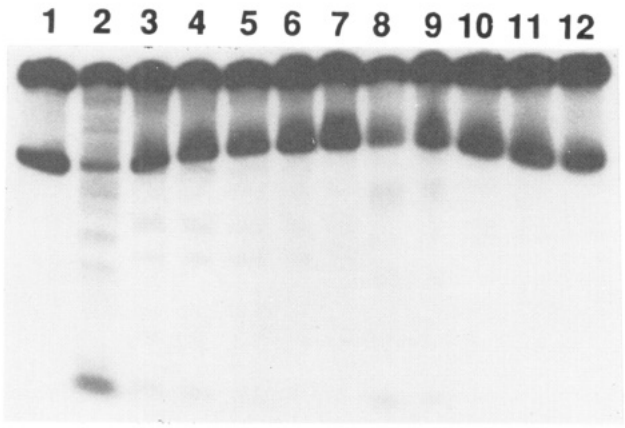

FIGURE 5: Effects of DNA ligands on DNA cleavage induced by commercial MT. The reaction mixture contained the ^{32}P 5′-end-labeled 337 bp fragment, 0.4 mM commercial MT, 0.2 mM $MgCl_2$, 1 μM per base of sonicated calf thymus DNA, and 5 μM DTPA in 200 μL of 10 mM sodium phosphate buffer at pH 7.9. After incubation at 37 °C for 60 min, followed by the piperidine treatment, the DNA fragments were analyzed by the method described in Experimental Procedures. DNA ligand was added to the reaction mixture where indicated. Lane 1, control; lane 2, MT; lane 3, MT + 20 μM chromomycin A_3; lane 4, MT + 40 μM chromomycin A_3; lane 5, MT + 100 μM chromomycin A_3; lane 6, MT + 200 μM chromomycin A_3; lane 7, MT + 400 μM chromomycin A_3; lane 8, MT + 20 μM berenil; lane 9, MT + 40 μM berenil; lane 10, MT + 100 μM berenil; lane 11, MT + 200 μM berenil; lane 12, MT + 400 μM berenil.

order to clarify whether the interaction of DNA with MT requires MT-induced DNA cleavage, the effects of DNA ligands (DNA binding agents) on DNA cleavage were examined. Chromomycin A_3 binds as a Mg(II)-coordinated dimer to G•C-rich sequences (Gao & Patel, 1990), and berenil is a minor groove binder with affinity for A•T-rich sequences (Zimmer & Wähnert, 1986; Portugal & Waring, 1987; Neidle *et al.*, 1987). Both chromomycin A_3 and berenil significantly inhibited commercial MT-induced DNA cleavage (Figure 5). On the other hand, chromomycin A_3 showed little effect on Cu_{12}-MT-induced DNA cleavage (data not shown).

Effect of Albumin on DNA Cleavage Induced by Cu(II) plus Cysteine and Commercial MT. Since copper ions administered are incorporated in the various protein systems of the body, experiments were performed in the presence of albumin for a simple protein model (Halliwell, 1988; Inoue & Kawanishi, 1987). Cu(II) plus cysteine induced DNA damage in the absence of albumin (Figure 6A, lane 2), and the addition of catalase inhibited DNA cleavage (data not shown). This can be explained by assuming that Cu(II) binds with DNA and catalyzes active oxygen production from H_2O_2. The addition of 5 μM albumin inhibited Cu(II) plus cysteine-induced DNA damage (Figure 6A, lane 7). These results suggest that Cu(II) binds to the protein and scarcely seems to participate in DNA damage. On the other hand, albumin showed no or little effect on commercial MT-induced DNA cleavage (Figure 6B).

