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Sequence Specificity of Heat-Labile Sites in DNA Induced by Mitomycin C†

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ABSTRACT: The sequence specificity of the mitomycin C–DNA interaction was directly determined by using DNA sequencing techniques and by using 3′- or 5′-end-labeled DNA fragments of defined sequence as substrates. Mitomycin C reduced with sodium borohydride induced heat-labile sites in DNA preferentially at specific sequences. The heat-labile sites were induced most preferentially at the dinucleotide sequence G-T (especilly Pu G-T), which was determined by scanning autoradiograms with a microdensitometer after gel electrophoresis. DNA was cleaved at the 3′ side of deoxyguanosines and of some deoxyadenosines by heat treatment. Oligonucleotides produced by heat treatment after reaction with reduced mitomycin C contained phosphoryl groups at the 5′ termini. The 3′ termini seemed not to have simple structures, judging from their electrophoretic mobilities. Oxygen radicals such as singlet oxygen and hydroxyl radical were possibly involved in the induction of heat-labile sites.

Mitomycin C, a potent anticarcinogenic antibiotic, interacts with DNA, resulting in a covalent binding of the drug to DNA, as well as in the formation of cross-links between the complementary strands of DNA (Iyer & Szybalski, 1963, 1964; Matsumoto & Lark, 1963). These DNA modifications, in which the former is predominant 10–20-fold over the latter (Szybalski & Iyer, 1964a), are believed to be essential for the cytotoxicity of mitomycin C (Iyer & Szybalski, 1963; Weiss et al., 1968, Kinoshita et al., 1971; Mercado & Tomasz, 1972). The aziridine and methylurethane moieties are suggested to be involved in the binding to DNA (Schwartz et al., 1963; Iyer & Szybalski, 1964; Hashimoto et al., 1982). The binding sites of mitomycin C in DNA are the O-6 position or the 2-amino group of guanine residues or the 6-amino group of adenine residues (Tomasz et al., 1983; Hashimoto et al., 1982). However, the details of the interaction of mitomycin C with DNA have yet to be elucidated.

Mitomycin C contains a quinone moiety beside aziridine and methylurethane. Reduction of mitomycin C, by chemical or enzymatic methods, followed by exposure to air results in the generation of superoxide anion and hydrogen peroxide (Handa & Sato, 1975; Tomasz, 1976). Oxygen radicals were generated not only by free mitomycin C but also by mitomycin C irreversibly bound to DNA (Tomasz, 1976). Lown et al. (1976) and Ueda et al. (1980, 1981, 1982) reported that chemically reduced mitomycin C induces single-strand scission in single-stranded and double-stranded DNAs. The DNA strand scission is considered to involve the oxygen radicals, such as hydroxyl radical and singlet oxygen, and mitomycin C semiquinone radical (Lown et al., 1976; Ueda et al., 1980, 1981, 1982).

DNA cleavage via a mechanism involving oxygen radicals is reported for some antitumor antibiotics such as bleomycin (Lown & Sim, 1977; Sausville et al., 1976). Strand scission by bleomycin occurs preferentially at specific sequences (Takeshita et al., 1978; D'Andrea & Haseltine, 1978) and at specific sites in DNA (Lloyd et al., 1978), and the sequence specificity of single-strand scission is related to the site-specific double-strand scission by bleomycin (Mirabelli et al., 1982).

We have investigated the interaction of mitomycin C with DNA by using DNA substrates of defined sequence. In this paper, we show that reduced mitomycin C induces heat-labile sites in DNA preferentially at specific sequences and that oxygen radicals are possibly involved in the induction of heat-labile sites.

Materials and Methods

Chemicals and Enzymes. Mitomycin C was kindly supplied by Kyowa Hakko Co. Ltd., Tokyo, Japan. The restriction enzymes *Hae*III, *Taq*I, and *Hin*fI and T4 polynucleotide kinase were obtained from Takara Shuzo Co. Ltd., the Klenow fragment of DNA polymerase I of *Escherichia coli* was from Bethesda Research Laboratories GmbH, and calf intestine alkaline phosphatase was from Boehringer Mannheim GmbH. [α-^{32}P]dTTP, [α-^{32}P]dCTP, and [γ-^{32}P]ATP (specific activity about 3000 Ci/mmol) were purchased from New England Nuclear, Du Pont, and Amersham International.

DNA Substrates. Three DNA fragments of defined sequence were obtained from bacteriophage ϕX174 replicative form DNA. Double-stranded ϕX174 replicative form DNA was prepared as previously described (Ueda et al., 1981) and digested with *Hae*III, and 194 and 234 base pair fragments [Z_7 and Z_8 fragments in the map reported by Sanger (Sanger et al., 1978)] were purified. Fragment Z_7 was digested with *Taq*I and was labeled by extension of the 3′ termini with Klenow polymerase in the presence of [α-^{32}P]dCTP (Maniatis et al., 1982). Fragment Z_8 was digested with *Hin*fI and labeled at the 3′ termini in the presence of [α-^{32}P]dTTP and unlabeled

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dATP and dCTP. Resulting 3′-end-labeled 56, 142, and 178 base pair fragments (C_{436}–C_{491}, C_{980}–T_{1121}, and C_{492}–C_{669} in the map reported by Sanger, respectively) were purified by electrophoresis on a 6% polyacrylamide gel.

