sequences, each of which directs the synthesis of the other, would be possible. A demonstration of complementary syntheses of this kind would have important implications for discussions of the origin of biological information storage.

We do not see any way of destabilizing the oligo(G) self-structure without greatly weakening the interaction of oligo(G) with C-containing monomers. However, the substitution of 7-deaza-G[17] (or 7-deaza-8-aza-G)[18] for G in a polynucleotide prevents the formation of multi-strand self-structures. We speculate that poly(7-deazaG) may form a stable double-helix with appropriate monomeric C derivatives and that it may be possible to develop pairs of complementary templates containing riboC and ribo-7-deaza-G residues.

We emphasize that the above suggestion is not directly relevant to the origins of life. We do not believe that 2-MeImpC, 2-MeImpG, and 7-deaza-G were present in large amounts on the primitive earth. However, the development of pairs of efficient, complementary templates would represent on important step toward the realization of a nonenzymatic replicating system that stores information.

(17) Seela, F.; Tran-Thi, Q.-H.; Franzen, D. *Biochemistry* **1982**, *21*, 4338-4343.
(18) Seela, F.; Driller, J. *Nucleic Acids. Res.* **1989**, *17*, 901-910.

Experimental Section

Materials and methods used in this work, with the exception of those described explicitly below, were the same as those described in part 1 of the series.[14]

Ribonuclease A was purchased from Boehringer Mannheim. Cytidine 5'-phosphoro(2-methyl)imidazolide (2-MeImpC), guanosine 5'-phosphoro(2-methyl)imidazolide (2-MeImpG), cytidine 5'-phosphoroimidazolide (ImpC), and guanosine 5'-phosphoroimidazolide (ImpG) were prepared by a published procedure.[19]

Ribonuclease A digestion of products was carried out as follows. An aliquot of the gel-purified product (3500 cpm) was dissolved in 14 μL of RNA sequencing buffer (7 M urea, 20 mM sodium citrate, 1 mM EDTA; pH 5). Ribonuclease A (5 units, 1 μL) was added and the solution was incubated at 37 °C. Aliquots (3 μL) were withdrawn after 10, 20, 40, 60, and 90 min and kept over a dry ice/ethanol mixture until they were analyzed by electrophoresis on polyacrylamide gels.

Reaction mixtures contained each relevant nucleoside phosphoroimidazolide at a concentration of 0.1 M.

Acknowledgment. This work was supported by Grant NAWG-1660 from the National Aeronautics and Space Administration. We thank Aubrey Hill for technical assistance and Sylvia Bailey for manuscript preparation.

(19) Joyce, G. F.; Inoue, T.; Orgel, L. E. *J. Mol. Biol.* **1984**, *176*, 279-306.

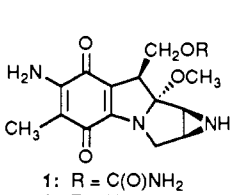
Recognition of Mitomycin C-DNA Monoadducts by UVRABC Nuclease

Harold Kohn,*,† Ven-Shun Li,† and Moon-shong Tang*,‡

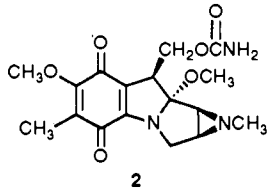
Contribution from the Department of Chemistry, University of Houston, Houston, Texas 77204-5641, and The University of Texas System M. D. Anderson Cancer Center, Science Park—Research Division, Smithville, Texas 78957. *Received December 20, 1991*

Abstract: The *Escherichia coli* UVRABC nuclease assay has been shown to be an excellent method to monitor mitomycin C-DNA monoalkylation transformations. Analysis of the mitomycin C-induced and 10-decarbamoylmitomycin C-induced DNA incision sites from five different DNA restriction fragments after UVRABC treatment revealed that all the drug-induced UVRABC incisions can be attributed to drug bonding at guanine residues and that bonding proceeded in a highly sequence selective manner. The densitometric data indicated that both the 5' and 3' nearest neighbor bases surrounding the guanine site affect its susceptibility to drug modification. Mitomycin lesions occurred predominantly at 5'CG sequences, and 5'CGG sequences were the preferred trinucleotide units for mitomycin C monoalkylation transformations. Potential contributing factors (i.e., C(10) carbamate moiety, C(8) hydroquinone hydroxyl group) responsible for the sequence selectivity of this process have been examined and a rationale offered for the DNA bonding specificity. The advantages of the UVRABC assay versus the previously employed λ-exo protocol for the detection of mitomycin C monoalkylation sites are discussed.

Mitomycin C (**1**) is a clinically significant antineoplastic agent.[1] Mechanisms have been advanced that suggest that drug function proceeds by initial reductive activation of **1** followed by attachment of mitomycin C to DNA to generate first mono-alkylated and then bis-alkylated products.[2] The ability of **1** to bond to complementary strands of DNA has been associated with its pronounced cytotoxicity and antitumor activity.[2a,f] Accordingly, reports have appeared concerning the DNA base sequence specificity for the formation of *cross-linked* mitomycin C-DNA adducts.[3-5] Evidence has been provided that these lesions are generated at complementary 5'CG sequences and explanations have been offered to account for this phenomenon. Recently, we demonstrated that the initial mitomycin C *monoalkylation* event to DNA oc-



1: R = C(O)NH$_2$
3: R = H



2

† University of Houston.
‡ The University of Texas System M. D. Anderson Cancer Center.

(1) Carter, S. K.; Crooke, S. T. *Mitomycin C. Current Status and New Developments*; Academic Press: New York, 1979.
(2) (a) Iyer, V. N.; Szybalski, W. *Science* **1964**, *145*, 55. (b) Remers, W. A. *The Chemistry of Antitumor Antibiotics*; Wiley: New York, 1979; Vol. 1, pp 221-276. (c) Franck, R. W.; Tomasz, M. In *The Chemistry of Antitumor Agents*; Wilman, D. E. V., Ed.; Blackie and Sons, Ltd.: Glasgow, Scotland, 1990; pp 379-394. (d) Fisher, J. F.; Aristoff, P. A. *Prog. Drug Res.* **1988**, *32*, 411. (e) Moore, H. W.; Czerniak, R. *Med. Res. Rev.* **1981**, *1*, 249 and references therein. (f) Zwelling, L. A.; Anderson, T.; Kohn, K. W. *Cancer Res.* **1979**, *39*, 365.
(3) Teng, S. P.; Woodson, S. A.; Crothers, D. M. *Biochemistry* **1989**, *28*, 3901.
(4) (a) Weidner, M. F.; Millard, J. T.; Hopkins, P. B. *J. Am. Chem. Soc.* **1989**, *111*, 9270. (b) Millard, J. T.; Weidner, M. F.; Raucher, S.; Hopkins, P. B. *J. Am. Chem. Soc.* **1990**, *112*, 3637. (c) Weidner, M. F.; Signurdsson, S. T.; Hopkins, P. B. *Biochemistry* **1990**, *29*, 9225.
(5) Borowy-Borowski, H.; Lipman, R.; Tomasz, M. *Biochemistry* **1990**, *29*, 2999.

0002-7863/92/1514-5501$03.00/0

curred preferentially at guanine residues within 5′CG and 5′GG sequences.[6] The sites of drug bonding were gleaned from the analysis of the λ exonuclease (λ-exo) digestion stop sites induced by mitomycin C modification in DNA restriction fragments. Our results differed in part from the findings obtained by Rupp and co-workers in a complementary study.[7] These investigators showed that the *Escherichia coli* UVRABC nuclease[8] was capable of incising *N*-methylmitomycin A (**2**)–DNA modification sites. This enzyme repair system has been previously utilized to detect DNA lesions produced by various agents and drugs.[9] Typically, this multisubunit endonuclease hydrolyzes the seventh or eighth phosphodiester bond 5′ and the fourth or fifth phosphodiester bond 3′ of the modified base providing a distinctive dual incision pattern. Analysis of the UVRABC incision sites on a **2**-modified DNA fragment indicated that drug attachment had occurred at most guanines within the DNA fragment. Significantly, no comment was made concerning the sequence specificity for the *N*-methylmitomycin A–DNA bonding event.