Site Specificity of DNA Cleavage Induced by Commercial MT, Cu_2Cd_5-MT, and Cu_{12}-MT. Figure 7 shows that Cu_{12}-MT frequently induced piperidine-labile sites at thymine residues. The preferred sites were the thymine residues of the 5′-GTC-3′ sequence, followed by the thymine residues between purine residues. The thymine residues of the 5′-CTG-3′ and 5′-CTC-3′ sequences showed little reactivity. Although the extent of cleavage at the positions of cytosine were variable according to the sequence, central cytosine

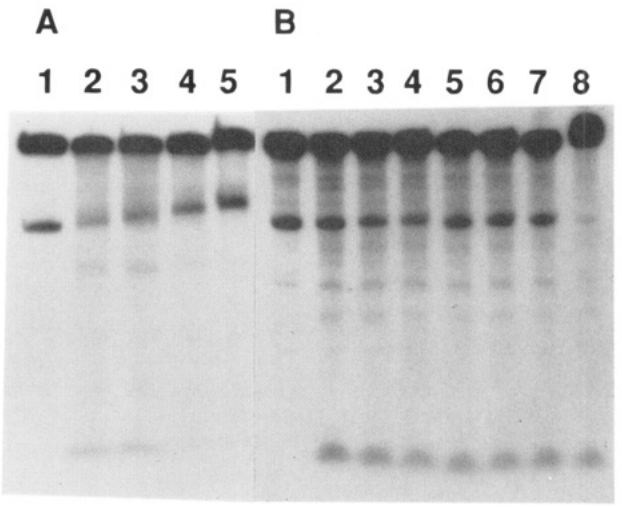

FIGURE 6: Effect of albumin on DNA cleavage induced by Cu(II) plus cysteine and commercial MT. The reaction mixture contained the ^{32}P 5′-end-labeled 337 bp fragment, 20 μM Cu(II) plus 20 μM cysteine (A) or 0.5 mM commercial MT (0.15 mM Cu) (B), indicated concentrations of albumin, 1 μM per base of sonicated calf thymus DNA, and 5 μM DTPA in 200 μL of 10 mM sodium phosphate buffer at pH 7.9. After incubation at 37 °C for 60 min, followed by the piperidine treatment, the DNA fragments were analyzed by the method described in Experimental Procedures. (A) Lane 1, control; lane 2, Cys + Cu(II); lane 3, Cys + Cu(II) + 0.2 μM albumin; lane 4, Cys + Cu(II) + 0.5 μM albumin; lane 5, Cys + Cu(II) + 1 μM albumin. (B) Lane 1, control; lane 2, MT; lane 3, MT + 2 μM albumin; lane 4, MT + 5 μM albumin; lane 5, MT + 10 μM albumin; lane 6, MT + 20 μM albumin; lane 7, MT + 50 μM albumin; lane 8, MT + 100 μM albumin.

residues of 5′-CCG-3′ sequence seemed to be cleaved extensively. The site specificity was similar to that obtained with the complexes of Cu(II) with cysteine, GSH, or Lys-Cys-Thr-Cys-Cys-Ala (56−61 residues of MT, data not shown). To clarify what active species participate in DNA cleavage, the pattern of DNA cleavage induced by Cu_{12}-MT was compared with that of H_2O_2 in the presence of Cu(I) or Cu(II). Cu_{12}-MT showed a cleavage pattern similar to that of Cu(I) plus H_2O_2, but not that of Cu(II) plus H_2O_2 (Figure 8).

Cu_2Cd_5-MT and commercial MT gave DNA cleavage with site specificity different from that of Cu_{12}-MT. Cu_2Cd_5-MT induced cleavage specifically at the central guanine residue of the 5′-GGT-3′ sequence, especially in the G•C-rich region (Figure 9). A similar pattern of DNA cleavage was obtained with commercial MT (Figure 10).

DISCUSSION

MT is expressed in various types of human tumors (Cherian *et al.*, 1994; Pattanaik *et al.*, 1994). MTs have been detected in the nucleus, as well as in the cytoplasm (Banerjee *et al.*, 1982; Tsujikawa *et al.*, 1991). Recently, copper was reported to be the major metal associated with the MT fraction in lung tumors (Hart *et al.*, 1993). It was reported that abnormal copper accumulation in the livers of LEC rats followed by Cu-MT induction is involved in hepatic carcinogenesis (Li *et al.*, 1991; Yamamoto *et al.*, 1993; Sakurai *et al.*, 1994). These reports suggest a role of MT in carcinogenesis.