[5′-^{32}P]DNA fragments were obtained by incubation of the Z_7 or Z_8 fragment with [γ-^{32}P]ATP and T4 polynucleotide kinase. After digestion with *Taq*I or *Hin*fI, 5′-end-labeled 55 and 139 base pair fragments were purified by electrophoresis on a 6% polyacrylamide gel.

Reaction Conditions. The standard reaction mixture (100 μL) contained 25 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer (pH 7.1), 0.1 mM mitomycin C, 0.5 mM sodium borohydride, and 3′- or 5′-^{32}P-labeled DNA fragment [approximately 50 ng (specific activity 2×10^3 cpm/ng)]. The reaction was started by the addition of a freshly prepared sodium borohydride solution, carried out for 15 min at 37 °C, and terminated by the addition of 4 μL of 0.5 M ethylenediaminetetraacetic acid (EDTA), 2 μL of 1 mg/mL tRNA, and 10 μL of 3 M sodium acetate (pH 5.2) followed by ethanol precipitation. The pellet was rinsed in 70% ethanol, dried, and resuspended in 40 μL of 10 mM Tris-HCl buffer (pH 8.1), and the suspension was heated for 5 min at 90 °C.

The heat-treated DNA was reprecipitated with ethanol, rinsed in 70% ethanol, dried, and dissolved in 5 μL of 80% (v/v) formamide–10 mM NaOH loading buffer for gel electrophoresis. DNA was heat denatured for 1 min at 90 °C and loaded on a 10% or 16% polyacrylamide slab gel for sequence analysis. Electrophoresis was at 25 mA. Autoradiography was done on a Fuji RX film at −70 °C.

Quantitative Sequence Analysis. The autoradiograms were scanned with a microdensitomer (Joyce Loebl & Co. Ltd. MK III CS double-beam recording microdensitometer). The relative positions of oligonucleotides produced by the reaction with reduced mitomycin C and subsequent heat treatment were determined by direct comparison with oligonucleotides produced by the chemical reactions of the Maxam–Gilbert procedure (Maxam & Gilbert, 1977). The relative amounts of produced oligonucleotides were measured quantitatively as follows: The relative peak heights on densitometric scans were measured and normalized relative to the average height of the peaks of products of the deoxyguanylate-specific reaction on the autoradiogram of each sequencing gel. DNA fragments shorter than decanucleotides were neglected because they were precipitated inefficiently by ethanol.

Analysis of 5′ Termini. The 5′ termini of the fragments resulting from breaks induced by reduced mitomycin C and subsequent heat treatment were analyzed for the presence of a phosphoryl group. For analysis of the 5′ termini of the DNA fragments resulting from dimethyl sulfate reaction, piperidine treatment in the Maxam–Gilbert procedure was replaced by hydrolysis with 0.1 N NaOH and followed by pH neutralization (Kross et al., 1982). DNA was precipitated with ethanol and resuspended in 40 μL of 10 mM Tris-HCl buffer (pH 8.1). DNA was heated for 5 min at 90 °C and quickly chilled. For dephosphorylation of DNA, 10 μL of 10 × CIP buffer [0.5 M Tris-HCl (pH 9.0), 10 mM $MgCl_2$, 1 mM $ZnCl_2$, and 10 mM spermidine] and 10 units of calf intestine alkaline phosphatase were added, and the mixture was incubated for 30 min at 37 °C and for 30 min at 56 °C. A second aliquat of the enzyme was added and the incubation was repeated at both temperatures (Maniatis et al., 1982). The reaction was terminated by phenol extractions and followed by ethanol precipitation. The pellet was rinsed in 70% ethanol, dried and dissolved in 5 μL of loading buffer for gel electrophoresis.