Several explanations can account for these divergent results.[10] First, the *N*-methylmitomycin A reaction may have been less selective than the corresponding mitomycin C process. Previous studies have demonstrated that **2** is biologically more potent than **1**.[3,11] A similar difference exists in the chemical reactivity of these two substrates and has been attributed in part to the increased ease in reduction of *N*-methylmitomycin A versus mitomycin C.[12-14] Second, the distinctive λ-exo stop site patterns observed by us may have reflected a preference by the enzyme to stop at mitomycin C–DNA lesions located at 5′CG and 5′GG sites over those at 5′AG and 5′TG loci, rather than a difference in the relative drug bonding occupancy at these sites. Third, UVRABC nuclease has been shown to be a more sensitive assay than λ-exo in detecting DNA–anthramycin adducts, suggesting that some modification sites may not be recognizable by λ-exo analysis.[15] Significantly, we note that anthramycin is structurally similar to **1**,[16] and both drugs preferentially bond to the N(2) position of guanine in DNA.[15,17]

In this report, we show that UVRABC nuclease recognizes and incises mitomycin C–guanine adducts typically seven bases 5′ and four bases 3′ to the drug lesion in the same fashion previously described for *N*-methylmitomycin A–guanine adducts.[7] Detailed analysis of the UVRABC incision patterns obtained from five different DNA restriction fragments modified with **1** conclusively demonstrated that initial drug bonding was highly specific for 5′CG sequences. These results were in agreement with our earlier report detailing the remarkable sequence dependency for the mitomycin C–DNA monoalkylation process.[6] Use of the UVRABC nuclease assay has also permitted the identification of additional **1** bonding sites not detected by the λ-exo protocol.

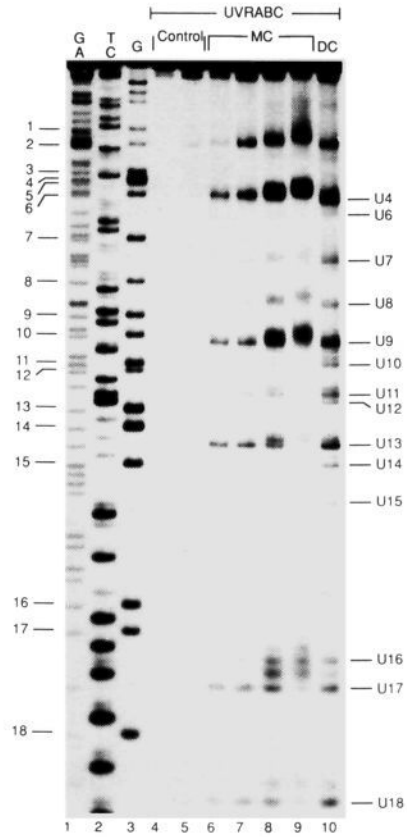


Figure 1. Autoradiogram of UVRABC nuclease cutting of a mitomycin C (**1**)-modified and 10-decarbamoylmitomycin C (**3**)-modified 3′ end-labeled 129 base pair fragment from pBR322 plasmid (top strand). Lanes 1–3, Maxam–Gilbert chemical sequencing reactions of G + A, T + C, and G, respectively; lane 4, DNA treated with 0.45 mM of **1** alone (control); lane 5, DNA treated with 0.45 mM of $Na_2S_2O_4$ alone (control); lanes 6–9, DNA modified with 0.15, 0.20, 0.30, 0.45 mM **1**, respectively, after reductive activation with $Na_2S_2O_4$ (1 equiv); lane 10, DNA modified with 0.45 mM **3** after reductive activation with $Na_2S_2O_4$ (1 equiv). The drug modification induced UVRABC nuclease incision bands (U4-U18) are labeled on the right side of the panel, and the numbers correspond to the guanine residues that are numbered on the left side of the panel.

Quantitative analysis of the observed incision sites has provided the preferred trinucleotide sequences for the mitomycin C–DNA monoalkylation process. Finally, new information has been ascertained concerning the origin of the sequence selectivity of this transformation.

Results

The preferred sites of mitomycin C modification and the DNA cleavage patterns after *Escherichia coli* UVRABC nuclease digestion were initially determined using a 3′ end-labeled (top strand) 129 base pair fragment isolated from pBR322 plasmid, which was modified with varying concentrations of **1**. DNA–drug bonding was accomplished using limiting amounts of sodium dithionite (0.6–1.0 equiv). This procedure has been shown to be sufficient to activate the C(1) site in mitomycin C but not the C(10) position.[6] A comparable protocol was employed for the determination of the drug-induced lesions for DNA modified with 10-decarbamoylmitomycin C (**3**). Compound **3** unlike **1** can only bond to DNA at the C(1) site in the drug due to the absence of the carbamate group at the C(10) position. The autoradiogram of the high-resolution denaturing polyacrylamide gel after UVRABC digestion of these DNA-modified fragments is provided in Figure 1.

Inspection of the autoradiogram revealed several prominent, distinct bands. These bands have been assigned to the UVRABC incision sites induced by mitomycin C–DNA lesions and have been labeled U4-U18. The band intensities were drug dose dependent.

(6) Li, V.-S.; Kohn, H. *J. Am. Chem. Soc.* **1991**, *113*, 275.
(7) Pu, W. T.; Kahn, R.; Munn, M. M.; Rupp, W. D. *J. Biol. Chem.* **1989**, *264*, 20697.
(8) (a) Howard-Flanders, P.; Boyce, R. P.; Theriot, L. *Genetics* **1966**, *53*, 1119. (b) Friedberg, E. C. *DNA Repair*; W. H. Freeman: New York, 1984; and references therein.
(9) (a) Sancar, A.; Sancar, G. B. *Annu. Rev. Biochem.* **1988**, *57*, 29. (b) Van Houten, B. *Microbiol. Rev.* **1990**, *54*, 18. (c) Sancar, A.; Franklin, K. A.; Sancar, G.; Tang, M.-s. *J. Mol. Biol.* **1985**, *184*, 725. (d) Sancar, A.; Rupp, W. D. *Cell* **1983**, *33*, 249. (e) Jones, B. K.; Yeung, A. T. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 8410. (f) Tang, M.-s.; Lee, C.-S.; Doisy, R.; Ross, L.; Needham-VanDevanter, D. R.; Hurley, L. H. *Biochemistry* **1988**, *27*, 893.
(10) An additional factor that may have contributed to the differences noted in these studies is the relative amounts of sodium dithionite employed for the activation of **1** and **2**, respectively.
(11) (a) Kunz, K. R.; Iyengar, B. S.; Dorr, R. T.; Alberts, D. S.; Remers, W. A. *J. Med. Chem.* **1991**, *34*, 2281. (b) Bradner, W. T.; Remers, W. A.; Vyas, D. M. *Anticancer Res.* **1989**, *9*, 1095, and references therein.
(12) Pan, S.-S.; Gonzalez, H. *Mol. Pharmacol.* **1990**, *37*, 960.
(13) McGuinness, B. F.; Lipman, R.; Goldstein, J.; Nakanishi, K.; Tomasz, M. *Biochemistry* **1991**, *30*, 6444.
(14) Subramaniam, S. M.Sc. Thesis, University of Houston, 1991.
(15) Walter, R. B.; Pierce, J.; Case, R.; Tang, M.-s. *J. Mol. Biol.* **1988**, *203*, 939.
(16) Rao, S. N.; Singh, U. C.; Kollman, P. A. *J. Med. Chem.* **1986**, *29*, 2484.
(17) (a) Hertzberg, R. P.; Hecht, S. M.; Reynolds, V. L.; Molineux, I. J.; Hurley, L. H. *Biochemistry* **1986**, *25*, 1249. (b) Hurley, L. M. *J. Antibiot.* **1977**, *30*, 349.

At low mitomycin concentrations (0.15–0.30 mM) the intensities of the bands increased with increasing concentration of **1** (Figure 1, lanes 6–8); however, at the highest drug concentration (0.45 mM) both the intensity and the clarity of the bands often decreased (Figure 1, lane 9). We have tentatively attributed this phenomenon to the multiple modification of a given DNA molecule by **1** at higher drug doses. We suspect that introduction of several mitomycin C lesion sites in the DNA fragment may effect the electrophoretic mobilities of the DNA incision fragments as well as influence the tertiary structure of the DNA and the ability of the UVRABC to process this modified DNA. Similar findings have been previously observed for other drug-modified DNA adducts.[15] Significantly, the different intensities of the bands in the autoradiogram within any given lane indicated that **1**– and **3**–DNA bonding had proceeded in a sequence-dependent manner.

Analysis of the cleavage patterns of the radioactive bands from the 3′ end-labeled mitomycin C-modifiied 129 base pair fragment permitted us to define the position of mitomycin C attachment. We noted that **1** induced UVRABC DNA incision to produce DNA fragments three-to-four nucleotides smaller than a guanine-terminating fragment in the Maxam–Gilbert chemical sequencing lane.[18] Significantly, both **1** and **3** exhibited similar incision patterns (i.e., Figure 1, lanes 7 and 10). Since compound **3** unlike **1** can only bond to DNA at the C(1) site in the drug, the near identity of these enzymatic incisions defines the site of DNA guanine attachment to the C(1) position in **1**.

