Articles

Effect of Site-Specifically Located Mitomycin C–DNA Monoadducts on in Vitro DNA Synthesis by DNA Polymerases†

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ABSTRACT: A series of site-specifically modified oligodeoxynucleotides were synthesized that contained either of the two known mitomycin C–DNA monoadducts. In vitro DNA synthesis was carried out on some of these templates using a modified bacteriophage T7 DNA polymerase (Sequenase), AMV reverse transcriptase, and two different varieties of *Escherichia coli* DNA polymerase I (Klenow fragment)—one that carries the normal 3′ → 5′ exonuclease activity and a mutant protein that lacks this enzymatic function. Regardless of the type of DNA polymerase being used, DNA synthesis was terminated nearly quantitatively at the nucleotide 3′ to each of these two monoadduct sites, although primer extension to full length of the template was noted with the unmodified control template. Substitution of Mn^{2+} for Mg^{2+} at a high concentration of the deoxynucleotide triphosphates resulted in incorporation of nucleotides opposite the adduct in the incubations with Sequenase or the 3′ → 5′ exonuclease-free Klenow fragment; however, primer extension beyond the adduct site did not take place. These studies demonstrated that the mitomycin monoadducts are strong blocks of replication and are likely to be toxic lesions in vivo. Since previous molecular modeling studies and molecular mechanical calculations indicated that the mitomycin adduction does not induce severe distortions at the site of adduction, a lack of base-pairing ability of the modified base in the extended product is unlikely to be the reason for the inhibitory effect. Instead, energy-minimized structural models indicated that additional hydrogen-bonding interactions have been introduced by the mitomycin moiety, and perhaps this increased thermodynamic stabilization of a distorted structure of the replication fork, in turn, may block the replication bypass. Experimental evidence of increased thermodynamic stability was provided by thermal melting of a template/primer complex that presumably a polymerase encounters in a typical replication fork. Consistently higher T_m of the adducted "replication fork" was noted when compared to its unmodified counterpart.

Mitomycin C (MC)[1] is a toxic antitumor antibiotic that has demonstrated activity against a variety of malignancies (Carter & Crooke, 1979). Alkylation of DNA by MC via either one- or two-electron reduction has been implicated in its ability to limit tumor cell growth (Iyer & Szybalski, 1964; Tomasz et al., 1974; Lown et al., 1976). MC reacts covalently to DNA both in monofunctional and bifunctional manners, generating, in the latter case, inter- or intrastrand DNA cross-links that are chemically stable (Szybalski & Iyer, 1967; Tomasz et al., 1986,a,b, 1987, 1988b; Bizanek et al., 1992a).

The major covalent adducts formed by MC both in vitro and in vivo have been isolated and characterized (Tomasz et al., 1986a,b, 1987, 1988b; Bizanek et al., 1992a). The structures of these adducts are displayed in Figure 1. Two monoadducts (**2** and **3**) and two bisadducts (**4** and **5**) are discerned. Bisadduct **4** represents interstrand cross-links, while bisadduct **5** originates from intrastrand cross-links in DNA. The MC moiety in all these adducts is attached covalently to the N^2-position of guanine (Gua). The relative proportions of formation of these adducts are dependent on the conditions of reductive activation of MC (Tomasz et al., 1987, 1988a). Recently, all of these adducts have been detected in intact cells (Bizanek et al., 1992b). Molecular modeling studies indicated that both the monoadducts and the interstrand cross-link can fit in the minor groove of duplex B-DNA without causing significant distortions (Tomasz et al., 1987; Remers et al., 1988; Teng et al., 1989; Millard et al., 1990). By contrast, the intrastrand cross-link which is formed 3.6-fold less frequently than the interstrand cross-link induces bending of DNA near the cross-link site (Bizanek et al., 1992a). A 2-D NMR study conclusively proved the structure and conformation of the interstrand cross-link (Norman et al., 1990).

The antitumor action of the drugs that covalently bind to DNA is believed to involve selective killing of tumor cells, and many studies with a variety of anticancer drugs indicate that

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[1] Abbreviations: MC, mitomycin C; DMC, 10-decarbamoylmitomycin C; MC–dG and DMC–dG, deoxyguanosine–N^2-monoadducts of MC and DMC, respectively; KF, Klenow fragment of *Escherichia coli* DNA polymerase I; KF (exo⁻), 3′ → 5′ exonuclease-deficient Klenow fragment; dG, 2′-deoxyguanosine; Gua, guanine; Ade, adenine; Cyt, cytosine; Thy, thymine; DTT, dithiothreitol; T_m, melting temperature; UV, ultraviolet; TEAA, triethylammonium acetate; SVD, snake venom phosphodiesterase; BSA, bovine serum albumin.

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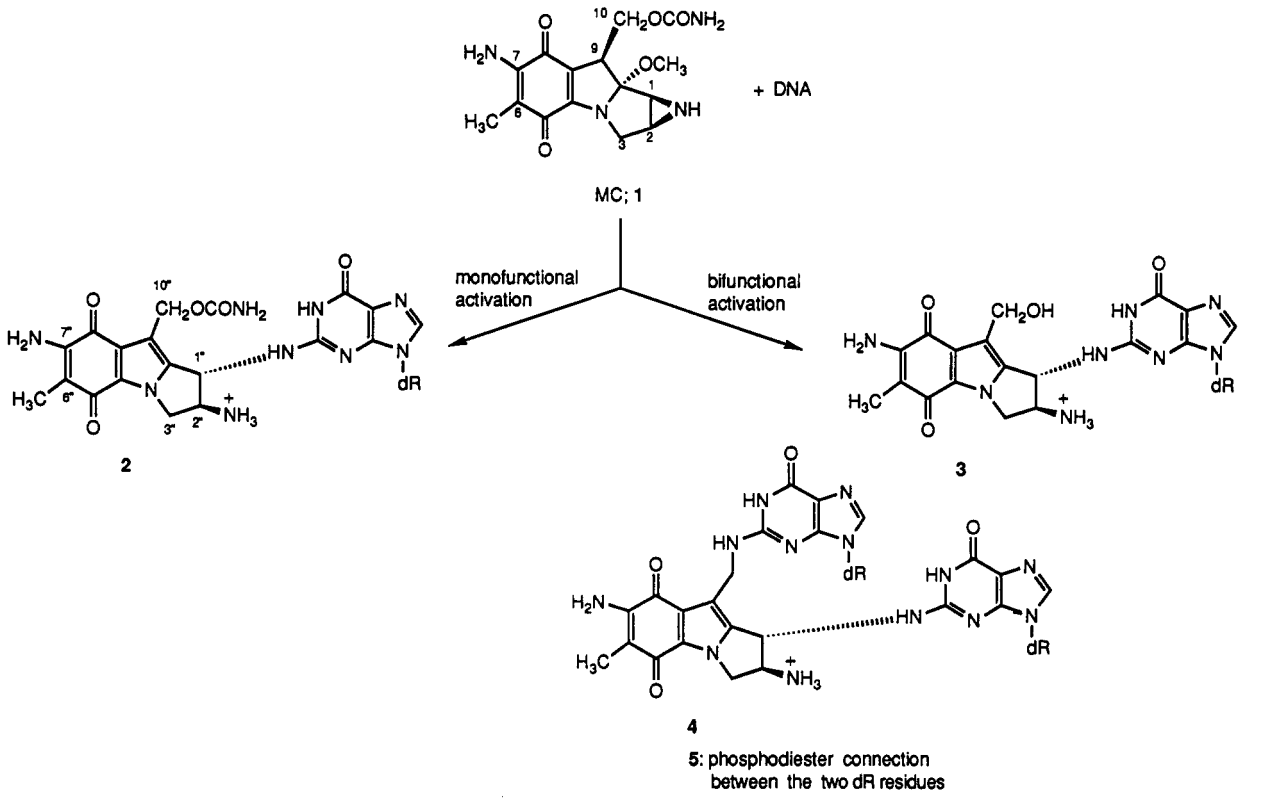

FIGURE 1: Formation of adducts of mitomycin C and DNA. dR = 2′-deoxyribos-1′-yl.

bifunctionality, and hence cross-linking, plays a crucial role [e.g., Erickson et al. (1980)]. Even though DNA cross-links were believed to be responsible for the antitumor effect of MC, various studies indicate that 10-decarbamoyl-MC (DMC), which is incapable of forming DNA cross-links, also possess potent antibiotic and cytotoxic effects (Otsuji & Murayama, 1972; Fujiwara et al., 1977; Carrano et al., 1979; Hoban et al., 1990). Interestingly, both MC and DMC form a guanine monoadduct of identical structure (**3**) in DNA upon reductive activation (Tomasz et al., 1988b).