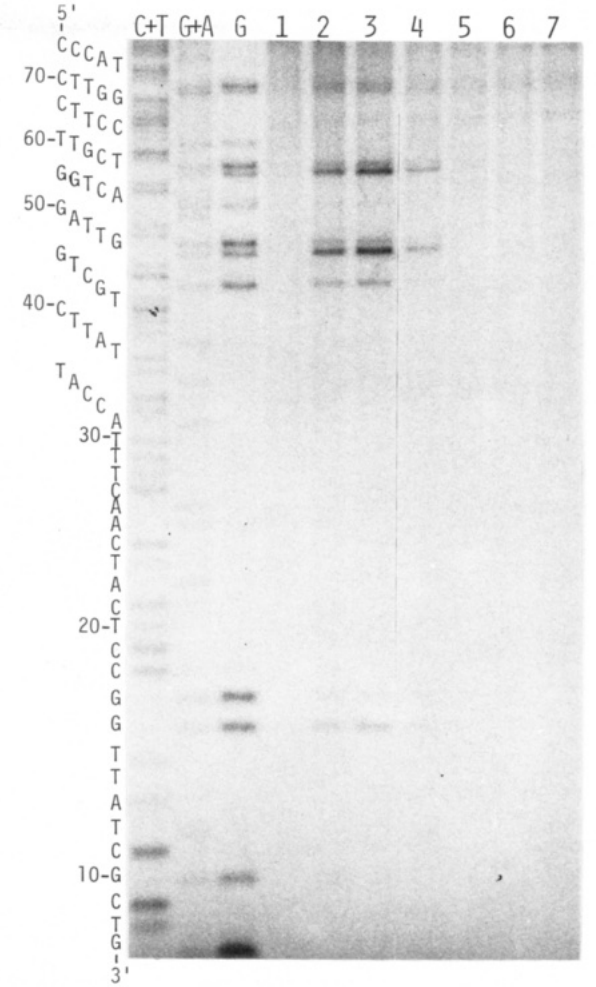

FIGURE 1: Induction of heat-labile sites in DNA by reduced mitomycin C. The 142 base pair 3′ end ^{32}P-labeled DNA was incubated with 0.1 mM mitomycin C and 0.5 mM sodium borohydride for 15 min at 37 °C. After the reaction, DNA was precipitated with ethanol, resuspended in 40 μL of 10 mM Tris-HCl buffer (pH 8.1), and heated for 3 min (lane 2) and for 5 min (lane 3) at 90 °C. The heat-treated DNA was reprecipitated with ethanol, dissolved in5 μL of loading buffer, and loaded on a 10% polyacrylamide gel for sequence analysis. Products without heat treatment were in lane 1. Products of reactions in the presence of 10 mM EDTA, in the absence of sodium borohydride, or in the absence of mitomycin C, followed by heat treatment, were in lanes 4, 5, and 6, respectively. Lane 7 contains untreated 142 base pair 3′-end-labeled DNA. Products of base-specific chemical reactions were in lanes C + T, G + A, and G. The sequence is indicated on the left side of the figure.

Results

Induction of Heat-Labile Sites in DNA by Reduced Mitomycin C. To investigate the interaction of mitomycin C with DNA, we used end-labeled DNA fragments of defined sequence as substrates. The DNA substrate used in Figure 1 was the 3′-end-labeled 142 base pair restriction fragment of ϕX174 replicative form DNA (C_{980}–T_{1121} in the map reported by Sanger). Oligonucleotides were clearly detected on the autoradiogram as the products of the reaction with reduced mitomycin C and subsequent heat treatment (Figure 1, lanes 2 and 3). These oligonucleotides were scarcely produced without heat treatment (Figure 1, lane 1) and were dependent on the time of heat treatment (Figure 1, lanes 2 and 3). All DNA samples were routinely denatured by heating at 90 °C for 1 min just before applying to the gel as described under Materials and Methods, but this heating procedure alone was not enough to cleave mitomycin C treated DNA. Heat treatments for 3–5 min in Tris-HCl buffer before the dena-

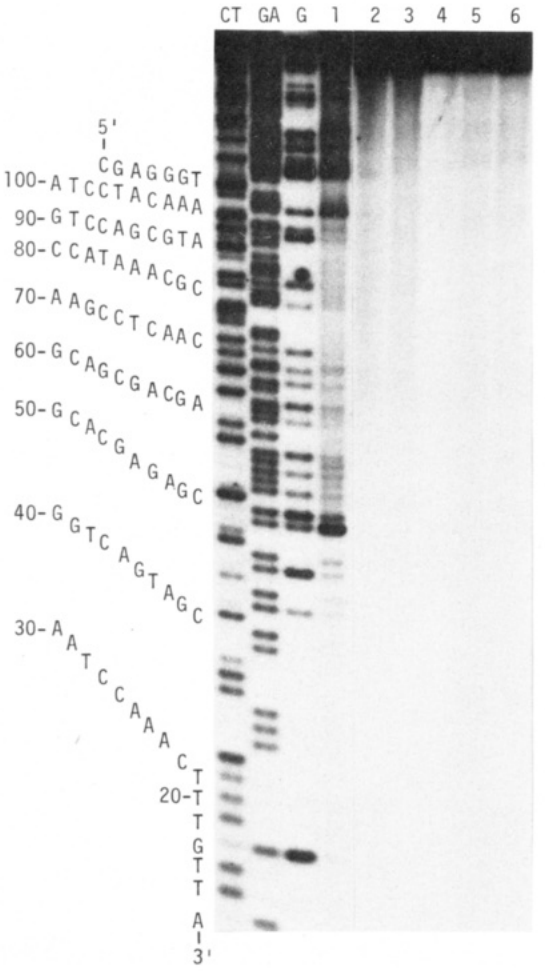

FIGURE 2: Inhibition by oxygen radical scavengers. The 178 base pair 3′ end ^{32}P-labeled DNA was incubated with 0.1 mM mitomycin C and 0.5 mM sodium borohydride (lane 1) in the presence of 0.1 M sodium benzoate (pH 7.5) (lane 2) or 0.1 M DABCO (pH 7.5) (lane 3) for 15 min at 37 °C. DNA was precipitated with ethanol, resuspended in 40 μL of 10 mM Tris-HCl buffer (pH 8.1), and heated for 5 min at 90 °C. The heat-treated DNA was reprecipitated with ethanol and analyzed on a 10% polyacrylamide gel for sequence analysis. Products of reactions in the absence of sodium borohydride or mitomycin C followed by heat treatment, were in lanes 4 and 5, respectively. Lane 6 contains untreated DNA. Products of base-specific chemical reactions were in lanes CT, GA, and G.

turation procedure (1 min) were required for chain cleavage of mitomycin C treated DNA. No oligonucleotides were produced by reaction with nonreduced mitomycin C (lane 5) or sodium borohydride alone (Figure 1, lane 6) and subsequent heat treatment.