Confirmation of the proposed mitomycin C–DNA bonding sites was accomplished by examining the drug-induced UVRABC incision sites from the 5′ end-labeled DNA fragment (top strand) from pBR322 plasmid containing the same 129 base pair (Figure 2). We observed a series of mitomycin C-induced incision sites (i.e., U3-U18) that were dependent upon the concentration of **1** (Figure 2, lanes 7–10). These radioactive bands uniformly migrated six-to-seven bases faster than a guanine band in the corresponding Maxam–Gilbert guanine-specific chemical cleavage reaction. Significantly, once again the band intensities within any given lane varied, suggesting that drug bonding was site specific.

The intensities of the bands induced by 0.2 mM mitomycin C after UVRABC nuclease digestion of the 3′ and 5′ end-labeled modified 129 base pair fragments were quantitatively determined by densitometric scanning. These results are graphically presented in Figure 3. We have not included in this histogram the U1 and U2 modification sites (not labeled in Figure 1) identified from the 3′ end-labeled DNA base pair and the U19 and U21 drug-bonding loci (not labeled in Figure 2) discerned from the 5′ end-labeled DNA fragment since these modification sites could not be detected from the data obtained from the corresponding 5′ and 3′ end-labeled DNA fragments, respectively. Figure 3 clearly showed that the degree of drug modification of the various guanine sites was site dependent. The most intense bands corresponded to guanines located within 5′CG sequences with one exception (U18 for G18), while the intensities of the bands at 5′GG, 5′TG, and 5′AG sites were considerably weaker. We noted that for any given guanine modification site, the relative intensity for the incision site generated from the 3′ end-labeled mitomycin C-modified DNA (lower panel) was approximately equal to the corresponding band obtained from the 5′ end-labeled mitomycin C-modified DNA (upper panel). These results indicated that in most cases UVRABC nuclease DNA cleavage on the 3′ and the 5′ sides surrounding the mitomycin–guanine modification site occurred with approximately equal efficiency.

The densitometric data in Figure 3 for the UVRABC nuclease cleavage patterns permitted us to compare this information with the results previously secured by us after digestion of the same mitomycin C-modified 129 base pair DNA fragment with λ-exo.[6] Several key observations were deduced. First, the most intense mitomycin C bonding sites determined from the UVRABC digests matched the most pronounced modification sites ascertained from the λ-exo stop sites. The excellent correspondence of these two

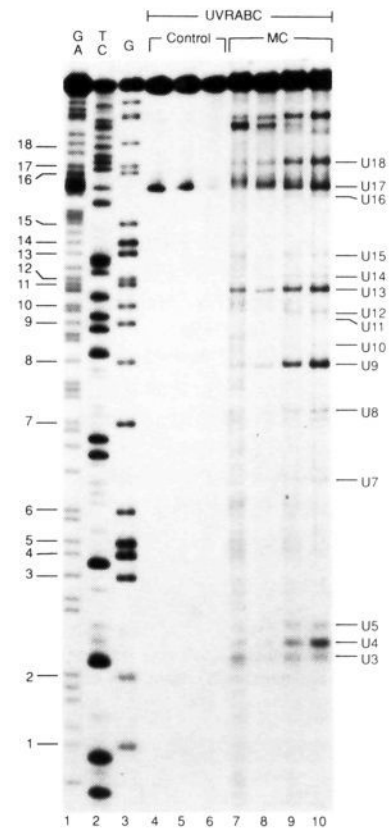


Figure 2. Autoradiogram of UVRABC nuclease cutting of a mitomycin C (**1**)-modified 5′ end-labeled 129 base pair fragment from pBR322 plasmid (top strand). Lanes 1–3, Maxam–Gilbert chemical sequencing reactions of G + A, T + C, and G, respectively; lane 4, DNA without drug modification (control); lane 5, DNA treated with 0.45 mM **1** alone (control); lane 6, DNA treated with 0.45 mM $Na_2S_2O_4$ alone (control); lanes 7–10, DNA modified with 0.15, 0.20, 0.30, and 0.45 mM **1**, respectively, after reductive activation with $Na_2S_2O_4$ (0.6 equiv). The drug modification induced UVRABC nuclease incision bands (U3-U18) are labeled on the right side of the panel, and the numbers correspond to the guanine residues that are numbered on the left side of the panel.

different enzymatic assays coupled with the dependency of band intensity on drug dosage provided strong evidence that recognition of the DNA–mitomycin C modification site by either UVRABC or λ-exo was not a function of how the DNA base sequence surrounding the individual guanine modification sites affected the processing of the DNA by the enzyme but rather was due to the percentage of drug occupancy at these bonding loci. Additional support for this contention is provided by a recent report by Tomasz and co-workers.[19] These investigators have confirmed our earlier observation that mitomycin C–DNA monoalkylation occurred preferentially at 5′CG sequences using yet a different assay. Second, UVRABC nuclease proved to be superior to λ-exo for the detection of mitomycin C–DNA lesions. We routinely observed that UVRABC analysis provided less ambiguity in the identification of drug modification sites than λ-exo. This phenomenon may be due in part to the nature of the enzyme–drug DNA recognition process for these two transformations. In separate studies we have shown that UVRABC nuclease incises only the drug-modified strand regardless of whether the drug modification stabilizes or destabilizes the DNA helix,[15] while λ-exo is occasionally stopped by drug lesion sites located on the opposite strand if the drug modification stabilizes the helix structure.[20] Furthermore, the former method is more sensitive, permitting the detection of minor drug lesions, and allowed the use of lower

(18) (a) Maxam, A. M.; Gilbert, W. *Methods Enzymol.* **1980**, *65*, 499. (b) Maxam, A. M.; Gilbert, W. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 560.

(19) Kumar, S.; Lipman, R.; Tomasz, M. *Fourth Chemical Congress of North America*; Medicinal Chem. Abstr. 14; New York, Aug., 1991.

(20) Pierce, J. R.; Nazimiec, M.; Tang, M.-s. *Biochemistry*, submitted for publication.

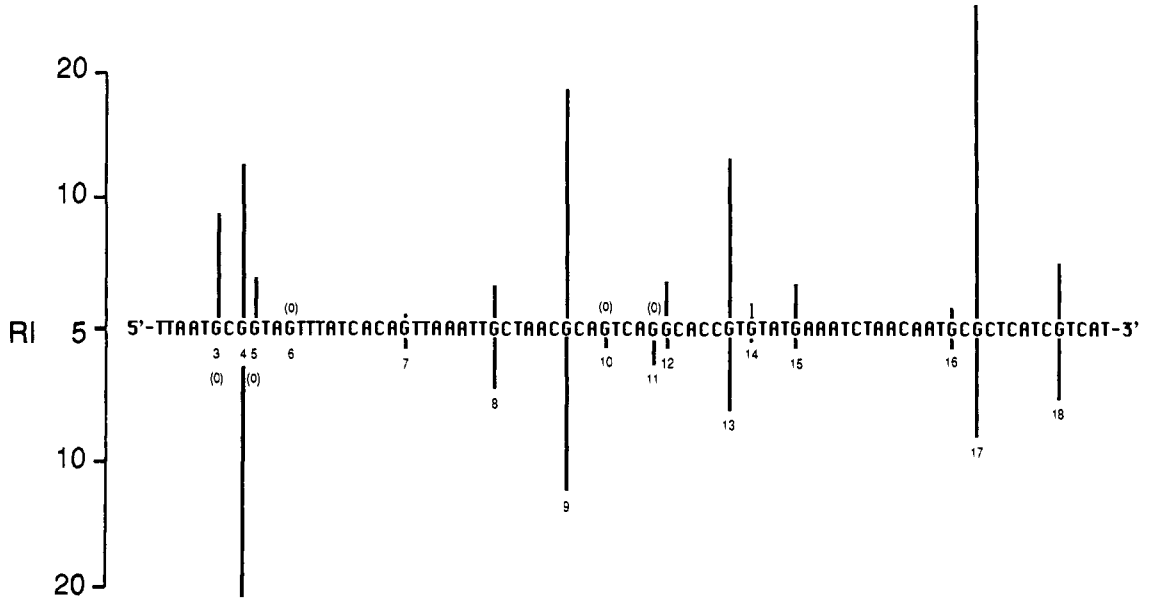


Figure 3. Relative intensity (RI) of UVRABC nuclease incision on the 5′ and 3′ side of mitomycin C–DNA adducts in the (*Eco*RI-*Bst*NI) 129 bp sequence. The intensity of UVRABC nuclease incision bands in Figure 1, lane 7, and Figure 2, lane 8, were scanned in a Bio Image Analyzer. The relative intensity (length of the bars) of each band was calculated as $B = I_i/\sum_{i=1}^{i} I_i$, where B is the relative intensity of each band and I_i is the reading of the individual band from the Bio Image Analyzer. A zero (0) below a numbered guanine signifies that no detectable drug bonding occurred at this site. Fifteen bands (two not shown) in the 3′ labeled DNA and fifteen bands (two not shown) in the 5′ labeled DNA were calculated. The upper panel and the lower panel represent the relative intensity of UVRABC nuclease incision sites at 5′ and 3′ of the drug–DNA adduct, respectively.

concentrations of **1** to be employed in the drug-bonding step. Third, the UVRABC derived data could be quantitatively assessed by densitometric analysis. This information was not obtained in the λ-exo experiments due to the processive nature of this enzymatic assay.