The chemistry of adduction of MC to DNA, and in particular the cross-linking reaction, has been studied in detail. Nevertheless, very little is known about the biological outcome of these adducts. Determination of covalent structures is an important step, but the structure alone cannot explain the mechanism of the antitumor effect of the drug. It is necessary to evaluate the biological response triggered by these structural modifications (Basu & Essigmann, 1988). The MC–DNA system may be compared to that of the antitumor platinum drugs in which DNA monoadducts, interstrand cross-links, and intrastrand cross-links are formed simultaneously. The analogous set of the MC adducts provides a similar opportunity to elucidate structure–activity relationships among these types of DNA lesions, a goal actively being pursued in the field of platinum drug research [e.g., Comess et al. (1992)].

As is the case with most antitumor agents, MC is also a carcinogen, and its potent direct-acting mutagenicity was shown in several systems including *Salmonella typhimurium* strains (Ames assay) and SOS chromotest (McCann et al., 1975; Levin et al., 1982; Quillardet et al., 1985). In the Ames assay, MC-induced mutagenesis is detectable only in the presence of the plasmid pKM101. Furthermore, MC mutagenesis is unique as it reverts only strains that have a functional *excision repair* system (Levin et al., 1982). The mechanism of MC mutagenesis, however, has not been elucidated.

To address which particular DNA-bound form of MC is responsible for a specific biological effect, we have initiated studies involving a defined single adduct-induced cytotoxicity and mutagenesis. In this publication we report the synthesis of small segments of DNA that had either of the two MC–DNA monoadducts (**2** or **3**, Figure 1) located at a preselected site. These site-specifically modified oligonucleotides have been used as probes for in vitro studies to determine whether the DNA polymerase can bypass the lesion and, in the case of a translesion bypass, whether the nucleotide incorporation was correct.

MATERIALS AND METHODS

Materials

MC was obtained from Bristol-Myers Squibb Co., Wallingford, CT. DMC was prepared from MC as previously described (Kinoshita et al., 1971). All DNA synthesis reagents were purchased from Applied Biosystems, Inc. Ultrapure-grade dNTPs were purchased from Pharmacia P-L Biochemicals. [γ-^{32}P]ATP was from Du Pont New England Nuclear. Klenow fragment (KF) and AMV reverse transcriptase were from Boehringer Mannheim and International Biotechnologies, respectively. 3′ → 5′ exonuclease-deficient Klenow fragment [KF (exo$^-$)] and a modified 3′ → 5′ exonuclease-free T7 DNA polymerase (Sequenase version 2.0) were purchased from U.S. Biochemicals. T4 polynucleotide kinase and T4 DNA ligase were obtained from Bethesda Research Laboratory. Sources for all other materials used were given in a previous publication (Borowy-Borowski et al., 1990a).

Methods

Oligodeoxynucleotides were synthesized on an Applied Biosystems, Inc., Model 380B, DNA synthesizer, using the phosphoramidite method. HPLC separations were performed

using reverse-phase columns (Beckman RPSC, C-3 Ultrapore, 10 × 250 mm, for oligonucleotide separations and Beckman C-18 ODS Ultrasphere, 4.6 × 250 mm, for nucleoside and MC–dG adduct analysis). Elution conditions were similar to those described in a prior publication (Kumar et al., 1992). Quantitative analysis of nonradiolabeled oligonucleotides was based on absorbance measurements in buffer solutions as described previously (Borowy-Borowski et al., 1990a). Denaturing polyacrylamide gel electrophoresis was carried out as described in Sambrook et al. (1989). The oligonucleotides were denatured by heating at 90 °C for 2 min and immediately loaded and electrophoresed on a 16% polyacrylamide–8 M urea gel in 89 mM Tris–borate and 2 mM EDTA (pH 8.0). The gels were electrophoresed in an IBI sequencing gel apparatus until the bromophenol blue dye ran out of the gel. Relative proportions of oligonucleotide products in the autoradiograms were analyzed by a Bio-Rad Model 620 video densitometer equipped with a Hewlett-Packard 3396 integrator.

Nucleoside and MC–dG or DMC–dG Composition Analysis. Unmodified or monoadducted oligonucleotides (1 A_{260} unit) were digested with SVD (1 unit) and *Escherichia coli* alkaline phosphatase (0.5 unit) in 0.2 mL of 0.1 M Tris–2 mM $MgCl_2$, pH 8.2, buffer, at 45 °C for 4 h. The digest was directly analyzed by HPLC. Peak areas were integrated. Molar ratios of each peak were calculated by dividing a peak area by ϵ_{254} of the corresponding nucleoside or nucleoside–MC adduct (dC, 6300; dG, 13 000; dI, 13 000; dT, 6600; dA, 13 300; adducts **2** and **3**, 24 000) (Kumar et al., 1992).

Synthesis of Nonadeoxynucleotides Containing MC and DMC at a Specific Site. A mixture of two complementary oligonucleotides, 5′-ACACGTCAT-3′ (**6**; 9-mer) and 5′-TIACGTIT-3′ (**7**; 8-mer; 1 mM each in mononucleotide units), and MC or DMC (4 mM) in 0.1 M potassium phosphate, pH 7.4, buffer (0.45 mL) was briefly heated at 50 °C and then allowed to cool slowly to 0 °C. An anaerobic $Na_2S_2O_4$ solution (40 mM) in the same buffer was prepared at the same time by purging the buffer before, during, and after the addition of the solid $Na_2S_2O_4$. An aliquot of this solution was added in one portion to the oligonucleotide–drug mixture to give 2 mM $Na_2S_2O_4$ concentration, and the solution was stirred, *exposed to air*, for 1 h at 0 °C. The reaction mixture was chromatographed immediately on a Sephadex G-50 column (2.5 × 56 cm), using 0.02 M NH_4HCO_3 as eluant. The first-eluting UV-absorbing peak contained modified and unmodified oligonucleotides as a mixture. After lyophilization of this fraction, it was separated into individual components by HPLC using a semipreparative column. The collected HPLC fractions were lyophilized and then desalted by passage through a 2.5 × 56 cm Sephadex G-25 (fine) column, with 0.02 M NH_4HCO_3 as eluant. The collected fractions were lyophilized.

Construction of 24-mers. Each of the site-specifically modified 9-mers MC-**6** and DMC-**6** and the unmodified control 9-mer **6** (~3 nmol) was ligated to 5′-phosphorylated 15-mer (~3 nmol) in the presence of a 20-nucleotide complementary oligomer (~3 nmol) which held the ligating oligomers together (Chart I). The mixture of the three oligonucleotides in 10 μL of 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, and 10 mM dithiothreitol (DTT) was heated to 80 °C, slowly cooled to 30 °C, and subsequently left at 4 °C for 30 min. T4 DNA ligase (2 units), ATP (final concentration 1 mM), and BSA (50 μg/mL) were added, and the mixture was incubated at 4 °C for 16 h. The reaction was stopped by the addition of a formamide–EDTA dye mixture, and the oligonucleotides were separated by electrophoresis on a 16% polyacrylamide–8 M urea gel. The ligated product bands were visualized by UV shadowing and excised. The 24-mers were desalted by using a Sep-pak cartridge (Waters), and the purity of the oligonucleotides was determined by ^{32}P-radiolabeling of a small fraction of the 24-mers followed by gel electrophoresis.