The biological significance of copper has recently attracted much interest in connection with carcinogenicity. A case-cohort study showed a relation between premorbid plasma copper levels, and the risk of developing breast cancer was

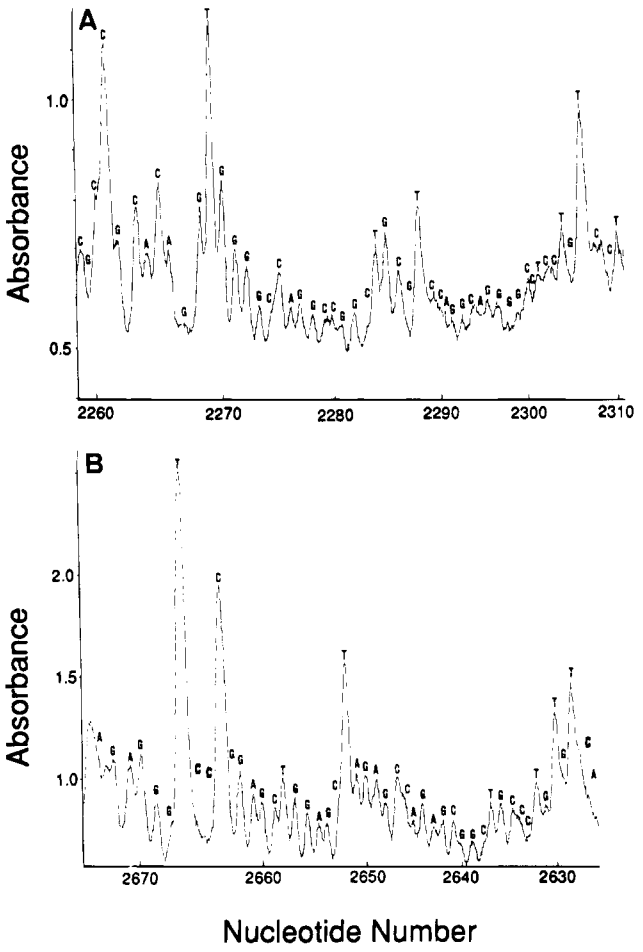

FIGURE 7: Site specificity of DNA cleavage induced by Cu_{12}-MT. (A) The ^{32}P 5′-end-labeled 98 bp fragment (*Ava*I* 2247−*Pst*I 2344) and 1 μM per base of sonicated calf thymus DNA in 200 μL of 10 mM sodium phosphate buffer at pH 7.9 containing 5 μM DTPA were incubated with 42 μM Cu_{12}-MT at 37 °C for 60 min. (B) The ^{32}P 5′-end-labeled 337 bp fragment (*Pst*I 2345−*Ava*I* 2681) was used. After the piperidine treatment, DNA fragments were electrophoresed on an 8% polyacrylamide/8 M urea gel, and the autoradiogram was obtained by exposing X-ray film to the gel. The relative amounts of oligonucleotides produced were measured by a laser densitometer (LKB 2222 UltroScan XL). The piperidine-labile sites of the treated DNA were determined by direct comparison with the same DNA fragment after undergoing DNA sequence reaction according to the Maxam−Gilbert procedure (Maxam & Gilbert, 1980). The horizontal axis shows the nucleotide number of human c-Ha-*ras*-1 protooncogene starting with the *Bam*HI site (Capon *et al.*, 1983).