The significant advantages accrued from the use of the UVRABC nuclease assay allowed us to determine the preferred nucleotide sequences for mitomycin C–DNA monoalkylation processes. Four additional DNA restriction fragments were selected for study to provide a statistical relevant number of drug-bonding opportunities. The DNAs selected were 3′ end-labeled 146 (bottom strand) and 237 (top strand) base pair fragments from pBR322 plasmid and 3′ end-labeled 142 (top strand) and 178 (bottom strand) base pair fragments from ØX174 RF I DNA. Each labeled DNA was treated with varying mitomycin C concentrations using limiting amounts of sodium dithionite. A similar reductive protocol was employed for 10-decarbamoylmitomycin C (**3**). Drug modification sites were determined by UVRABC analysis and then compared to the corresponding stop sites obtained with λ-exo. The histograms corresponding to the autoradiograms for these experiments identifying the specific drug modification sites and the relative intensities of the radiolabeled bands at low **1** concentrations are given in Figure 4a–d. The autoradiograms are provided in the supplementary material.

Similar results were obtained in all four sets of experiments. First, distinctive mitomycin C-induced UVRABC incision sites were observed that were drug dose dependent. In all cases DNA cleavage occurred three-to-four bases proximal to a 3′ guanine residue. In several instances dual incision bands were detected (i.e., Figure 4a, for the G9 site; Figure 4b, for the G9 site; Figure 4c, for the G8, G12, and G18 sites; Figure 4d, for the G4 site). This phenomenon has been previously observed for other drug–DNA adducts and has been attributed to the plasticity of the UVRABC incision process.[15] Second, the UVRABC digestion product patterns for the 10-decarbamoylmitomycin C-modified DNAs were nearly identical to those obtained for the mitomycin C-modified DNAs, indicating that the C(10) carbamate substituent (i.e., $C(O)NH_2$) in **1** did not significantly alter the selectivity of the mitomycin C–DNA bonding process. Third, the radiolabeled bands obtained after the UVRABC treatment were considerably sharper than those observed in the λ-exo experiments. The enhanced clarity of the UVRABC treated lanes permitted us to confidently assign several modification sites that could not have been identified solely on the basis of the λ-exo experiments. Fourth, the proposed major mitomycin–guanine modification sites determined by UVRABC analysis consistently correlated with those sites identified by the λ-exo assay. Fifth, the λ-exo stop sites detected for the 10-decarbamoylmitomycin C-modified DNAs mirrored those observed for the corresponding mitomycin C-modified DNAs with one exception. In the 237 base pair fragment from pBR322 plasmid, λ-exo analysis of the mitomycin C-modified DNA failed to reveal one drug bonding site (Figure 4b, G17) that was observed after treatment of the corresponding 10-decarbamoylmitomycin C-modified DNA with λ-exo. This modification site, however, was identified after UVRABC analysis of both the **1**- and **3**-modified DNAs. Sixth, slightly increased electrophoretic mobilities were generally observed for the radiolabeled products generated from the 10-decarbamoylmitomycin C-modified DNAs after λ-exo analysis versus the corresponding adducts obtained from the mitomycin C-modified DNAs. This phenomenon may be due to the decreased molecular weights of the 10-decarbamoylmitomycin-modified DNA fragments versus the analogous mitomycin C–DNA fragments, and/or that these two drugs induce λ-exo to stop at different positions in the DNA.

The utilization of five different radiolabeled base pair fragments afforded a random distribution of bases and base sequences. The combined DNA sequences accessible from the autoradiograms spanned over 385 bases and included 97 guanines. Each autoradiogram comprising an approximately 80 base pair length of readable region revealed the presence of drug-induced UVRABC incision bands that differed markedly in their relative intensities. Densitometric scanning of the radiolabeled bands permitted a quantitative assessment of the relative mitomycin C bonding occupancy at each of the corresponding DNA lesions. The percentage intensities of the individual UVRABC incision sites within each restriction fragment were determined. In order to compare the relative intensities of the drug-induced UVRABC incision bands among the five 3′ end-labeled DNA fragments, the percentage intensities of UVRABC incision at each site within the different DNA fragments were standardized to the average intensity observed for the 5′NuCGC modification sites when Nu = T, A, G, and then the data were normalized to the most intense band. These three tetranucleotide sequences were selected as the "standard unit" since they frequently appeared in the DNA sequences of the five restriction fragments and because of their