Chart I: Sequences of Oligonucleotides

5′- ACACG*TCAT

6: unmodified 9-mer
MC-**6**: modified by MC at G*
DMC-**6**: modified by DMC at G*

5′- TIACG*TIT

7: unmodified 8-mer
MC-**7**: modified by MC at G*
DMC-**7**: modified by DMC at G*

5′- ACACG*TCATTACC

8: unmodified 13-mer
MC-**8**: modified by MC at G*
DMC-**8**: modified by DMC at G*

5′- TIACG*TITA

9: unmodified 9-mer

5′- ACACG*TCATTACC (**8**)
3′- AGTAATGG (**10**)

8/10: unmodified template/primer
MC-**8/10**: modified by MC at G*
DMC-**8/10**: modified by DMC at G*

5′- ACACG*TCATTTAGAGATT (**11**)
3′- AGTAAATCTCTAA (**12**)

11/12: unmodified template/primer
MC-**11/12**: modified by MC at G*
DMC-**11/12**: modified by DMC at G*

DNA Polymerase Reactions. The primed template was obtained by annealing about a 5-fold molar excess of the site-specifically modified as well as control 24-mer templates (~10 ng) to a complementary 5′-^{32}P-labeled 15-mer (2–3 ng) in 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 50 mM NaCl, and 10 mM β-mercaptoethanol. The mixture was heated to 90 °C for 2 min and then left at 37 °C for 20 min for annealing to occur. After addition of DTT to 2 mM and BSA to 100 μg/mL final concentration, a mixture of nucleotide triphosphates (dATP, dGTP, dTTP, and dCTP), the concentration of which varied for each experiment, was added. Depending upon the experiment, either $MgCl_2$ to 8 mM or $MnCl_2$ (in 15 mM sodium citrate buffer, pH 7.5) to 2.5 mM was added. Aliquots of these solutions were transferred and incubated with 2–5 units of KF or KF (exo$^-$). After the desired time of incubation at 37 °C, 5 μL of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) was added. After being denatured at 90 °C for 2 min, a portion was loaded onto a 16% polyacrylamide sequencing gel that contained 8 M urea.

In the case of Sequenase version 2.0, the buffer used was 36 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 50 mM NaCl, and 1 mM DTT. After annealing, a mixture of dNTPs, $MgCl_2$ or $MnCl_2$, and DTT to 2 mM concentration was added, and

aliquots of this mixture were incubated with 2–5 units of Sequenase version 2.0. The buffer for the AMV reverse transcriptase was similar, but the pH was changed to 8.3 at which the avian enzyme works more efficiently.

To determine the nucleotide preferentially incorporated opposite the adducts, instead of a 15-mer a 19-mer was used, and only a single nucleotide was added to the reaction mixture. The rest of the procedures remained identical as above.

Molecular Modeling. All-atom minimizations were performed on a VAX station 3100 using the program MacroModel V3.0 (Mohamadi et al., 1990). The AMBER force field and charges (Weiner et al., 1984) were used in the absence of solvent and counterions, with dielectric R_{ij} and with cutoffs of 8 Å (van der Waals) and 13 Å (electrostatic) in the united atom mode. Minimizations were conducted by using a block-diagonal Newton Raphson routine followed by a Polak–Ribiere conjugate gradient. The structure of the unmodified template/primer **11/12** was generated in two steps: First, the single-stranded segment 5′-ACACG and the duplex portion 5′-TCATTAGACATT·5′-AATCTCTAATGA in the B-form were produced separately by the MacroModel GROW mode. The 5′-ACACG structure was then connected as its 3′-end by phosphate linkage to the 5′-end of the 5′-TCATTAGACATT strand of the duplex visually, using the Evans and Sutherland 390 graphic system. This structure was energy-minimized (rms = 0.032 kJ mol^{-1} Å^{-2}). Generation of the structures of the drug-modified template/primers (MC-**11/12** and DMC-**11/12**) was carried out by connecting the energy-minimized mitosene[2] or 10-decarbamoylmitosene unit (Bizanek et al., 1992a) to the energy-minimized template/primer **11/12** by forming a crude bond between C-1″ of the mitosene and N^2 of the guanine marked with the asterisk in **11/12**. The mitosene was docked in two opposing orientations with respect to the DNA helical axis: either the 10″-carbamate or the 2″-C-NH$_3^+$ group points in the 5′-direction of the template strand. The structures were then again energy-minimized.

Thermal Denaturation of Oligonucleotides. A Varian Cary 3 spectrophotometer, equipped with "thermal applications" accessories, was utilized. Duplex oligonucleotides at 1.1 μM concentration of each strand in 0.1 M NaCl–0.015 M Tris–0.001 M EDTA, pH 7.5, buffer were heated at a rate of 0.5 °C/min. T_ms were calculated from the data by the "average" method of the computer data processing software supplied by Varian.

RESULTS

Synthesis and Characterization of Oligonucleotides Containing MC–dG (**2**) *and DMC-dG* (**3**) *at a Specific Site.* We reported recently that substoichiometric amounts of $Na_2S_2O_4$ and aerobic conditions result quantitatively in *monofunctional* activation of MC, leading exclusively to monoadduct formation (Figure 1; **2**) and that Gua in the 5′-CpG sequence in a duplex structure is required for optimal monoalkylation yields (Kumar et al., 1992). Similar structural requirements were found for the formation of monoadduct **3** upon activation of DMC. Accordingly, monoalkylated oligonucleotides were synthesized by the above method of Kumar et al. (1992). All modified oligonucleotides prepared in this work contain MC or DMC at a Gua in a 5′-CpG sequence. For synthesis of MC-**6**, the 9-mer oligonucleotide **6** was annealed to **7** to provide a duplex substrate for the alkylation. The guanines in both strands were alkylated, as expected, as seen from the product profile on HPLC (Figure 2a): MC-**6** and MC-**7** were formed in 19% and 17.5% yields, respectively, as calculated from the areas of the HPLC peaks relative to the unmodified strands. Identification of MC-**6** and MC-**7** was based on quantitative nucleoside and MC–nucleoside adduct analysis: The HPLC-purified products MC-**6** and MC-**7** were digested with SVD and *E. coli* alkaline phosphatase, and the mixture was directly analyzed by HPLC (Figure 3). The composition of MC-**6** (Figure 3b) was dC:dG:dT:dA:**2** = 3.0:<0.1:2.0:2.9:1.1 (calculated 3:0:2:3:1), and that of MC-**7** (HPLC not shown) was dC:dG:dI:dT:dA:**2** = 1.2:<0.1:1.8:2.9:1.0:1.0 (calculated 1:0:2:3:1:1). These results identified and distinguished the two

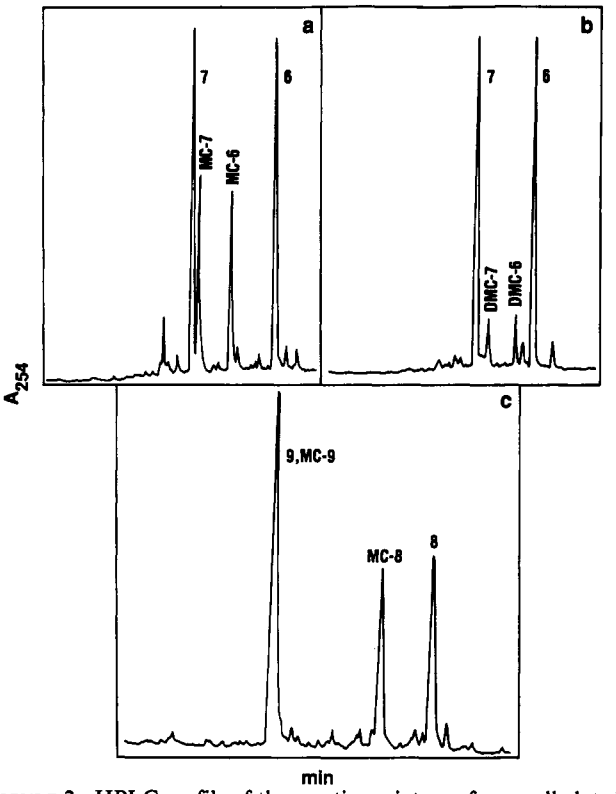

FIGURE 2: HPLC profile of the reaction mixture of monoalkylated oligonucleotides by MC and DMC. (a) Monoalkylation of **6/7** by MC. HPLC conditions: 5–15% acetonitrile in 0.1 M TEAA in 120 min; 5 mL/min flow rate. Elution times (minutes): **7**, 36; MC-**7**, 37; MC-**6**, 43; **6**, 52. (b) Monoalkylation of **6/7** by DMC. HPLC conditions: 5–15% acetonitrile in 0.1 M TEAA in 90 min. Elution times (minutes): **7**, 26.5; DMC-**7**, 29; DMC-**6**, 34; **6**, 37. (c) Monoalkylation of **8/9** by MC. HPLC conditions: same as in (a). Elution times (minutes): **9**, MC-**9**, 37; MC-**8**, 57; **8**, 67.