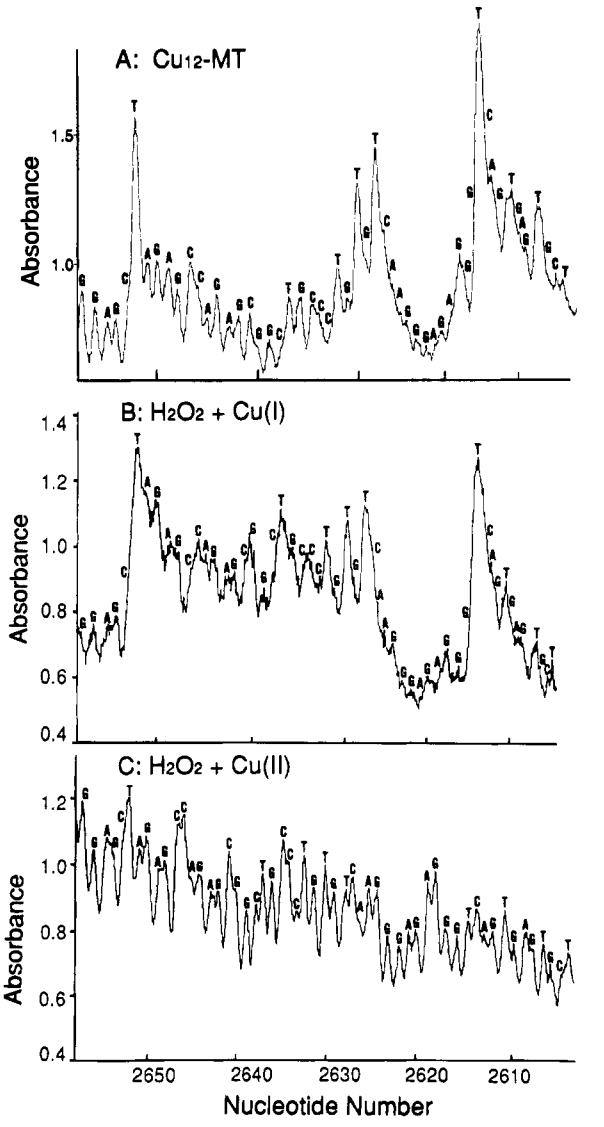

FIGURE 8: Comparison of the site specificity of DNA cleavage by Cu_{12}-MT with that of H_2O_2 plus Cu(I) and H_2O_2 plus Cu(II). (A) The ^{32}P 5′-end-labeled 337 bp fragment (*Pst*I 2345−*Ava*I* 2681) and 1 μM per base of sonicated calf thymus DNA in 200 μL of 10 mM sodium phosphate buffer at pH 7.9 containing 5 μM DTPA were incubated with 42 μM Cu_{12}-MT at 37 °C for 60 min. (B) The ^{32}P 5′-end-labeled 337 bp fragment (*Pst*I 2345−*Ava*I* 2681) and 50 μM per base of sonicated calf thymus DNA in 200 μL of 10 mM sodium phosphate buffer at pH 7.9 containing 5 μM DTPA were incubated with 0.5 mM H_2O_2 plus 0.5 mM Cu(I) at 37 °C for 1 min. (C) The ^{32}P 5′-end-labeled 337 bp fragment (*Pst*I 2345−*Ava*I* 2681) and 50 μM per base of sonicated calf thymus DNA in 200 μL of 10 mM sodium phosphate buffer at pH 7.9 containing 5 μM DTPA were incubated with 1 mM H_2O_2 plus 0.5 mM Cu(II) at 37 °C for 10 min. After piperidine treatment, the DNA fragments were analyzed by the method described in the legend to Figure 8. The horizontal axis shows the nucleotide number of human c-Ha-*ras*-1 protooncogene starting with the *Bam*HI site (Capon *et al.*, 1983).

observed (Overvad *et al.*, 1993). The intratesticular injection of $CuSO_4$ or $CuCl_2$ induced testicular tumors in mice and chicken (Magos, 1991). $CuSO_4$ showed clastogenic effects on the bone marrow chromosomes of mice *in vivo* (Agarwal *et al.*, 1990). Tkeshelashvili *et al.* (1991) reported a mutation spectrum of Cu-induced DNA cleavage. It is well-known that free Cu(II) ion induces strong DNA cleavage in the presence of H_2O_2. The main active species causing DNA cleavage are more likely copper−peroxide complexes, with reactivity similar to singlet oxygen and/or •OH, rather than •OH (Yamamoto & Kawanishi, 1989). However, these studies were performed in the absence of proteins. Since copper ions administered induce MT and most of the copper ions bind to it (Hamer, 1986), we have investigated whether copper-containing MTs induce DNA cleavage.

Müller *et al.* (1991) reported that Cd,Zn-MT induced strand breaks to isolated DNA and speculated that the DNA cleavage might be caused by a radical species formed by the cysteine residues of MT charged with heavy metal ions. However, the commercial rabbit MT I used in this work contained 0.3 mM Cu/1 mM MT. The present data show that Cd_7-MT containing no copper has no ability to cause DNA cleavage and that commercial MT causes site-specific DNA cleavage. The DNA cleavage was efficiently inhibited by EDTA, but not by bathocuproine. These results indicate that DNA cleavage was due to the metal contained in commercial MT.