Inhibition by Oxygen Radical Scavengers and Metal-Chelating Agents. Oxygen radicals and metal ions are involved in mitomycin C induced DNA strand scission (Lown et al., 1976; Ueda et al., 1980, 1981, 1982). We examined the involvement of oxygen radicals and metal ions in the induction of heat-labile sites by reduced mitomycin C. The DNA substrate used in Figure 2 was the 3′-end-labeled 178 base pair restriction fragment of φX174 replicative form DNA (C_{492}–C_{669} in the map reported by Sanger). Oligonucleotides were produced by the reaction with reduced mitomycin C and subsequent heat treatment (Figure 2, lane 1). The production of oligonucleotides was inhibited by sodium benzoate (Figure 2, lane 2), which scavenges the hydroxyl radical (Dorfman & Adams, 1973), and 1,4-diazabicyclo[2,2,2]octane (DABCO) (Figure 2, lane 3), which scavenges singlet oxygen (Ouannès & Wilson, 1968). DABCO completely inhibited the production of oligonucleotides even at a concentration of 10 mM,

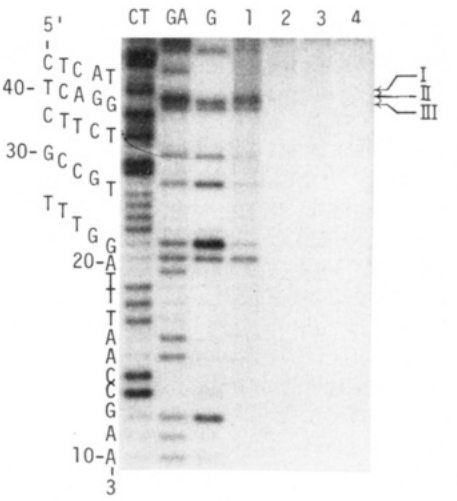

FIGURE 3: Base sequence specific interaction of mitomycin C with DNA. The 56 base pair 3′ end ^{32}P-labeled DNA was incubated with 0.1 mM mitomycin C and 0.5 mM sodium borohydride (lane 1), 0.1 mM mitomycin C alone (lane 2), or 0.5 mM sodium borohydride alone (lane 3). After the reaction, DNA was heated for 5 min at 90 °C, and the products were analyzed on a 16% polyacrylamide gel as described under Materials and Methods. Lane 4 contains untreated DNA. Products of base-specific chemical reactions were in lanes CT, GA, and G. Arrows I, II, and III indicate oligonucleotides described in the text.

and 10 mM sodium benzoate also had an inhibitory effect (data not shown). EDTA, a metal-chelating agent, also inhibited the production of oligonucleotides (Figure 1, lane 4). The production of oligonucleotides was almost completely inhibited also by 10 mM diethylenetriaminepentaacetic acid (DETAPAC), a metal-chelating agent, and partially by 1 mM DETAPAC (data not shown). The high concentrations of these salts were reported to inhibit the binding of mitomycin C to DNA (Lipman et al., 1978). But the inhibitory effect of salts seems negligible when the inhibitors are used below 10 mM, because 10 mM sodium chloride did not inhibit the production of oligonucleotides (unpublished results).

Sequence Specificity of Heat-Labile Sites. Autoradiograms in Figures 1 and 2 made us expect that reduced mitomycin C induced heat-labile sites preferentially at specific sequences in DNA. To examine this possibility, we used another DNA substrate (a 3′-end-labeled 56 base pair DNA fragment) (Figure 3) and scanned autoradiograms in Figures 1–3 with a microdensitomer (Figure 4). The relative amounts of produced oligonucleotides were measured quantitatively as described under Materials and Methods, and the relative positions were determined by direct comparison of the products of the mitomycin C reaction with the products of the deoxyguanylate-specific chemical reaction (Figure 5). DNA was cleaved at the 3′ side of deoxyguanosines and of some deoxyadenosines by the heat treatment of DNA reacted with reduce mitomycin C. Reduced mitomycin C induced heat-labile sites extensively at the sequence GGT, GAGGGT, and AAGT, followed by induction at the sequences GGC, GT, GAGAGC, AGT, AGGC, and GGA. In dinucleotide sequences, G-T sequences (especially Pu G-T) were cleaved most extensively by the reaction with reduced mitomycin C subsequent heat treatment.