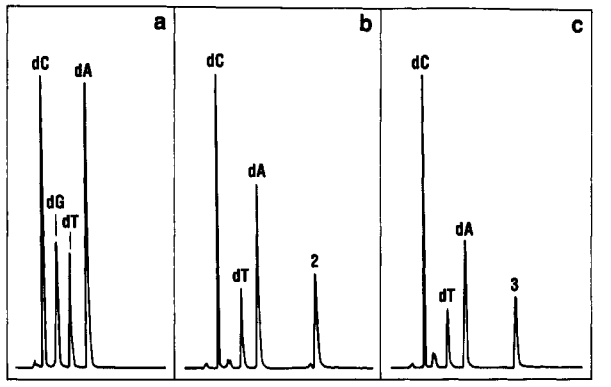

FIGURE 3: HPLC profiles of enzymatic digests of monoalkylated and parent oligonucleotides. HPLC conditions: 6–18% acetonitrile in 0.03 M phosphate, pH 5.4, in 30 min; 1 mL/min flow rate. Elution times (minutes): dC, 3.3; dG, 4.8; dT, 6.3; dA, 8.2; **2**, 14.8; **3**, 14.1. (a) Parent oligonucleotides **6**. (b) Monoalkylated oligonucleotide MC-**6**. (c) Monoalkylated oligonucleotide DMC-**6**.

[2] Mitosene refers to the DNA-bound form of the mitomycin (e.g., structure **2** in Figure 1).

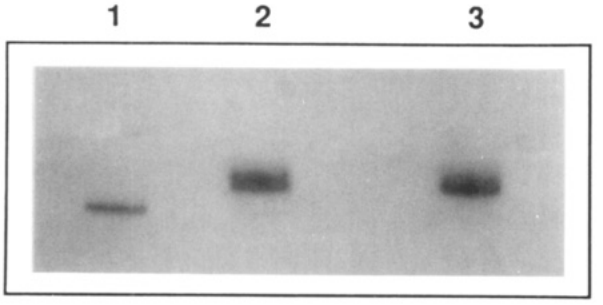

FIGURE 4: Autoradiogram showing the γ-^{32}P end-labeled 24-mer templates used in this study. The oligonucleotides were heated to 90 °C for 1 min and immediately loaded and electrophoresed on a 16% polyacrylamide–8 M urea gel. The gels were electrophoresed until the bromophenol blue dye ran out of the gel. Lanes: 1, control 24-mer; 2 and 3, MC–dG- and DMC-dG-containing 24-mers, respectively.

MC-modified strands unambiguously. Only MC-**6** was utilized in the present work. Synthesis of DMC-**6** was analogous to that of MC-**6**, using DMC instead of MC for the alkylation (Figure 2b). Identification and characterization of the DMC-**6** product by digestion was analogous to that of MC-**6**. The yield of monoalkylated products by DMC was considerably lower than that by MC (DMC-**6**, 12%; DMC-**7**, 8.5%). Only DMC-**6** was utilized in this work.

Synthesis of the modified 13-mers MC-**8** and DMC-**8** was carried out by first annealing **8** and **9**, to achieve the duplex state of the 5′-CpG target site (see above). The alkylation reactions, product isolation, and characterization were done using the same protocol as for MC-**6** and DMC-**6**. The isolation of MC-**8** by HPLC is shown in Figure 2c. Its yield was 45% compared to a 15% yield for DMC-**8** (data not shown). The results of nucleoside composition analysis were satisfactory for both oligonucleotides.

Each of the site-specifically modified 9-mers MC-**6** and DMC-**6** and the unmodified control 9-mer **6** (~3 nmol) was ligated to the 5′-phosphorylated 15-mer 5′-TAGAGATTG-GTAGGG (~3 nmol) in the presence of T4 DNA ligase and a 20-nucleotide complementary oligomer, 5′-ACCAA-TCTCTAATGACGTGT (~3 nmol), which held the ligating oligomers together; the resultant 24-mers 5′-ACA-CG*TCATTAGAGATTGGTAGGG (where G* denotes MC–dG, DMC–dG, or dG) were purified by electrophoresis on a denaturing polyacrylamide gel. Analysis of the 24-mers by polyacrylamide gel electrophoresis indicated a slightly slower mobility of MC- and DMC-adducted oligomers compared to the unmodified control 24-mer, as expected (Figure 4). Interestingly, the adducted oligonucleotides always provided a somewhat broad band when compared to the unmodified 24-mer. Whether this was due to the presence of multiple conformations of the adducted nucleoside is not known. It is unlikely that this was due to degradation of the adducted oligomer, because HPLC analysis revealed that the adducted 9-mer remained unaffected following gel electrophoresis in a similar manner. Overexposure of these autoradiograms revealed that a small proportion of unmodified 24-mer also accompanied the adducted 24-mers. If these bands were repurified, the same characteristics were noted upon subsequent gel electrophoresis. This is not surprising because MC and DMC modifications of dG have been shown to reverse modification by hot piperidine, or in the presence of reducing agents (Borowy-Borowski et al., 1990a). At a neutral pH and in the absence of reducing agents, it is likely that the reversal may still take place, albeit at a much slower rate.

In Vitro DNA Replication System. We studied the ability of several DNA polymerases to replicate past MC–dG and

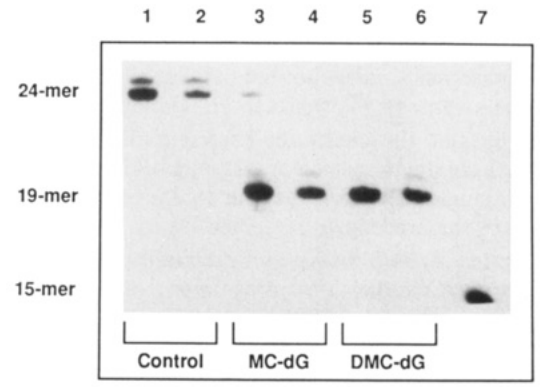

FIGURE 5: An overexposed autoradiogram of the electrophoretic separation of DNA products synthesized by KF on a primed template. The template contained dG, MC–dG, or DMC–dG at position 20 from the 3′-end. The primed 24-mers were incubated for 15 min with 2–3 units of KF and 1 mM dNTPs in the presence of Mg^{2+} (8 mM) (lanes 1, 3, and 5) or Mn^{2+} (2.5 mM in 15 mM sodium citrate buffer, pH 7.5) (lanes 2, 4, and 6). Other conditions have been described in the text. Lanes: 1 and 2, polymerase reaction of control template; 3 and 4, the same for MC–dG-containing 24-mer; 5 and 6, the same for DMC–dG-containing 24-mer; 7, 15-mer primer.

DMC–dG adducts in vitro. The replication system used the following primed template in which the adducted nucleotide has been denoted as G*:

15-mer primer 3′- ATCTCTAACCATCCC^{32}p -5′

24-mer template 5′- ACACG*TCATTAGAGATTGGTAGGG -3′

A 24-nucleotide site-specifically modified template was hybridized to a 5′-^{32}P-labeled complementary 15-mer primer. The MC or DMC adduct was located at template position 20 from the 3′-end, and the 3′-terminus of the primer was four nucleotides away from adduct site in the template. In each experiment a control template was used, which was synthesized in a similar manner but contained an unmodified dG in place of the adduct.