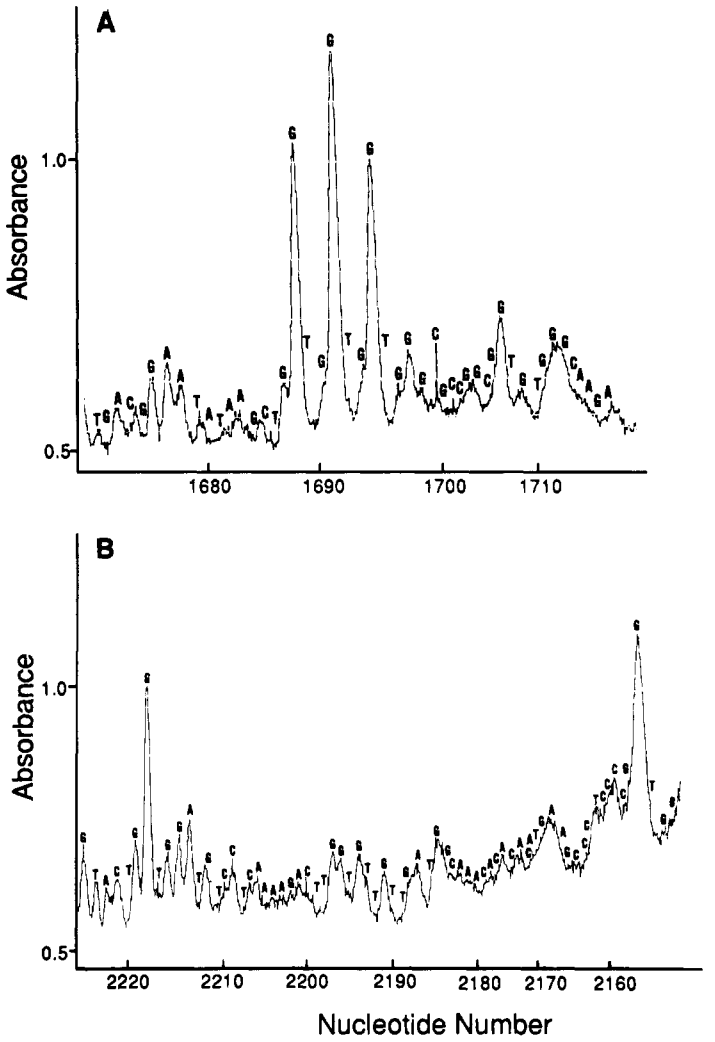

FIGURE 9: Site specificity of DNA cleavage induced by Cu_2Cd_5-MT. (A) The ^{32}P 5′-end-labeled 261 bp fragment (*Ava*I* 1645−*Xba*I 1905) and 1 μM per base of sonicated calf thymus DNA in 200 μL of 10 mM sodium phosphate buffer at pH 7.9 containing 5 μM DTPA were incubated with 0.22 mM Cu_2Cd_5-MT at 37 °C for 60 min. (B) The ^{32}P 5′-end-labeled 341 bp fragment (*Xba*I 1906−*Ava*I* 2246) was used. After piperidine treatment, the DNA fragments were analyzed by the method described in the legend to Figure 8. The horizontal axis shows the nucleotide number of human c-Ha-*ras*-1 protooncogene starting with the *Bam*HI site (Capon *et al.*, 1983).

In order to examine the effect of copper content on MT-induced DNA cleavage, we obtained Cu-reconstituted MT (Table 1). MT exists as a two-domain protein with the seven Cd(II) ions coordinated tetrahedrally in two polynuclear clusters: a three-metal cluster coordinated by six terminal cysteine thiolate ligands and three bridging cysteine thiolates and a four-metal cluster coordinated by the six terminal and five bridging cysteine thiolates (Boulanger *et al.*, 1982; Furey *et al.*, 1986). Cu(I) ions bind in a cluster as Cu_{12}-MT with a distribution of six metal ions per cluster (Nielson *et al.*, 1985). The Cu(I) ions appear to be bound in a trigonal geometry, at least with the β-domain (Nielson & Winge, 1984). Cd(II) was sequestered preferentially in the α-domain, and reciprocally, copper was found to bind preferentially in the β-domain (Nielson *et al.*, 1985; Nielson & Winge, 1985; Kille *et al.*, 1992).