Structure of Termini of the Breaks. The structure of termini of the breaks was analyzed to investigate the chemical nature of the heat-labile sites induced by reaction with reduced mitomycin C. The electrophoretic mobilities of oligonucleotides produced from 3′-end-labeled DNA by reaction with reduced mitomycin C, followed by heat treatment, were

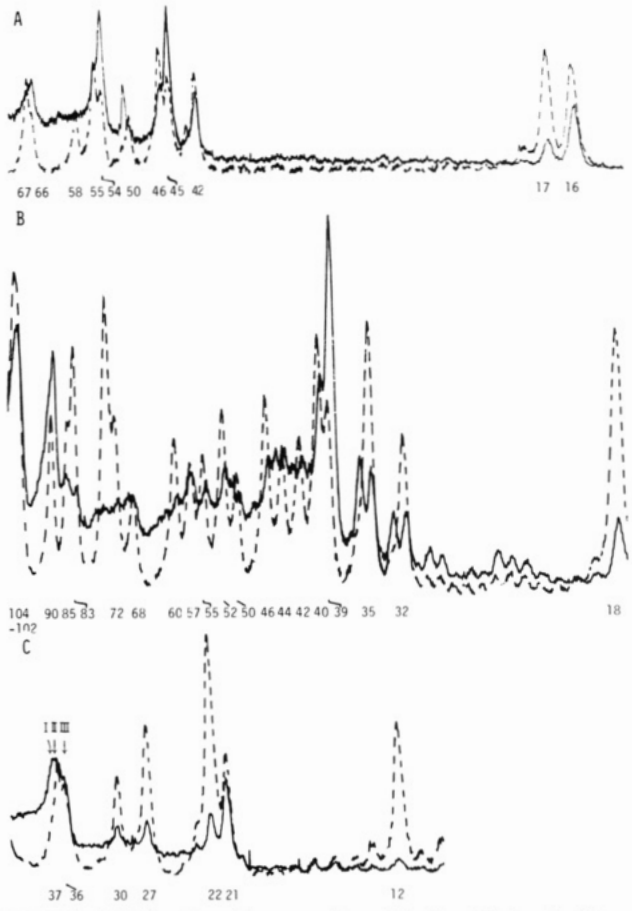

FIGURE 4: (A) Densitometric scans of lane 3 (—) and G (---) in Figure 1. (B) Densitometric scans of lanes 1 (—) and G (---) in Figure 2. (C) Densitometric scans of lanes 1 (—) and G (---) in Figure 3. Numbers correspond to the position of deoxyguanylate in the fragments from the 3′ terminus. Arrows I, II, and III indicate oligonucleotides described in the text.

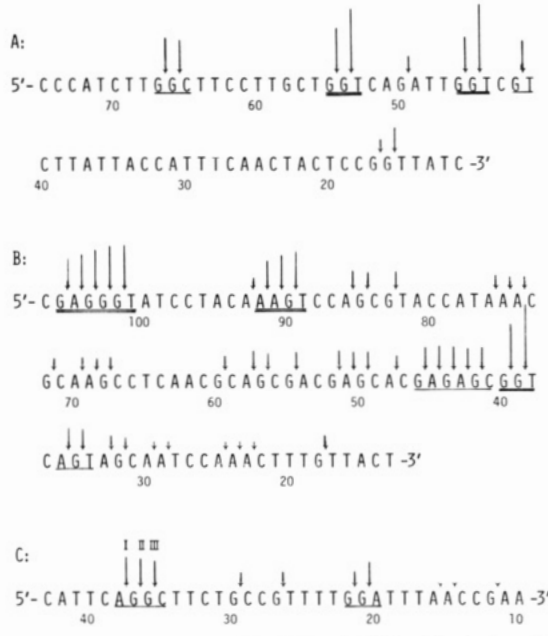

FIGURE 5: Sequence specificity of heat-labile sites induced in a 3′-end-labeled DNA fragment 142 (A), 178 (B), and 56 (C) base pairs long. These fragments were used in Figures 1, 2, and 3, respectively. Arrows locate sites determined from the densitometric scans in Figure 4. The length of the arrows indicates the relative extent of cleavage at a particular site as measured from densitometric scans. Sequences doubly underlined are the sites extensively cleaved, and followed by sequences singly underlined.

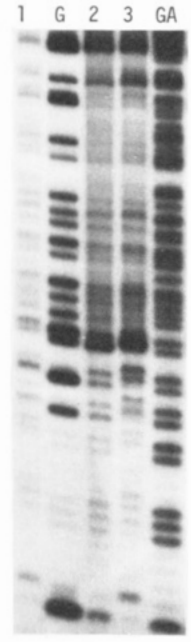

FIGURE 6: Structure of 5′ termini of the breaks. The 178 base pair 3′ end ^{32}P-labeled DNA was reacted with 0.1 mM mitomycin C and 0.5 mM sodium borohydride and subsequently heated for 5 min at 90 °C. DNA was treated with calf intestine alkaline phosphatase as described under Materials and Methods (lane 3). Original products of the mitomycin C reaction were in lane 2. Products of the dimethyl sulfate reaction, in which piperidine treatment was replaced by 0.1 N NaOH hydrolysis followed by pH neutralization, were treated with calf intestine alkaline phosphatase (lane 1). Original products of base-specific chemical reactions were in lanes G and GA.

the same as some of those produced by chemical degradation with the Maxam–Gilbert procedure (Figures 1–3). The comigration of the products suggested that the 5′ termini of the breaks are phosphorylated (Maxam & Gilbert, 1980).