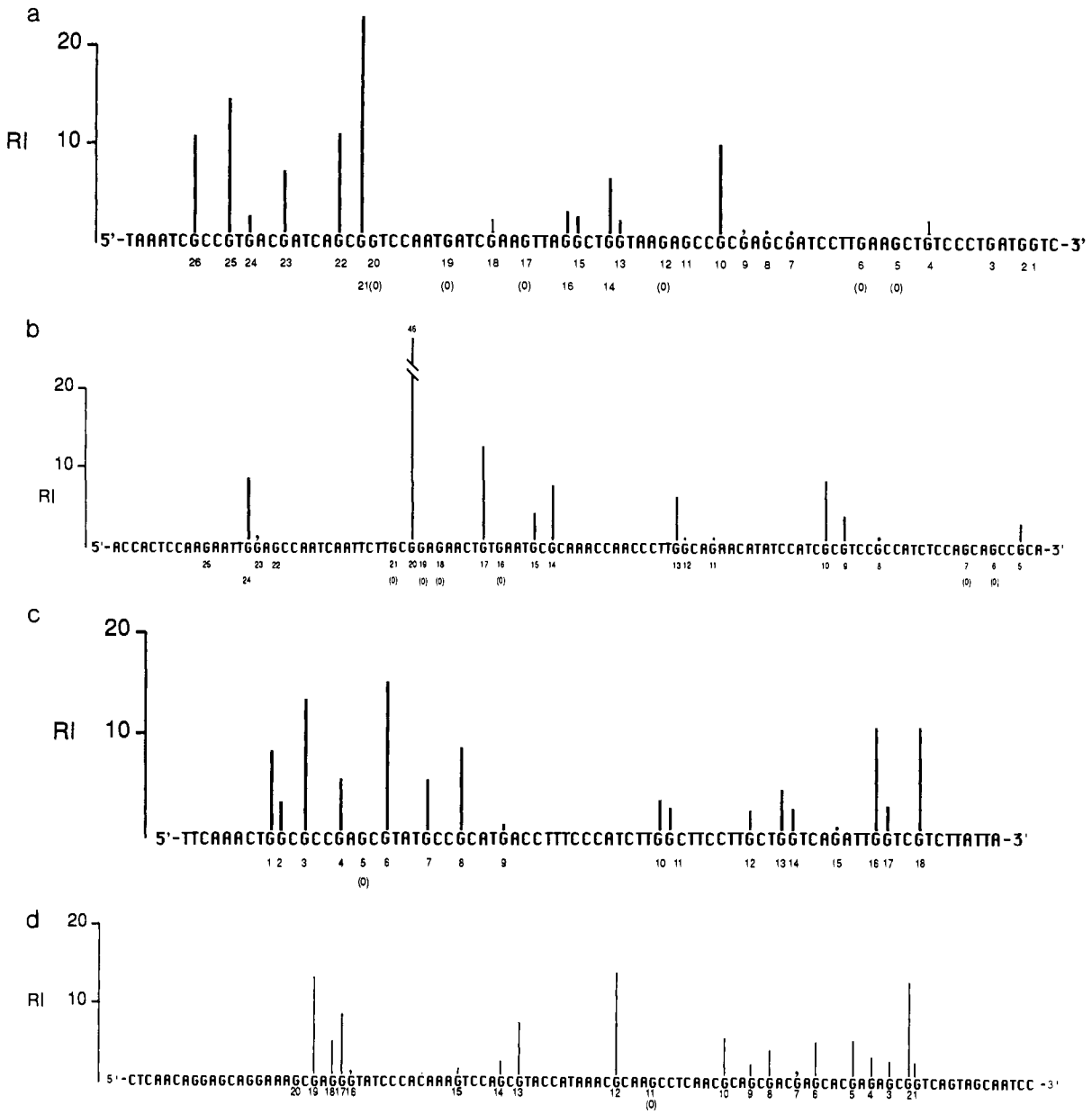


Figure 4. Relative intensities (RI) of UVRABC nuclease incision sites on the 3′ side of mitomycin C-DNA adducts in (a) 146 bp and (b) 237 bp sequences of pBR322 plasmid DNA and (c) 142 bp and (d) 178 bp sequences of ØX 174 RF I DNA. The intensity of UVRABC nuclease incision bands in either 0.15 or 0.20 mM mitomycin C modified fragments (see Tables Ia,b and II) was scanned in a Bio Image Analyzer and calculated the same as described in Figure 3. The length of the bars represents the relative intensity of UVRABC nuclease incision at each drug-modified guanine residue. A zero (0) below a numbered guanine signifies that no detectable drug bonding occurred at this site.

uniformity in band intensity. We have not included in this "standard set" the corresponding data for 5′CCGC sequences since this modification site gave rise to incision bands that were generally less than 10% as intense as those detected when the initial 5′ base was either T, A, or G. A rationale for this observation is provided in the Discussion Section.

In sections a and b of Table I we have listed the average intensities of the observed cleavage bands for the dinucleotide sequences 5′NuG* and 5′G*Nu′, respectively, where G* is the established drug modification site. Included in Table Ia,b is a fifth entry which lists the average relative intensities for the 5′CG* and 5′G*G bonding sites, respectively, but excludes the data observed for 5′GCG*G tetranucleotide sequences. We have observed that this unit is extensively modified and may have biased the data. Inspection of these two tables revealed that the bases flanking the guanine (G*) modification site played a critical role in the drug bonding process. We conclude that cytosine is the preferred nucleotide 5′ to the drug bonding site and that the reactivity order for the 5′ base is C > T > G, A. Correspondingly, we noted that placement of a guanine residue 3′ to the drug bonding locus led to increased mitomycin C-DNA modification. The order of reactivity for the 3′ base was G > T, C > A. Significantly, these patterns were observed even when the 5′GCG*G data were not included in the data analyses. Further examination of the densitometric data permitted the determination of the preferred trinucleotide sequences for the mitomycin C-DNA monoalkylation process. In order to facilitate comparison of the various bonding sites we have normalized the data to the most intense trinucleotide sequence and ranked the sequences from most to least intense. This information is presented in Table II. A similar ordering was observed when the initial "standard unit" was selected as 5′CGT instead of 5′NuCGC when Nu = T, A, G. The intensities of the bands associated with the 5′CGT modification site were fairly uniform throughout the data set. The relative intensities based on this "standard unit" have been placed in parentheses in Table II. The densitometric data for the trinucleotide sequences can be grouped into four categories: highest bonding (100%), strong bonding (35–19%), weak bonding (14–7%), and very weak bonding (4–3%). The data clearly showed that 5′CG*G was the optimal base sequence for mitomycin C

Table I. Relative Intensities Observed for the 5′ Base (Nu) and 3′ Base (Nu′) Flanking the Mitomycin C Guanine (G*) Modification Site

(a) 5′ Base

entry no.	NuG*	no. of sites	rel intensities[a]
1	AG*	25	18 ± 5
2	CG*	31	100 ± 16
3	GG*	14	22 ± 6
4	TG*	22	34 ± 8
5	CG*[b]	27	76 ± 8

(b) 3′ Base

entry no.	G*Nu′	no. of sites	rel intensities[a]
1	G*A	21	24 ± 7
2	G*C	35	43 ± 7
3	G*G	14	115 ± 33
4	G*T	22	44 ± 10
5	G*G[c]	10	56 ± 8

[a] The relative intensities were calculated from the densitometric data obtained from the UVRABC nuclease treated 3′ end-labeled DNA fragments modified with either 0.2 mM **1** (pBR322-derived fragments: 129 and 237 base pairs; ϕX 174 RF I-derived fragments: 142 and 178 base pairs) or 0.15 mM **1** (pBR322 plasmid: 146 base pairs). The data were scaled to the "standard unit" 5′NuCGC where Nu = T, A, G (see Results Section for discussion on treatment of data). [b] The relative intensity observed for the 5′CG* sites when the data for the 5′GCG*G bonding sites were excluded in the calculation. [c] The relative intensity observed for the 5′G*G sites when the data for the 5′GCG*G bonding sites were excluded in the calculation.

Table II. Relative Intensities Observed for the Mitomycin C Trinucleotide Bonding Sites 5′NuG*Nu′

sequence	no. of sites	normalized rel intensities[a]
CG*G	4	100 ± 22 (100 ± 23)
CG*T	7	35 ± 16 (24 ± 2)
CG*C	12	33 ± 17 (27 ± 14)
GG*G	1	31 ± 0 (25 ± 0)
TG*G	6	24 ± 14 (16 ± 18)
TG*T	3	21 ± 50 (25 ± 37)
CG*A	8	19 ± 32 (13 ± 24)
AG*G	3	14 ± 17 (10 ± 14)
TG*C	6	10 ± 27 (9 ± 23)
AG*C	12	8 ± 61 (5 ± 32)
GG*C	5	7 ± 14 (5 ± 2)
GG*T	7	7 ± 31 (4 ± 23)
AG*A	5	4 ± 52 (4 ± 31)
AG*T	5	4 ± 30 (3 ± 19)
TG*A	7	3 ± 46 (2 ± 25)
GG*A	1	0 (0)

[a] The relative intensities were calculated from the densitometric data obtained from the UVRABC nuclease treated 3′ end-labeled DNA fragments modified with either 0.2 mM **1** (pBR322-derived fragments: 129 and 237 base pairs; ϕX 174 RF I-derived fragments: 142 and 178 base pairs) or 0.15 mM **1** (pBR322 plasmid: 146 base pairs). The data were scaled to the "standard unit" 5′NuCGC where Nu = T, A, G and then normalized to the most intense trinucleotide sequence (see Results Section for discussion on treatment of data). The numbers in parentheses are the normalized relative intensities observed using 5′CGT as the "standard unit".

bonding. Correspondingly, significant drug modification was observed at 5′CG*T, 5′CG*C, 5′GG*G, 5′TG*G, 5′TG*T, and 5′CG*A sequences, while weak, very weak, or no bonding was detected at all other trinucleotide sequences.

Discussion

The UVRABC nuclease assay readily permitted the detection of strong as well as weak mitomycin C (**1**)- and 10-decarbamoylmitomycin C (**3**)-DNA modification sites. Typically, we observed clear, distinct incision bands that were drug dose dependent. In most instances, bond cleavage occurred four bases to the 3′ side and seven bases to the 5′ side of the drug modification site. Occasionally, these incision sites appeared at three and six bases distal to the mitomycin C-guanine lesion, respectively. This cleavage pattern was identical to that reported by Rupp and co-workers for *N*-methylmitomycin A (**2**)-modified DNA,[7] indicating that the structural differences in compounds **1-3** did not appreciably alter the ability of this multisubunit enzyme repair system to recognize these DNA lesions.