The order and site specificity of DNA cleavage by Cu_2Cd_5-MT, commercial MT, and Cu_{12}-MT are summarized in Table 1. The mechanism for DNA cleavage induced by Cu_2Cd_5-MT and commercial MT is completely different from that for Cu_{12}-MT-induced DNA cleavage. DNA cleavage induced by Cu_2Cd_5-MT and commercial MT was not increased by piperidine treatment. DNA cleavage was not inhibited by the addition of catalase. Therefore, DNA cleavage is explained singly by nonoxidative cleavage of the deoxyribose phosphate backbone. On the other hand, the piperidine effect observed with Cu_{12}-MT indicated that Cu_{12}-MT induced base damage and/or liberation in addition to cleavage of the deoxyribose phosphate backbone. Oxidative cleavage of the backbone induced by Cu_{12}-MT may be due to the abstraction of a hydrogen atom from the deoxyribose ring. As a similar reaction, 4′-carbon−hydrogen bond cleavage was reported with iron bleomycin-mediated DNA cleavage (Stubbed & Kozarich, 1987). When base damage and/or liberation are caused, piperidine goes on to catalyze β-elimination of phosphates from the empty sugar to finally break the DNA strand (Maxam & Gilbert, 1980).

Cu_{12}-MT frequently induced piperidine-labile sites at thymine residues. The most preferred sites were the thymine residues of the 5′-GTC-3′ sequence. The site specificity was similar to that obtained with Cu(II) in the presence of cysteine, GSH, or Lys-Cys-Thr-Cys-Cys-Ala (56−61 residues of MT), suggesting the importance of cysteine residues as a reducing group. The site specificity was also similar to that obtained with Cu(I) plus H_2O_2. Bathocuproine, a Cu(I)-specific chelating agent, and catalase showed inhibitory effects on Cu_{12}-MT-induced DNA cleavage. These results indicate that Cu(I) and H_2O_2 participate in DNA cleavage

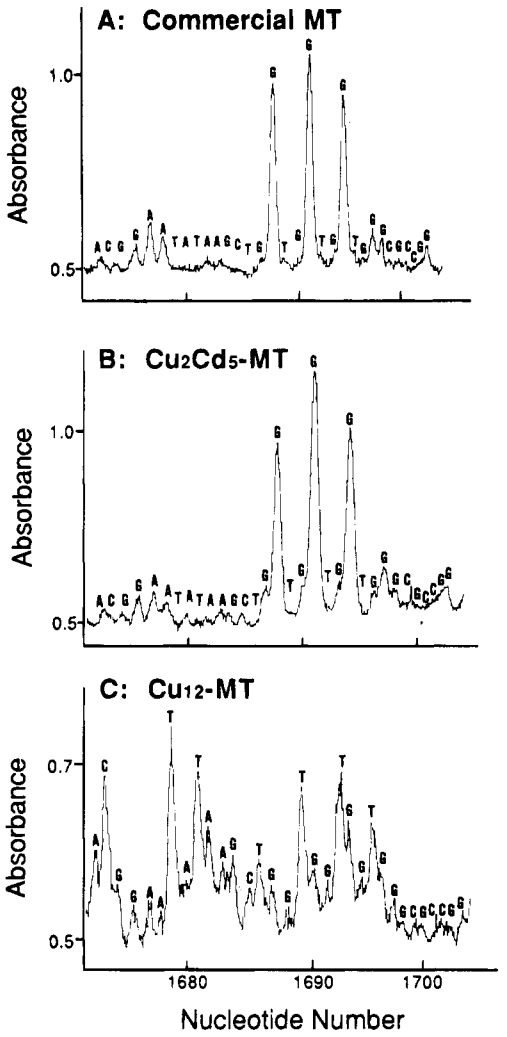

FIGURE 10: Comparison of the site specificity of DNA cleavage by commercial MT with that of Cu_2Cd_5-MT and Cu_{12}-MT. The ^{32}P 5′-end-labeled 261 bp fragment (*Ava*I* 1645−*Xba*I 1905) and 1 μM per base of sonicated calf thymus DNA in 200 μL of 10 mM sodium phosphate buffer at pH 7.9 containing 5 μM DTPA were incubated with several kinds of MT at 37 °C for 60 min: (A) 0.5 mM commercial MT; (B) 0.22 mM Cu_2Cd_5-MT; (C) 42 μM Cu_{12}-MT. After piperidine treatment, the DNA fragments were analyzed by the method described in the legend to Figure 8. The horizontal axis shows the nucleotide number of human c-Ha-*ras*-1 protooncogene starting with the *Bam*HI site (Capon *et al.*, 1983).