To determine the structure of 5′ termini of the breaks, we treated the produced oligonucleotides with calf intestine alkaline phosphatase which removes 5′-phosphoryl groups. Treatment of the products of a dimethyl sulfate (deoxyguanylate-specific) reaction with the enzyme resulted in retardation of the electrophoretic mobilities (Figure 6, lane 1). Treatment of the oligonucleotides produced by reaction with reduced mitomycin C and subsequent heat treatment resulted in a similar retardation (Figure 6, lane 3). The retardation of the electrophoretic mobilities is expected to be due to the removal of 5′-phosphoryl groups from the oligonucleotides and the resulting change of the charge/mass ratio of the molecule. These results indicate that the 5′ termini of the oligonucleotides produced by the reaction with reduced mitomycin C and subsequent heat treatment contain phosphoryl groups.

Structure of 3′ Termini of the Breaks. For analysis of the structure of 3′ termini of the breaks, 5′-end-labeled DNA was used. The same strand of the fragment used in Figure 3 was labeled at the 5′ terminus and used as a substrate (Figure 7). Oligonucleotides were produced when the DNA was reacted with reduced mitomycin C and then heat treated (Figure 7, lane 1). Reduction of mitomycin C and the subsequent heat treatment were a prerequisite for the production of oligonucleotides (Figure 7, lanes 3 and 2, respectively) as well as in the reaction of 3′-end-labeled substrates. Sodium benzoate, DABCO, and EDTA inhibited the reaction also in this case (data not shown).

Heat treatment of the DNA fragment bearing a heat-labile site results in the production of two olgionucleotides. The oligonucleotide indicated by arrow I in Figure 7A,B and that

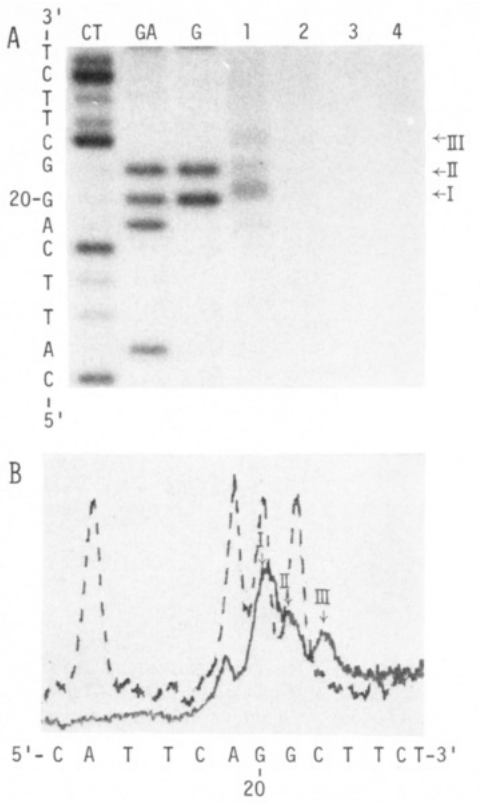

FIGURE 7: Structure of 3′ termini of the breaks. (A) The same strand of the fragment used in Figure 3 was labeled at the 5′ terminus, and the 5′-end-labeled 55 base pair DNA was incubated with 0.1 mM mitomycin C and 0.5 mM sodium borohydride. DNA was heated for 5 min at 90 °C (lane 1) as described under Materials and Methods, and the products were analyzed on a 16% polyacrylamide gel for sequence analysis. Products without heat treatment were in lane 2. Products of reactions in the absence of sodium borohydride or mitomycin C, followed by heat treatment, were in lanes 3 and 4, respectively. Products of base-specific chemical reactions were in lanes CT, GA, and G. (B) Densitometric scans of lanes 1 (—) and GA (---). G_{21}, which is the 21st nucleotide from the 5′ end, corresponds to G_{37} in Figure 3 in the fragment. Arrows I, II, and III indicate oligonucleotides described in the text.

indicated by arrow I in Figures 3 and 4 are considered to have resulted from the cleavage at a heat-labile site. Oligonucleotides indicated by arrow II or arrow III in Figures 3, 4, and 7 are also considered result from cleavage at a heat-labile site. The products from 5′-end-labeled DNA had lower electrophoretic mobilities than the dimethyl sulfate reaction products and the formic acid reaction products, although the products from 3′-end-labeled DNA had the same mobilities as the products of the chemical reactions of the Maxam–Gilbert procedure (Figures 3 and 4). Broad peaks on the scan of the autoradiogram of the products from the 5′-end-labeled DNA (Figure 7B) suggest that the 3′ termini of the breaks do not have simple structures. The lower electrophoretic mobilities and the broadness of peaks of the products from the 5′-end-labeled DNA were confirmed with another fragment (a 5′-end-labeled 139 base pair fragment) (data not shown).

Discussion

We have investigated the interaction of mitomycin C with DNA by using a sequencing technique. The results demonstrate that reduced mitomycin C induces heat-labile sites in DNA at specific sequences. The breaks occur at the 3′ side of deoxyguanosines and of some deoxyadenosines by heat treatment of DNA reacted with reduced mitomycin C. The dinucleotide sequence G-T (especially Pu G-T) is the most preferred for induction of heat-labile sites. Mono-, di-, and trinucleotides of deoxyadensine are rather resistant to induction of heat-labile sites. DNA sequencing techniques revealed the sequence-specific interaction with DNA of several antitumor agents, e.g., bleomycin (Takeshita et al., 1978; D'Andrea & Haseltine, 1978), neocarzinostatine (Hatayama et al., 1978), hedamycin (Bennett, 1982), actinomycin D, and netropsin (Lane et al., 1983). The sequence specificity of the mitomycin C–DNA interaction reported in this paper is remarkable compared to other agents, although mitomycin C is a rather small molecule.