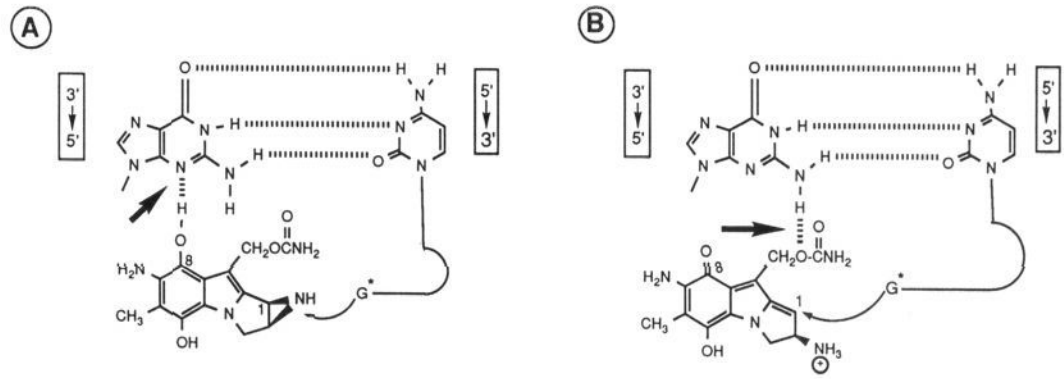
The endonuclease activity of the UVRABC protocol permitted the use of both 3′ and 5′ end-labeled DNA strands. This feature allowed the unambiguous assignment of all the guanine modification sites in the 129 base pair fragment isolated from pBR322 plasmid. A total of ten dual incision sites were noted in the other four restriction fragments of which four of these corresponded to 5′GG sequences (i.e., Figure 4a, G13,14; Figure 4b, G12,13; Figure 4c, G13,14, G16,17). This phenomenon prevented us from conclusively identifying the guanine modification site within these 5′GG dinucleotide sequences since the radiolabel was incorporated at only a single end in these DNAs.

The densitometric data provided clear evidence that the nearest neighbors (or the complementary bases) surrounding the guanine modification site played a profound role in the mitomycin monoalkylation bonding event. The 5′CGG sequence was the most prone to drug bonding, while noticeable amounts of mitomycin C modification proceeded at 5′CGT, 5′CGC, 5′GGG, 5′TGG, 5′TGT, and 5′CGA sequences. The remaining nine trinucleotides containing a central guanine were not appreciably alkylated. Few exceptions to these preferred trinucleotide sequences were observed. Every 5′CGG unit was extensively alkylated, and only two 5′CGC (Figure 4b) and four 5′CGA (Figure 4, a and d) sequences were not appreciably modified. In these instances, DNA proximity effects may have influenced the extent of drug bonding. These exceptions can be classified into two groups. In one set, a 5′CGG (i.e., Figure 4b, G5) or a 5′CGC (i.e., Figure 4a, G7) appeared one base displaced on the *complementary* (unlabeled) strand. These trinucleotide sequences are among the most preferred for mitomycin C bonding (Table II). Accordingly, DNA alkylation may have preferentially taken place on this strand, thereby diminishing the likelihood of **1** bonding to the guanine located in the same general vicinity on the labeled strand. A similar phenomenon may have accounted for the substantially lower amounts of drug modification detected for the G8 (Figure 4b) and the G9 (Figure 4a) guanines. In these instances, strong mitomycin C bonding sites were observed on the same strand two-to-four bases from these guanines. We suspect, once again, that the juxtaposition of mitomycin C bonding sites in close proximity to one another may influence the extent of DNA modification at these sites even at the low levels of drug-DNA bonding utilized in this study.

The distinctive mitomycin C monoalkylation bonding preference for 5′CG sequences was in accord with our earlier report that this transformation was sequence specific. In this previous investigation, analysis of the **1**-induced λ-exo stop sites led to the conclusion that mitomycin C bonding had proceeded at both 5′CG and 5′GG sequences. A similar finding has been described by Tomasz and co-workers using a combined enzymatic and HPLC assay.[19] These investigators have reported that the order of reactivity for the base 5′ to the guanine modification site was C > G ≫ A, T, while the corresponding reactivity order for the base 3′ to the guanine bonding site in 5′CGNu′ was C > T > A, G.[21] The enhanced sensitivity of the UVRABC assay versus the λ-exo procedure coupled with the larger number of bases scanned in the present study has permitted us to quantify the order of reactivity of the 5′ bases flanking the guanine modification site. Our results indicated cytosine was the preferred base and that the order of reactivity for 5′NuG is Nu = C > T > G, A (Table Ia). Information has also been gained concerning the order of reactivity of the 3′ base adjacent to the guanine modification site (Table Ib). We observed the order of reactivity for the dinucleotide

(21) A similar bonding pattern has been defined for the interstrand cross-linking process in which bis-alkylation preferentially proceeded at 5′CGPyr sequences.[5]

Scheme I. Proposed Binding Interactions Responsible for Mitomycin C–DNA Monoalkylation Sequence Specificity



Key hydrogen bond is indicated by an arrow, and G* is the initial guanine monoalkylation site: (A) hydrogen bond is between the carbon-8 hydroxyl hydrogen of activated 1 and the N(3)-pyrimidine nitrogen atom; (B) one of two key hydrogen bonds is between the guanine-2-amino hydrogen and the carbon-10 oxygen of activated 1.

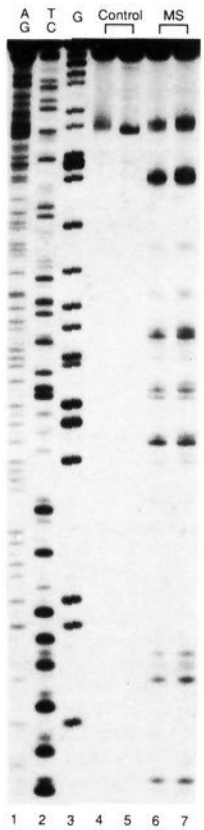


Figure 5. Autoradiogram of UVRABC nuclease cutting of a 7-aminoaziridinomitosene (**4**)-modified 3′ end-labeled 129 base pair fragment from pBR322 plasmid (top strand). Lanes 1–3, Maxam–Gilbert chemical sequencing reactions of G + A, T + C, and G, respectively; lane 4, DNA modified with **4** (0.09 mM) without UVRABC treatment (control); lane 5, DNA modified with **1** (0.2 mM) without UVRABC treatment (control); lanes 6 and 7, DNA modified with 0.045 and 0.09 mM **4**, respectively.

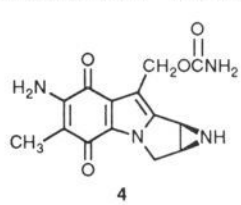
sequence 5′GNu′ to be Nu′ = G > T, C > A. These patterns are not in agreement with the preferred base orders found by the Tomasz research group. The origin of these differences is not known. Of interest, sections a and b of Table I indicated that the preference for 5′CG* and 5′G*G sites is further amplified by the 5′ base proximal to the 5′CG*G unit. Significantly, enhanced drug bonding was noted at these sites within the tetranucleotide sequence 5′GCG*G. Our data do not permit us to state whether this sequence represents "hot spots" for mitomycin C monoalkylation processes because of the insufficient number and kind of base pair combinations that existed within the DNA sequences examined. These results indicated that future studies on this transformation should examine whether drug bonding specificity can be further magnified by the incorporation of a specific base *Nu* at the 5′*Nu*CGG site.

The findings listed in Tables I and II suggested several empirical observations concerning the mitomycin C–DNA monoalkylation transformation. First, the data indicated that nearby guanines greatly facilitated covalent attachment of **1** to DNA, while cytosine and thymine units promoted drug bonding to a lesser extent and adenines had little or no effect. Second, the data suggested that the location of the nearby base in relation to the guanine modification site was also critical. We noted that within any given trinucleotide base pair sequence

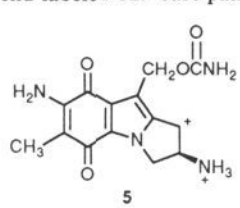
$$5'Nu_1G^*Nu_2$$
$$Nu_4C\ \ Nu_3 5'$$

where G* was the mitomycin C monoalkylation site, drug bonding increased when the nearby guanine was located at $Nu_4 > Nu_2 > Nu_1 > Nu_3$.

What is the primary factor responsible for this base and site specificity? Previously, we suggested that drug bonding at guanine within 5′CG sequences was promoted by the formation of a hydrogen bond between the carbon-8 hydroxyl group in the activated mitomycin hydroquinone (semiquinone) species and the N(3) pyrimidine nitrogen atom of the guanine residue on the complementary 5′CG strand prior to the carbon-1 alkylation event (Scheme IA).[6] The patterns observed in Table II are consistent with this scenario. This hypothesis has now been tested. We have reported the synthesis of 7-aminoaziridinomitosene (**4**).[22] This compound readily reacted with nucleophiles (i.e., water, alcohols, amines) under nonreductive conditions to give both cis and trans carbon-1 substituted products presumably through the ring-opened intermediate **5**. Treatment of the 3′ end-labeled 129 base pair



4



5

fragment from pBR322 plasmid with **4** followed by UVRABC nuclease digestion led to a series of incision products (Figure 5) that was virtually identical to those observed with **1** under limiting reductive conditions (Figure 1). The similarity of these results diminishes the likelihood that the hydrogen bond interaction

(22) Han, I.; Kohn, H. *J. Org. Chem.* **1991**, *56*, 4648.