Replication Block by MC–dG and DMC–dG. In this replication system, we considered three major possibilities. DNA synthesis may be terminated without incorporation of a nucleotide opposite the adduct; i.e., the progress of the polymerase may be blocked 3′ to the adduct site. This should produce a 19-nucleotide product. Alternatively, the replication may be obstructed after incorporation of a nucleotide opposite the adduct, which should generate a 20-nucleotide product. The third possibility would be partial or complete extension of the primer to a 24-nucleotide product, suggesting a postlesion bypass (or read-through) product. We have analyzed these products of DNA synthesis by using denaturing polyacrylamide gel electrophoresis.

When either KF or KF (exo$^-$) was used in the presence of Mg^{2+}, DNA synthesis was blocked at the nucleotide 3′ to the adduct site (Figures 5 and 6). While the unmodified template produced full-length 24-nucleotide product,[3] both MC–dG

[3] For extension of the primer on the control template, KF (exo$^-$) produced some 23-nucleotide product in addition to the full-length product in the presence of Mg^{2+}. When the metal ion was changed to Mn^{2+}, about half of the product was full-length product whereas the other half had an extra nucleotide (i.e., a 25-nucleotide product). For Sequenase version 2.0, synthesis of the control 24-mer template proceeded to the full length, and in ~50% of the product the polymerases introduced an extra nucleotide in the presence of Mg^{2+}, a phenomenon also noted by others (Reardon et al., 1990). In the presence of Mn^{2+}, however, about half of the product had been extended two extra nucleotides to a 26-mer whereas the other half was a 25-nucleotide product.

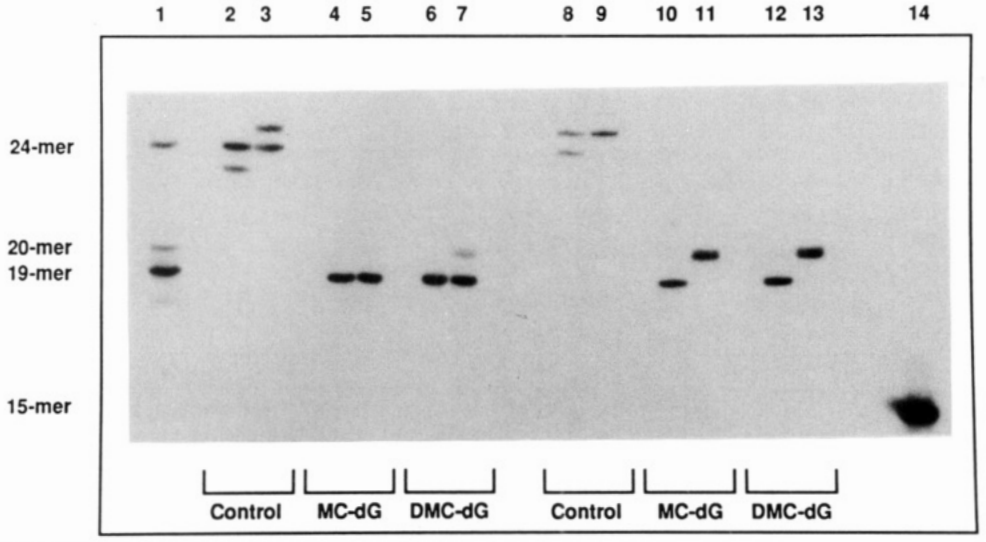

FIGURE 6: Electrophoretic separation of DNA products synthesized by KF (exo⁻) on a primed template. The template contained dG, MC–dG, or DMC–dG at position 20 from the 3′-end. The primed 24-mers were incubated for 1 min (lanes 2–7) or 2 h (lanes 8–13) with 2–3 units of KF (exo⁻) and 1 mM dNTPs in the presence of Mg^{2+} (8 mM) (lanes 2, 4, 6, 8, 10, and 12) or Mn^{2+} (2.5 mM in 15 mM sodium citrate buffer, pH 7.5) (lanes 3, 5, 7, 9, 11, and 13). Buffers and other conditions have been described in the text. Lanes: 1, standard DNA marker containing 18, 19, 20, and 24 nucleotides; 2, 3, 8, and 9, polymerase reaction of control template; 4, 5, 10, and 11, the same for MC–dG-containing 24-mer; 6, 7, 12, and 13, the same for DMC–dG-containing 24-mer; 14, 15-mer primer.

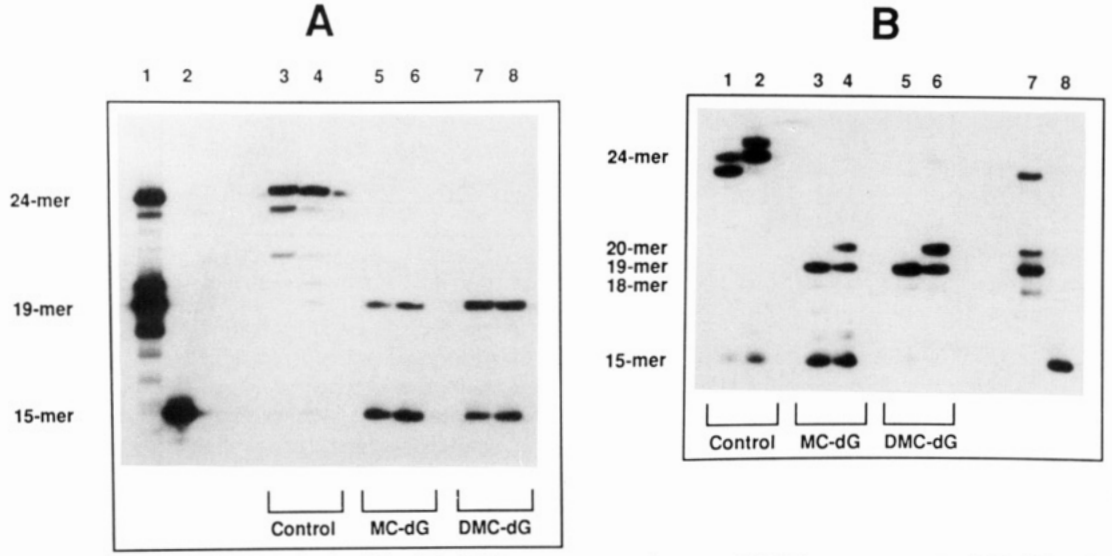

FIGURE 7: Analysis of DNA products synthesized by (A) AMV reverse transcriptase and (B) Sequenase version 2.0. The primed 24-mers were incubated for 15 min with 2–3 units of AMV reverse transcriptase (A) or Sequenase (B) and 1 mM dNTPs in the presence of Mg^{2+} (8 mM) (lanes 3, 5, and 7, panel A; lanes 1, 3, and 5, panel B) or Mn^{2+} (2.5 mM in 15 mM sodium citrate buffer, pH 7.5) (lanes 4, 6, and 8, panel A; lanes 2, 4, and 6, panel B). (A) lanes: 1, DNA marker standard; 2, 15-mer primer; 3 and 4, polymerase reaction of control 24-mer; 5 and 6, the same for MC–dG-containing 24-mer; 7 and 8, the same for DMC–dG-containing 24-mer. (B) Lanes: 1 and 2, polymerase reaction of control 24-mer; 3 and 4, the same for MC–dG-containing 24-mer; 5 and 6; the same for DMC–dG-containing 24-mer; 7, DNA marker standard; 8, 15-mer primer.

and DMC–dG blocked synthesis. Neither the amount of dNTPs (from 100 μM to 1 mM) nor the amount of DNA polymerase (2–10 units) influenced the lesion-induced replication block (or the amount of postlesion synthesis product). Increasing the time of incubation from 1 min to 2 h did not increase the full-length product either (Figure 6; lanes 4 and 6 represent 1-min incubations with MC–dG and DMC–dG containing templates, respectively, whereas lanes 10 and 12 represent 2-h incubations for the same). We always detected a small proportion (<2%) of the full-length product, but they were most likely generated from a small contaminating unmodified template that was formed by reversal by MC or DMC modification. The proportion of the full-length product did not increase or decrease by changing the conditions of the replication system. Replacement of Mg^{2+} with Mn^{2+} has been demonstrated to increase postlesion synthesis of a variety of DNA damages (Strauss et al., 1982). We investigated the possibility of such a bypass. In the presence of Mn^{2+}, only a little extension product (<2%) was noted for KF (Figure 5, lanes 4 and 6). However, a significant amount of 20-nucleotide product was detected when KF (exo⁻) was used (Figure 6; lanes 5 and 7 represent 1-min incubations with MC- and DMC-adducted 24-mers, respectively, whereas lanes 11 and 13 were the same with 2-h incubations). This suggests an important role of editing in the arrest of synthesis provoked by the MC or DMC adducts. The 3′ → 5′ exonuclease activity of the Klenow fragment could recognize the nucleotide opposite the adduct as "unnatural" and excised it. Perhaps more significantly, the polymerase was unable to extend the primer beyond the adduct site despite this incorporation of a nucleotide opposite the adducted nucleotide.