Table 1: Two Mechanisms of DNA Cleavage by MT

kind of MT	DNA cleavage: backbone cleavage	DNA cleavage: base alteration	type of site specificity[a]	mechanism of DNA cleavage
Cu_{12}-MT	+	+	I	oxidative
Cu_2Cd_5-MT	+	−	II	nonoxidative
commercial MT	+	−	II	nonoxidative
Cd_7-MT	−	−		

[a] I: Thymine residue of the 5′-GTC-3′ sequence. II: Central guanine residue of the 5′-GGT-3′ sequence.

and also provide indirect evidence for the autoxidation of Cu(I) contained in Cu_{12}-MT or Cu(I) released from Cu_{12}-MT. Relevantly, autoxidation of the Cu(I)−thiolate oligonuclear binding centers of MT was observed, leading to the release of some Cu(II) and probably superoxide (O_2^-) (Beltramini *et al.*, 1989; Da Costa Ferreira *et al.*, 1993; Felix *et al.*, 1993). However, although Cu_{12}-MT itself may continuously release copper under aerobic conditions, Cu(I) contained in Cu_{12}-MT did not appear to be autoxidized

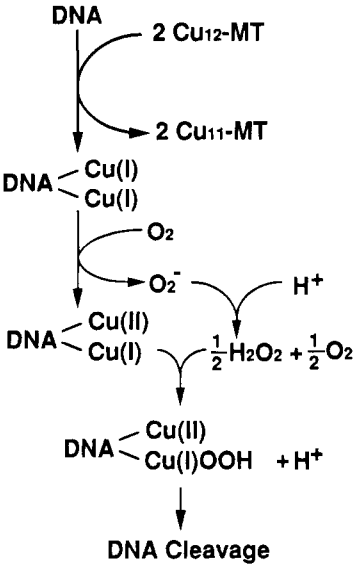

FIGURE 11: Possible mechanism of DNA damage induced by Cu_{12}-MT.

rapidly enough to cause DNA cleavage. On the other hand, since Cu(I) has high affinity with DNA (Prütz *et al.*, 1990), it may be considered that Cu(I) is transferred from Cu_{12}-MT to DNA, resulting in the promotion of autoxidation of Cu(I). This is supported by the observations that Cu(I) strongly induced DNA cleavage and that the cleavage pattern was similar to that of Cu(I) plus H_2O_2 (data not shown).

A possible mechanism of Cu_{12}-MT-induced DNA cleavage as shown in Figure 11 can be envisioned as accounting for most of the observations. As the first step, O_2^- and H_2O_2 are generated by the autoxidation of Cu(I) released from Cu_{12}-MT and react with another Cu(I) to form an active species causing DNA cleavage. Since the typical •OH scavenger, ethanol, did not inhibit Cu_{12}-MT-induced DNA cleavage, the bound •OH such as Cu(I)OOH can be considered as an •OH-generating species causing DNA cleavage. Recently, it was pointed out that free •OH is extremely short-lived and travels a short distance in water in a cell (Pryor, 1986; Tchou & Grollman, 1993). Cu_{12}-MT-induced DNA cleavage was not inhibited by DNA ligand (chromomycin A_3). This result suggests that the specific interaction of Cu_{12}-MT with DNA was not necessary for DNA cleavage.

On the other hand, the present results show that DNA ligands such as chromomycin A_3 and berenil significantly inhibit commercial MT-induced DNA cleavage. Albumin showed no or little effect on commercial MT-induced DNA cleavage. In addition, when denatured single-stranded DNA was treated with commercial MT, cleavage decreased. These results suggest that the interaction of DNA with MT is essential for Cu_2Cd_5-MT- and commercial MT-induced DNA cleavage. Thus, it is considered, on the basis of analyses of site specificity of DNA cleavage, that these Cu-MTs interact with the 5′-GGT-3′ sequence in the G•C-rich region to induce cleavage of the deoxyribose phosphate backbone.

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