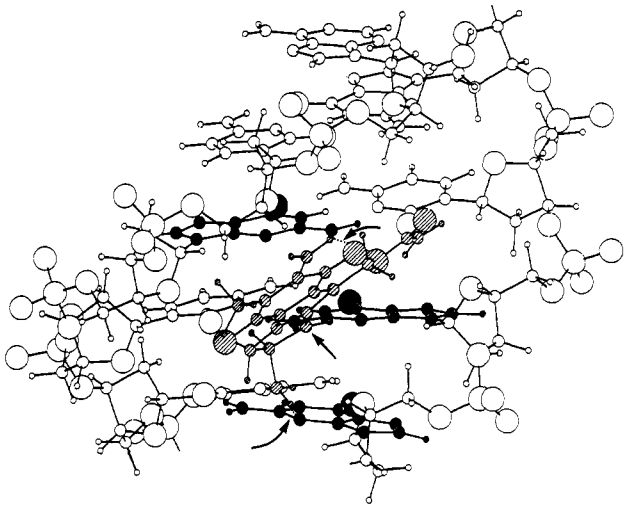


Figure 6. Ball-and-stick model of idealized representation of activated mitomycin C species in the presence of the hexanucleotide duplex TGCGGA·TCCGCA prior to covalent bonding at C-1 in mitomycin C (shaded figure), showing the formation of the key hydrogen bonds (curved arrows) between the *N*(2)-amino guanine proton on the complementary strand and the C-10 oxygen of the carbamate group in the activated **1** and the *N*(2)-amino group in activated **1** and the *N*(3) pyrimidine nitrogen on the 3′ adjacent guanine on the bonding strand. The key guanine groups are indicated in black and the initial C(1) mitomycin C alkylation site is denoted by a straight arrow.

proposed in Scheme IA is responsible for the drug bonding specificity displayed by **1**. Moreover, the present data do not permit us to conclusively identify the critical interactions responsible for the observed DNA sequence selectivity. Use of computer aided model building studies indicated that prior to bonding of the activated mitomycin species to G* within 5′CG*G·5′CCG base pairs, both the C-10 oxygen of the carbamate moiety and the *N*(2)-ammonium group in the activated mitomycin species are well-situated to hydrogen bond to the *N*(2)-amino guanine on the complementary strand and the *N*(3) pyrimidine nitrogen on the 3′ adjacent guanine on the bonding strand, respectively. This notion is presented in part in Scheme IB, and an idealized representation of this interaction is provided in Figure 6 for the *N*(2)-amino deprotonated analogue of the activated mitomycin species. This argument suggests that the preference for 5′CG*G sequences for mitomycin C monoalkylation transformations stems from an additive effect provided by both guanines situated near the guanine modification site. This hypothesis can be tested and studies are now in progress aimed at delineating the significance of these proposed interactions. The DNA sequence selectivity exhibited by 7-aminoaziridinomitosene (**4**) also suggests that a comparable reactive intermediate generated by an autocatalytic process may be partially responsible for the DNA base sequence specificity observed in the sodium dithionite-mediated reactions involving **1**.[23] Finally, the biological significance for the preferred drug bonding at 5′CGG remains obscure. We do note that putative promoter regions of certain genes are characteristically rich in cytosine and guanine[24] leading to the provocative suggestion that these DNA sequences serve as the primary sites for mitomycin C action.

Conclusions

Detailed analysis by the UVRABC assay of five different end-labeled restriction fragments modified with limiting amounts of mitomycin C and 10-decarbamoylmitomycin C revealed that the formation of mitomycin–DNA monoadducts proceeded in a highly sequence specific manner. Drug modification proceeded preferentially at 5′CG sequences. Further analysis indicated that preferred trinucleotide sequences existed for this transformation. Of these, 5′CG*G was the most prone to drug bonding. The UVRABC data were compared directly to the results obtained with the λ-exo assay. The former method proved superior for the identification of the specific DNA lesion sites and for the quantification of the extent of drug bonding. The successful demonstration of a reliable method to monitor the mitomycin C–DNA monoalkylation event at the nucleotide level should allow the identification of the key interactions responsible for this remarkably specific DNA transformation.

Experimental Section

Materials. Mitomycin C (**1**) (pure) was supplied by Bristol-Myers Squibb Co. (Wallingford, CT). 10-Decarbamoylmitomycin C (**3**) was prepared as previously described.[25] Sodium dithionite was purchased from Fisher Scientific Co. Restriction enzymes, T4 polynucleotide kinase, and DNA polymerase I (Klenow fragment) were obtained from New England BioLabs. Bacterial alkaline phosphatase, λ-exo, and NACS Prepacs convertible columns (NACS Pacs) were purchased from BRL. All other chemicals and electrophoretic materials were obtained either from Sigma or Bio-Rad. The [α-^{32}P]TTP, [α-^{32}P]dCTP, and [γ-^{32}P]ATP (specific activity approximately 3000 Ci/mmol) were purchased from Du-Pont-New England Nuclear.

DNA Fragments Isolation and ^{32}P End Labeling. (a) DNA from pBR322 Plasmid. Plasmid pBR322 was purified by cesium chloride density centrifugation and dialyzed extensively against TE buffer (10 mM Tris·HCl, pH 8.0, 1 mM EDTA). The following procedure was utilized for the isolation of the 129, 146, and 237 base pair fragments with ^{32}P labeled at one of the 3′ ends. The pBR322 plasmid was first digested with *Bst*NI and the bands corresponding to 1857 and 383 base pair fragments were isolated from a 1.4% agarose gel and cleaned by passing through a NACS Pac followed by ethanol precipitation. The 1857 base pair fragment was labeled at the 3′ termini in the presence of [α-^{32}P]TTP and Klenow fragment (5 units) in 10 mM Tris·HCl, pH 8.0, 5 mM $MgCl_2$, and 7.5 mM DTT and incubated at 20 °C (30 min). The labeled DNA fragment was precipitated with ethanol and digested with *Eco*RI. The desired 129 base pair fragment was purified by electrophoresis on a 5% polyacrylamide gel. The 383 base pair fragment was labeled at both the upper and the lower 3′ ends with [α-^{32}P]TTP, precipitated, and then digested with *Nar*I. The resulting 146 base pair (labeled at the lower strand 3′ end) and the 237 base pair (labeled at the upper strand 3′ end) fragments were purified as above.

The following procedure was employed for the isolation of the 129 base pair fragment labeled with ^{32}P at its 5′ end. The pBR322 plasmid was first digested with *Eco*RI/*Bam*HI, and the 375 base pair fragment was isolated from a 1% agarose gel. This fragment was extracted with phenol–chloroform and precipitated with ethanol. The 375 base pair fragment was then treated with bacterial alkaline phosphatase at 60 °C for 1 h (BAP reaction), extracted with phenol–chloroform, and precipitated with ethanol. The purified fragment was then exposed to 100 μCi of [γ-^{32}P]ATP in the presence of T4 polynucleotide kinase (20 units) in 50 mM Tris·HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine HCl, and 0.1 mM EDTA, pH 8.0, and incubated at 37 °C (30 min). The labeled fragment was precipitated with ethanol and dried in a vacuum centrifuge. Digestion with *Bst*NI afforded a 129 base pair 5′ end-labeled fragment, which was isolated and purified by a 5% polyacrylamide gel.

(b) DNA from Bacteriophage ØX174 RF I DNA. Double strand ØX174 RF I DNA was digested with *Hae*III and purified by electrophoresis on a 5% preparative polyacrylamide gel. The bands corresponding to the 194 and 234 base pair fragments were isolated. The 194 base pair fragment was digested with *Hin*fI and labeled at the 3′ end with Klenow fragment in the presence of [α-^{32}P]TTP and unlabeled dATP and dCTP. The 234 base pair fragment was digested with *Taq*I and labeled at the 3′ end in the presence of [α-^{32}P]dCTP. The resulting 142 and 178 base pair fragments were purified by a 5% polyacrylamide gel.

Mitomycin C (1)- and 10-Decarbamoylmitomycin C (3)–DNA Bonding with Sodium Dithionite. The procedure previously employed for drug bonding was utilized,[6] except the reductive activation of **1** was carried out at 0 °C to minimize possible cross-linking of the DNA.