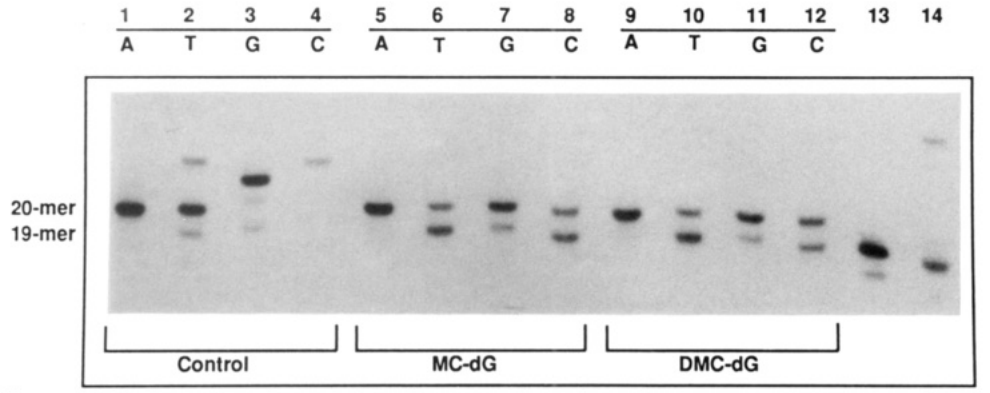

FIGURE 8: Autoradiogram of the electrophoretic separation of the extension products of a 19-mer primer by KF (exo$^-$) in the presence of Mn^{2+} (2.5 mM) and a 1 mM concentration of only one dNTP for 2 h. The dNTPs used are shown in the figure as A, T, G, and C for dATP, dTTP, dGTP, and dCTP, respectively. Lanes: 1–4, polymerase reaction of control 24-mer; 5–8, the same for MC–dG-containing 24-mer; 9–12, the same for DMC–dG-containing 24-mer; 13, 19-mer primer; 14, DNA marker standard. The percentages of 19-mer that was extended to the 20-nucleotide product were A:T:G:C = 99:39:78:45 for MC–dG and A:T:G:C = 99:36:84:57 for DMC–dG as determined by a densitometer.

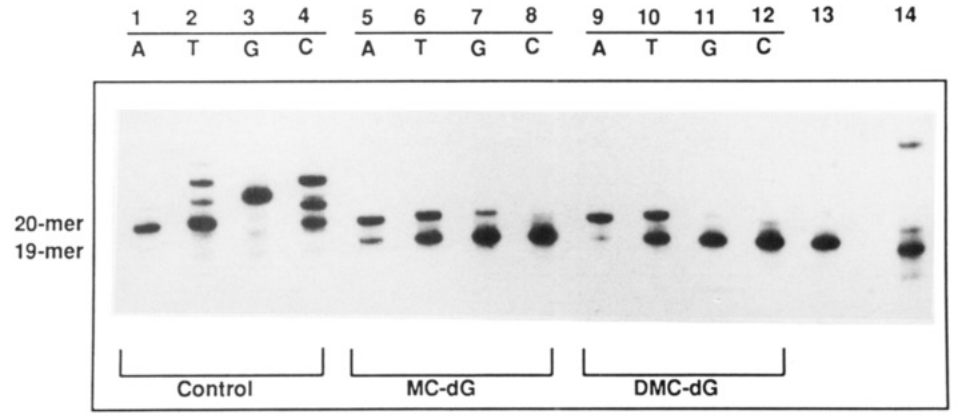

FIGURE 9: Autoradiogram of 19-mer primer extension as described in Figure 8 with the exception that Sequenase (and the necessary buffer) was used in lieu of KF (exo$^-$). The percentages of 19-mer that was extended to the 20-nucleotide product were A:T:G:C = 81:29:12:2 for MC–dG and A:T:G:C = 94:37:3:5 for DMC–dG as determined by a densitometer.

That MC–dG and DMC–dG constitute a major block of DNA synthesis was confirmed by performing similar experiments with AMV reverse transcriptase and Sequenase version 2.0. AMV reverse transcriptase was unable to replicate beyond the nucleotide 3′ to the adduct site regardless of whether Mg^{2+} or Mn^{2+} was used up to 1 mM concentration of dNTPs (Figure 7A). Sequenase, on the other hand, behaved like the KF (exo$^-$) in that a significant incorporation opposite the adduct sites was noted in the presence of Mn^{2+} ion (Figure 7B).

Nucleotide Incorporation opposite MC–dG and DMC-dG. In the presence of Mn^{2+}, the incorporation of a nucleotide opposite the adduct was significant for both KF (exo$^-$) and Sequenase. We wanted to determine whether there was any preference of incorporation of the correct nucleotide (dCTP) opposite the Gua adduct at a high dNTP concentration and in the presence of Mn^{2+}. For this experiment, we used a 19-mer primer that extended 3′ to the MC- or DMC-adducted nucleotide, and the primer/template complex was incubated with the polymerase, Mn^{2+}, and just one nucleotide triphosphate. For the unmodified template, KF (exo$^-$) extended each of the four nucleotides, and nearly 100% extension was observed with dATP (Figure 8, lane 1) and dCTP (Figure 8, lane 4). Extension was terminated after incorporation of dATP opposite Gua in the template strand (Figure 8, lane 1). In the case of dTTP, the major proportion of the primer also extended one more nucleotide although a small portion was extended two steps (Figure 8, lane 2). A significant proportion of incorporation of dGTP occurred twice, probably because the next base was Cyt (Figure 8, lane 3). It is not clear why dCTP was incorporated three times since only the first incorporation was correct (Figure 8, lane 4). It appeared that, in the absence of 3′ → 5′ exonuclease activity, the polymerase stops synthesis only after at least one base–base mismatch has been created. Sometimes multiple mismatches are necessary to arrest DNA synthesis. By contrast, both MC–dG and DMC–dG allowed only partial extension of dNTPs with the exception of dATP, which was incorporated almost quantitatively. For Sequenase, the results were qualitatively similar. Again, for the unmodified template, Sequenase was able to extend each of the four nucleotides (Figure 9). In each case, however, the extension of the 19-mer to a larger fragment was nearly quantitative. In contrast to that, both MC–dG and DMC–dG allowed only partial extension of the primer in the presence of dATP, dTTP, and dGTP, but virtually no extension was observed when the correct nucleotide, dCTP, was present (Figure 9, lanes 5–12). These experiments demonstrate that, even under conditions when forced misincorporation of any nucleotide can be induced quantitatively opposite dG, a preference in incorporation of the correct nucleotide dCTP opposite either MC–dG or DMC–dG was not observed.