Covalent binding of mitomycin C to DNA is thought to be preceded by a noncovalent association, presumably of an intercalation type, between DNA and the mitomycin C semiquinone (Tomasz et al., 1974). The association facilitates covalent bond formation betwen the O-6 position or the 2-amino group of guanine and C-1 of mitomycin C (Tomasz et al., 1983; Hashimoto et al., 1982). Mitomycin C reduced with sodium borohydride under the conditions used in this study formed a complex with calf thymus DNA, and the binding ratio was about 100 nucleotides/antibiotic (data not shown), which was detected by the ultraviolet absorbance at 310 nm and by using $\epsilon_{310} = 11\,500$ (Tomasz et al., 1974). A derivative of mitomycins, 7-methoxymitosene, which cannot bind to DNA via the aziridine ring, did not induce heat-labile sites in DNA (unpublished results). These results also suggest that covalent binding of mitomycin C at the C-1 position to DNA is essential for the induction of heat-labile sites.

Substitution at certain sites on bases in DNA, e.g., 7-alkylguanine, increases the rate of hydrolysis of the N-glycosidic bond to yield apurinic sites (Lawely & Brookes, 1963) and then produces strand breaks. Mitomycin C does not alkylate the N-7 positon (Tomasz, 1970) but the alkylates the O-6 position or the 2-amino group of guanine residues in DNA (Tomasz et al., 1983; Hashimoto et al., 1982). It is not known that O^6-alkylguanine produces apurinic sites. The inhibitory effects of oxygen radical scavengers and metal-chelating agents suggest the involvement of oxygen radicals such as singlet oxygen and hydroxyl radical in the induction of heat-labile sites by mitomycin C. DNA cleavage via a mechanism involving oxygen radicals and metal ions is reported for some antitumor drugs such as bleomycin and phenanthroline (Sigman et al., 1979). The DNA cleavage by bleomycin (Giloni et al., 1981; Wu et al., 1983) and by phenanthroline (Uesugi et al., 1982) is considered to be caused by oxidation of C-4′ and C-1′, respectively, of the deoxyribose moiety. Mitomycin C induces heat-labile sites in DNA but does not cleave DNA under the conditions used. The detailed mechanism of sequence-specific induction of heat-labile sites needs further study.

We have reported that reduced mitomycin C induces DNA strand scission in covalently closed circular DNA (Ueda et al., 1980, 1981, 1982). However, the present results demonstrate that the subsequent heat treatment is required for DNA cleavage in the reaction with DNA fragments of 50–200 base pairs long. The mechanism of the above two reactions of mitomycin C closely resemble each other, e.g., in the inhibitory effects of radical scavengers and metal-chelating agents. As the binding ratio was about 100 nucleotides/antibiotic under our experimental conditions, more than 50 heat-labile sites are supposed to be induced in closed circular phage DNA (ϕX174 DNA is 5386 bases long) if one bound mitomycin C molecule produces one heat-labile site. The damage is though to be very unstable, because we only needed a short period (3–5 min) of heating at neutral pH to produce strand scission. It is predictable that one of those damages spontaneously produces

strand scission in long DNA molecules. The higher order structures of DNA such as supercoiling may also be involved in the production of stand scission.

TA102, a new strain for the *Salmonella*/microsome mutagenicity test (Ames test) which detects oxidative mutagens with high sensitivity, detects also bleomycin and mitomycin C (Levin et al., 1982). This suggests that the mutagenicity of those antibiotics involves oxygen radicals. The surrounding sequence of the mutation site of the strain is AAG-T-AA. This sequence is interesting because the dinucleotide sequence GT is one of the most preferred sites for cleavage by bleomycin (Takeshita et al., 1978; D'Andorea & Haseltine, 1978) and is the most preferred site for induction of heat-labile sites by reduced mitomycin C. The dinucleotide sequence GT is also known as the sequence, in the alternatingly repeating sequence, potentially forming the Z form (Hamada & Kakunaga, 1982). Poly(dG-dT) sequences are highly dispersed in the human genome and could be involved in the regulation of gene expression (Hamada et al., 1982). Mitomycin C possibly induces heat-labile sites at specific sequences and causes DNA strand scission in cellular DNA.

This is the first paper reporting the sequence-specific interaction of mitomycin C with DNA which produce the heat-labile sites. Although the properties of mitomycin C–DNA complexes have been eagerly studied (Kaplan & Tomasz, 1982; Szybalski & Iyer, 1964b; Tomasz et al., 1974), no loss of a cross-linked fraction or no appreciable decrease of the molecular weight of DNA was observed even if those complexes were heated. The differences in the in vitro activation systems of mitomycin C may be important: namely, other authors used sodium hydrosulfite ($Na_2S_2O_4$) under anaerobic conditions, and we used sodium borohydride under aerobic conditions. Although whether the reported heat-labile damages play an important role in the actions of mitomycin C needs further study, the heat lability showed us the sequence specificity of the mitomycin C–DNA interaction. Nevertheless, it is of biochemical and chemical interests to reveal the mechanism of the sequence-specific mitomycin C–DNA interaction.