7-Aminoaziridinomitosene (4)–DNA Bonding. Specific amounts of **4**[22] (freshly prepared) were dissolved in DMSO as a stock solution just prior

(23) A comparable DNA bonding profile was observed after UVRABC analysis for the 3′ end-labeled 129 base pair fragment from pBR322 plasmid (top strand) modified with **1** using either 1 equiv of sodium dithionite added in a single increment (Tris·HCl, pH 7.4) or xanthine oxidase/NADH (Tris·HCl, pH 7.4).

(24) (a) Dynan, W. S. *Trends Genet.* **1986**, *2*, 196. (b) Park, J. H.; Hershey, H.; Taylor, M. W. In *Molecular Genetics of Mammalian Cells*; Malacinski, G. M., Ed.; MacMillan Publishing Co.: New York, 1986; pp 79–98.

(25) Kinoshita, S.; Uzu, K.; Nakano, K.; Takahashi, T. *J. Med. Chem.* **1971**, *14*, 109.

to use. The stock solution was further diluted with DMSO and used immediately. The DMSO solution (10 μL) was added with a microsyringe to a solution (90 μL) containing the ^{32}P-labeled DNA in Tris·HCl buffer (25 mM, pH 7.4) to give a final concentration of 0.045 and 0.09 mM. After incubation at room temperature (1 h), the DNA was precipitated.

λ-Exo Digestion of Mitomycin C (1)-, 10-Decarbamoylmitomycin C (3)-, and 7-Aminoaziridinomitosene (4)-Modified DNA. The method used was the same as described previously.[6,15]

Purification of UVRA, UVRB, and UVRC Proteins. UVRA, UVRB, and UVRC proteins were isolated from *E. coli* K12 strain CH296 (*recA*, *endA*/F'lacI^{Q}) carrying plasmids pUNC45 (*uvrA*), pUNC211 (*uvrB*), and pDR3274 (*uvrC*).[26] The methods of purification were the same as described previously.[27]

UVRABC Nuclease Reactions. The UVRABC nuclease reactions were carried out in a reaction mixture (25 μL) containing 50 mM Tris·HCl (pH 7.5), 0.1 mM EDTA, 10 mM $MgCl_2$, 1 mM ATP, 100 mM KCl, 1 mM dithiothreitol, 15 mM UVRA, 15 mM UVRB, 15 mM UVRC, and substrate DNA. The mixtures were incubated at 37 °C (1 h), and the reactions were stopped by phenol-chloroform extractions followed by ethanol precipitation in the presence of aqueous NH_4OAc (2.5 M). The precipitated DNA was recovered by centrifugation and washed with 80% ethanol.

DNA Sequencing, DNA Sequencing Gel Electrophoresis, and Autoradiography. The 3' and 5' end-labeled DNA fragments were sequenced by the method of Maxam and Gilbert.[18] The ^{32}P-labeled fragments with or without various enzyme treatments were suspended in sequencing tracking dye (80% v/v deionized formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue), heated at 90 °C (3 min), and quenched in an ice bath. The samples were applied to a 8% denaturing sequencing gel in parallel with the Maxam and Gilbert sequencing reactions. After electrophoresis the gels were exposed to Kodak X-Omat AR film with intensifying screen at -70 °C.

Densitometric Scanning. The intensities of UVRABC nuclease incision bands were determined with a Bio-Image Visage 100 System consisting of a high-resolution digitizing camera and whole band analysis software.

Acknowledgment. We thank Dr. M. Nazimiec and Ms. A. Pao for UVR protein purification and Dr. A. Sancar (University of North Carolina-Chapel Hill) for providing *E. coli* strains containing UVR gene plasmids. We express our appreciation to Dr. B. M. Pettitt and Mr. P. Schiltz for their help in providing the idealized representation of the activated mitomycin C complex with DNA and Dr. M. Nazimiec for calculations. Grateful acknowledgment is made to Drs. A. M. Casazza and W. Rose and the Bristol-Myers Squibb Co. (Wallingford, CT) for the generous gift of mitomycin C. We thank the National Institutes of Health (RO1CA29756, H.K.; ES03124, M.-s T.), the Robert A. Welch Foundation (E607, H.K.), and the American Cancer Society (CH-485, M.-s T.) for their support of this study.

Supplementary Material Available: Autoradiograms of UVRABC nuclease cutting and λ-exo digestion of mitomycin C (**1**) and 10-decarbamoylmitomycin C (**3**)-modified 3' end-labeled DNAs: Figure 7a, 146 base pair fragment from pBR322 plasmid (bottom strand); Figure 7b, 237 base pair fragment from pBR322 plasmid (top strand); Figure 7c, 142 base pair fragment from ØX 174 RF I DNA (top strand); Figure 7d, 178 base pair fragment from ØX 174 RF I DNA (bottom strand) (7 pages). Ordering information is given on any current masthead page.

(26) Thomas, D. C.; Levy, M.; Sancar, A. *J. Biol. Chem.* **1985**, *260*, 9875.
(27) Tang, M.-s.; Nazimiec, M. E.; Doisy, R. P.; Pierce, J. R.; Hurley, L. H.; Alderete, B. E. *J. Mol. Biol.* **1991**, *220*, 855.

Site-Specific Labeling of DNA Sequences Containing Phosphorothioate Diesters

Jacqueline A. Fidanza, Hiroaki Ozaki, and Larry W. McLaughlin*

Contribution from the Department of Chemistry, Boston College, 140 Commonwealth Avenue, Chestnut Hill, Massachusetts 02167. *Received December 19, 1991*

Abstract: A phosphorothioate diester can be incorporated site-specifically into DNA sequences to provide a nucleophilic site that is amenable to alkylation by labels containing haloacetamide, aziridine sulfonamide, or γ-bromo-α,β-unsaturated carbonyl functionalities. Labeling reactions proceed most efficiently at 50 °C in the pH range 5.0-8.0 and require a number of hours for completion. HPLC techniques employing reversed-phase columns can be used to rapidly purify the labeled materials, and a variety of examples are shown including the purification of a site-specifically labeled 30-mer. Sequences containing a single diastereomeric phosphorothioate (R_p or S_p) can be prepared by synthesizing the appropriate dNp(s)N dimer block followed by resolution of the diastereomers and incorporation of either the S_p or R_p dimer into the sequence of interest. Oligodeoxynucleotides labeled in this manner are quite stable near neutral pH values, but the phosphorothioate triesters formed undergo hydrolysis under alkaline conditions. Double-stranded sequences containing the labeled phosphorothioate are less prone to base catalyzed hydrolysis than single-stranded sequences. Duplex structures containing a single backbone label are shown to have thermal stabilities that are generally very similar to those of the unlabeled sequences suggesting that little structural perturbation is present for sequences labeled by this technique. Labeling internucleotidic phosphorothioate diesters provides a rapid and simple procedure for the introduction of fluorophores, spin labels, or other moieties site-specifically without significant changes in standard phosphoramidite DNA synthesis techniques.

Introduction

A wide variety of techniques has been developed for the functionalization of oligodeoxynucleotides with reporter groups (for a recent review see Goodchild[1]). The 3'- or 5'-termini of short DNA fragments are amenable to chemical modification, and a number of procedures have described the incorporation of an appropriate amino or thiol tether that subsequently can be modified with the moiety of interest.[2] The use of end labeling procedures has been valuable in a number of subsequent studies, but this approach is less optimal in cases where the reporter group would be more effective if present internally within the sequence. The introduction of prosthetic groups to internal sites of a sequence

* Author to whom correspondence should be addressed.

(1) Goodchild, J. *J. Bioconjugate Chem.* **1990**, *1*, 165-187.
(2) For a recent selection of procedures that involve terminal labeling, see: (a) Ansorge, W.; Sproat, B.; Stegemann, J.; Schwager, C.; Zenke, M. *Nucleic Acids Res.* **1987**, *15*, 4593-4602. (b) Tyagi, S. C.; Wu, F. Y.-H. *J. Biol. Chem.* **1987**, *262*, 10684-10688. (c) Connolly, B. A. *Nucleic Acids Res.* **1987**, *15*, 3131-3139. (d) Zuckermann, R.; Corey, D.; Schultz, P. *Nucleic Acids Res.* **1987**, *15*, 5305-5321. (e) Thuong, N. T.; Chassignol, M. *Tetrahedron Lett.* **1987**, *28*, 4157-4160. (f) Asseline, U.; Thuong, N. T. *Nucleosides and Nucleotides* **1988**, *7*, 431-455. (g) Asseline, U.; Thuong, N. T. *Tetrahedron Lett.* **1988**, *30*, 2521-2524.

0002-7863/92/1514-5509$03.00/0