Molecular Modeling of the Replication Fork. To investigate the structural basis for the severe replication block induced by MC–dG and DMC–dG, we have carried out molecular modeling and molecular mechanical calculations. Figure 10 shows the models of unmodified, MC-modified, and DMC-modified template/primer complexes (**11/12**, MC-**11/12**, and DMC-**11/12**). The drug residue is oriented with the 2″-NH_3^+ group pointing in the 5′-direction of the template strand. The free energy values were −6250 kJ/mol (rms

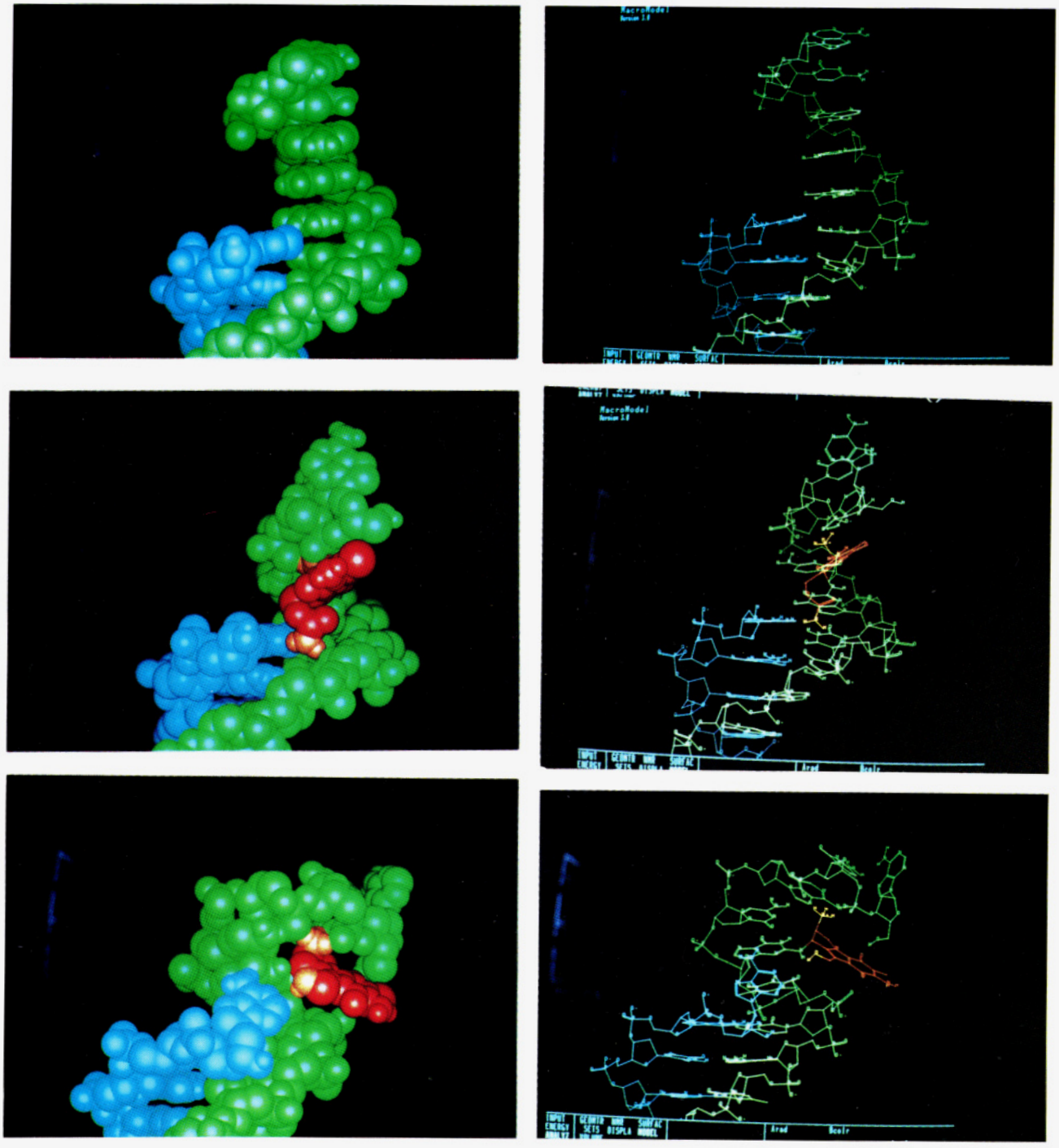

FIGURE 10: Computer-generated models of monoalkylated and unmodified template/primers: (a, top) unmodified **11**/**12**; (b, middle) MC-**11**/**12**; (c, bottom) DMC-**11**/**12**. Of the duplex region only the upper three base pairs are included in the pictures. Color code: blue, primer strand; green, template strand; orange, mitosene residue; yellow, 2″-NH_3^+ and 2″-NH_2 of the 10″-carbamate in (b) and 2″-NH_3^+ and 2″-OH of the 10″-hydroxymethyl in (c).

0.032), –6548 kJ/mol (rms 0.077), and –6606 kJ/mol (rms 0.046), respectively. Of the duplex region, only the upper three base pairs are included in the pictures. Notable structural changes as compared to the structure of the parent **11**/**12** in the vicinity of the modified site are as follows:

MC-**11**/**12**: The 10″-OC(O)NH_2 group of the bound mitosene forms H-bonds with N^3 of the terminal A of the primer and with O4′ of C(7) of the template. The 2″-NH_3^+ group has three H-bonds (1.63–1.77-Å distance) to various electronegative atoms on the template strand. While the position of the 3′-OH of the primer terminus is not altered, base-pair formation of a nucleotide triphosphate to the MC-modified Gua would be blocked by the large 10″-carbamate group which is stabilized firmly by its H-bonds in this obstructive conformation.

DMC-**10**/**11**: In this case the 10″-substituent of the mitomycin is not a carbamate but a hydroxyl group. Nevertheless, it is in H-bonding position (1.79 Å) to O^2 of T(6) in the template. This interaction apparently disrupts the terminal primer/template AT base pair; the primer terminal A is displaced from its stacked base-paired position, resulting in a large displacement of the 3′-OH of the primer terminus from its original position. The interactions of the 2″-NH_3^+ group with three oxygen atoms of phosphate groups (1.61–1.68-Å O to H distances) upstream on the template strand undoubtedly contribute to the stability of this structure which

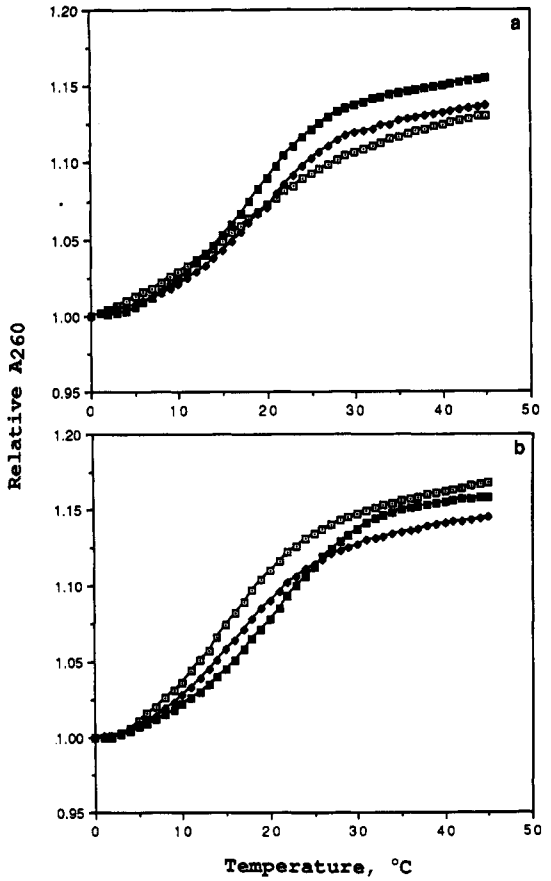

FIGURE 11: Thermal melting curves of monoalkylated oligonucleotides: (a) template/primer complexes **8/10** (square with white dot), MC-**8/10** (◆), and DMC-**8/10** (square with black dot); (b) duplex oligonucleotides **6/7** (square with black dot), MC-**6/7** (square with white dot), and DMC-**6/7** (◆).

is severely distorted at the template/primer terminus.

Thermal Denaturation of Template/Primer Complexes. Experimental evidence of an increased stabilization induced by MC–dG was obtained by generating a part of the replication fork that was used for thermodynamic comparisons of the adducted and unadducted complex. T_ms were determined at 1.9 μM concentration. The synthetic MC-modified template/primer MC-**8/10** exhibited increased duplex stability relative to **8/10** (ΔT_m 0.8–1.0 °C in several independent determinations) (Figure 11a). This finding is consistent with the theoretical model (Figure 10b), which indicates favorable interaction of the bound drug with atoms *in both strands* at the template/primer junction (see above). The DMC-**8/10** oligonucleotide shows a smaller elevation of T_m (0.0–0.5 °C).