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Non-Ohmic Proton Conductance of Mitochondria and Liposomes†

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ABSTRACT: Direct measurements of the proton/hydroxyl ion flux across rat liver mitochondria and liposome membranes are reported. H^+/OH^- fluxes driven by membrane potential ($\Delta\psi$) showed nonlinear dependence on $\Delta\psi$ both in mitochondria and in liposomes whereas ΔpH-driven H^+/OH^- flux shows linear dependence on ΔpH in liposomes. In the presence of low concentrations of a protonophore the H^+/OH^- flux was linearly dependent on $\Delta\psi$ and showed complex dependence on ΔpH. The nonlinearity of H^+/OH^- permeability without protonophore is described by an integrated Nernst–Plank equation with trapezoidal energy barrier. Permeability coefficients depended on the driving force but were in the range 10^{-3} cm/s for mitochondria and 10^{-4}–10^{-6} cm/s for liposomes. The nonlinear dependence of H^+/OH^- flux on $\Delta\psi$ explains the nonlinear dependence of electrochemical proton gradient on the rate of electron transport in energy coupling systems.

It is generally accepted that the electrochemical proton gradient $\Delta\tilde{\mu}_{H^+}$ across the mitochondrial inner membrane is the intermediate which couples respiration to ATP synthesis (Mitchell, 1961, 1979). However, a complete quantitative description of proton fluxes in this system has proven difficult and controversial. The measured coupling ratios for proton transport by the respiratory chain differ by as much as 3-fold (Hinkle, 1981). Even the usual assumption that there is an invariant coupling ratio has been challenged, and the concept of variable stoichiometry and molecular slipping of proton pumps has been proposed (Rottenberg, 1979a; Walz, 1979; Westerhoff et al., 1981; Pietrobon et al., 1981). An important factor in these determinations which has not been well studied is the kinetics of proton permeability across the mitochondrial inner membrane. Direct measurements of proton permeability were made by Mitchell & Moyle (1967). An indirect method was devised by Nicholls (1974), who measured the magnitude of $\Delta\tilde{\mu}_{H^+}$ formed by respiration when the respiration rate was partially inhibited with malonate. If it is assumed that the coupling ratio of proton transport by the respiratory chain is constant, then this analysis gives the relative proton permeability as a function of $\Delta\tilde{\mu}_{H^+}$. The result was a nonlinear curve with increasing permeability coefficient at high values of $\Delta\tilde{\mu}_{H^+}$. Similar observations have been made with submitochondrial particles (Sorgato & Ferguson, 1979) and bacteria (Kell et al., 1978; Jackson, 1982; Clark et al., 1983). An alternative interpretation of Nicholl's results proposed by Pietrobon et al. (1981, 1983) is that the coupling ratio of respiration-driven proton transport decreased at high values of $\Delta\tilde{\mu}_{H^+}$ and the proton permeability coefficient is constant. To distinguish between these two interpretations, we have directly measured the equivalent proton flux, J_{H^+/OH^-}, driven by membrane potential, $\Delta\psi$, and pH gradient, ΔpH, in rat liver mitochondria and liposomes.

Materials and Methods

Rat liver mitochondria were prepared according to Pedersen et al. (1978) in 0.25 M sucrose. Respiratory control ratios of 6–10 were obtained with succinate as substrate. Myxothiazol was a generous gift from Dr. W. Trowitzsch (Gesellschaft für Biotechnologische Forschung, Braunschweig).

Potassium-loaded sonicated liposomes (for the measurement of $\Delta\psi$-induced proton flux) were made from lipids derived from either soybean [acetone-washed asolectin (Kagawa & Racker, 1971)] or beef heart mitochondria (Kagawa et al., 1973). Lipids were suspended in a medium containing from 135 to 500 mM KCl and 0.5 mM MOPS[1] at pH 7.1 (with 10% w/w potassium cholate in the case of mitochondrial lipids) at a concentration of 50–70 mg/mL and sonicated for about 30 min until clear. The pH was readjusted to 7.1, and the external K^+ was removed by passage through a (30 × 1.5 cm) Sephadex G-50 column equilibrated with a medium containing 230 mM sucrose, 20 mM LiCl, 0.5 mM LiMOPS, and 0.4 mM Li_2EGTA at 4 °C.

Liposomes for the measurement of ΔpH-driven proton flux were prepared by sonicating the lipids in 100 mM potassium citrate, pH 5.0, followed by passage through the Sephadex column equilibrated with 0.1 mM MES, pH 5.0. Imposition of ΔpH and the measurement of proton flux are described in the legend to Figure 6.

Proton flux measurements were carried out in a well stirred and thermostated chamber fitted with a combination pH and K^+-sensitive electrodes connected to electrometers and a dual channel recorder. The half-times of response for both electrodes were less than 1 s. The rates of change in the external

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[1] Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NEM, *N*-ethylmaleimide; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

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