Thermal Denaturation of MC-Modified Duplex Oligonucleotides. The T_ms of the two modified duplexes, MC-**6/7** and MC-**7/6**, as well as the unmodified control duplex **6/7** were determined, at 1.1 μM concentration each (Figure 11b). The modified structures had higher T_ms than the control duplex (16.44, 19.29, and 16.06 °C, respectively).

DISCUSSION

The major covalent adduct formed between MC and DNA is the guanine-N^2-linked monoadduct **2** (Figure 1). This product is also the precursor to all other observed adducts (**3–5**). The formation of these secondary products from **2** is determined by the nature of the DNA base directly adjacent to the adducted Gua on the 5′-side. Thus interstrand cross-links (**4**) are formed exclusively at 5′-CG (Teng et al., 1989; Millard et al., 1990; Borowy-Borowski et al., 1990b) and intrastrand cross-links (**5**) at 5′-GG (Bizanek et al., 1992a). The decarbamoylated monoadduct **3** is formed from **2** mostly at 5′-AG and 5′-TG sequences, by hydrolysis of the 10″-carbamate of **2** in its reduced (activated) state (Tomasz et al., 1987, 1988a). The cross-link:monoadduct ratio of MC is higher and more variable than previously estimated (0.05–0.10; Szybalski & Iyer, 1967), since both the DNA sequence and the conditions of reductive activation were shown to modulate this ratio (Tomasz et al., 1987, 1988a). Nevertheless, monoadducts are generally formed in comparable proportions to the cross-links. In addition, both **2** and **3** were shown to occur together in DNA in cell-free systems and in intact cells (Tomasz et al., 1988a; Bizanek et al., 1992b). Despite the abundance of MC monoadducts, the antitumor properties of MC have been attributed to its ability to form cross-links.

Formation of the monoadduct **2** is highly sequence dependent, with the sequence 5′-CG providing the best yield for the alkylation of Gua (Li & Kohn, 1991; Kumar et al., 1992). The base 3′ to the Gua, on the other hand, has only a relatively modest effect. On the basis of these data, we have chosen the 5′-CG for our studies, because this would represent the predominant proportion of the monoadduct **2** formed in DNA.

The replication studies described in this paper, contrary to current belief, suggest a likely role of monoadducts in MC-induced cytotoxicity. DNA polymerase replication studies of DNA fragments carrying other site-specifically located adducts induced by carcinogens or antitumor drugs have been performed. All of these, including the C8-guanine adduct of 2-aminofluorene (O'Connor & Stöhrer, 1985; Michaels et al., 1987), N^3-ethylthymine (Grevatt et al., 1991), O^2-ethylthymine (Grevatt et al., 1992), the cis-syn thymine dimer (Taylor & O'Day, 1990), the synthetic model of abasic site (Takeshita et al., 1987), 8-oxo-7-hydroguanine (Kuchino et al., 1987; Shibutani et al., 1991), thymine glycol (Clark & Beardsley, 1989), the N^2-guanine and N^6-adenine adducts of 7-(bromomethyl)benz[*a*]anthracene (Reardon et al., 1990), the N^3-adenine adduct of CC-1065 (Sun & Hurley, 1992), and both monofunctional and bifunctional adducts of *cis*-diamminedichloroplatinum(II) (Pinto & Lippard, 1985; Comess et al., 1992), have shown translesion bypass although the degree of bypass varies widely depending upon the structure of adduct and the conditions of the replication study. By contrast, all the DNA polymerases used in this study are blocked 3′ to the adduct site in the presence of Mg^{2+}. In many other in vitro studies, translesion bypass increased with a higher concentration of dNTPs (Grevatt et al., 1991, 1992; Sun & Hurley, 1992). In the present study, however, no such effect was observed. In the presence of Mn^{2+}, which has been demonstrated to increase translesion bypass past many other adducts (Grevatt et al., 1991, 1992), some incorporation opposite the adduct occurred by two polymerases that lack the 3′ → 5′ exonuclease activity. However, in each case the incorporation was terminated there. This suggests that the polymerase was unable to "read" the MC-adducted nucleotide; i.e., a normal Watson–Crick base pairing capability of Gua may no longer be there in the adducted Gua. However, molecular modeling studies indicate that MC monoadduct **2** is relatively nondistortive of duplex B-DNA. It fits snugly in the minor groove, leaving the base pairing of the modified Gua with Cyt intact (Tomasz et al., 1986a,b; Remers et al., 1988; Arora et al., 1990). DNA melting studies (Chawla et al., 1988) and the results of the present oligonucleotide melting experiments (Figure 11b) support this model. Thus lack of the proper base-pairing capacity of the adduct in duplex DNA

is not likely to be the case. Instead, we hypothesize that the unusually severe replication block caused by the MC monoadducts in comparison with other DNA lesions is inherent in the unique noncovalent, hydrogen-bonding capacities of the mitomycin residue, which may induce additional interaction in DNA with other nucleotides. This increased thermodynamic stability of a distorted structure at the replication fork, in turn, results in the polymerase block. In a series of molecular mechanics modeling studies of MC–DNA adducts (Rao et al., 1986; Remers et al., 1986) strikingly large drug–polynucleotide interaction energies, due to H-bonds between polar functional groups of MC and DNA, were noted as a characteristic general feature of mitomycin–DNA complexes. Similar conclusions were reached by others (Tomasz et al., 1987; Arora et al., 1990). The two polar functional groups of the bound drug, i.e., the 2″-NH_3^+ and 10″-carbamate, were seen as the main interactive elements. Thus, they are likely to interact with polar sites of the template/primer or the template/primer/polymerase (ternary complex) through H-bonding and electrostatic interactions. The 10″-OH group of DMC has similar properties. Covalent adducts which are capable of bypass (see list above) are largely hydrophobic in nature and lack the capacity for similar, polar interactions. The present results of the modeling of the MC- and DMC-modified template/primer complexes qualitatively support these expectations. Thus, the MC-**11/12** model (Figure 10b) shows a H-bond-stabilized structure in which the bulky 10″-carbamate blocks directly the access to the base-pairing functions of the modified Gua. The DMC-**11/12** model (Figure 10c), although different in detail, presents a stabilized structure in which base pairing by the dNTP to Gua may be unhindered but the primer terminus is severely out of place. The significance of these model structures[4] lies primarily in their demonstration of the interactive power of the functional groups of the mitosene residue bound at a template Gua at the template/primer junction leading to stabilized distortion. The actual structures may be different, especially in the protein/template/primer ternary complex. It is interesting that both a Glu residue and an Asp residue were identified at the template nucleotide site of the Klenow fragment (Yadav et al., 1992, and references therein). Interaction of the 2″-NH_3^+ with the $-COO^-$ of these residues may play a role in the distortion. The slightly but consistently higher T_m of the template/primer MC-**8/10** (and perhaps DMC-**8/10**) compared to the T_m of the unmodified one provides experimental indication of thermodynamically stabilized structures consistent with our hypothesis. It would be interesting to also test other polar covalent adducts for high DNA polymerase blocking activity.

These results are consistent with the observation that DMC, which cannot form a cross-link, is cytotoxic in vitro. They are also consistent with the observation that MC mutagenesis needs an intact excision repair system and the presence of the plasmid pKM101. In the absence of excision repair, the adducts may not be bypassed and survival would be too low. On the other hand, an error-prone repair system like pKM101 in *S. typhimurium* strains (or an inducible SOS type of processing in *E. coli*) may be necessary for both survival and mutagenesis—a characteristic noted with other polymerase-blocking lesions in DNA. Indeed, the existence of an inducible pathway that leads to enhanced mutagenesis appears to be applicable to mammalian cell systems as well. Dixon and co-workers have demonstrated that pretreatment of monkey cells with MC resulted in enhanced mutagenesis of UV-damaged DNA (Roilides et al., 1988).

In conclusion, the results of this study do not necessarily diminish the importance of the MC-induced cross-links in DNA. An important question that needs to be addressed now is, what are the relative rates of repair of monoadducts compared to that of cross-links in vivo? An answer to this question is crucial in our understanding of the relative importance of these MC–DNA adducts.

[4] Comparably low-energy minima were obtained for the models having the mitosene docked in the opposite orientation in the DNA. These models (not shown) resemble those above in many aspects.

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