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5-Methylcytosine in CpG Sites and the Reactivity of Nearest Neighboring Guanines Toward the Carcinogen Aflatoxin B_1-*8,9*-Epoxide

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The reactivity of guanines in an oligonucleotide containing mutational hot spots within the *p53* gene (codons 248 and 249), 5′-CCG$_1$G$_2$AG$_3$G$_4$CCCA-3′, toward dimethyl sulfate (DMS) and aflatoxin B$_1$-*8,9*-epoxide (AFB$_1$-*8,9*-epoxide) was investigated by a modified Maxam–Gilbert technique. 5-Methylcytosine in the CpG site of codon 248 did not appear to modulate the reactivity of target guanines G$_1$, G$_2$, G$_3$, and G$_4$ toward either genotoxin when compared to the sequence containing a nonmethylated CpG site. A similar experiment was conducted in which a 0.5-kb fragment of the human *HPRT* gene containing exon 1 and several CpG sites was treated with UV-activated aflatoxin B$_1$. Results showed that guanine adduct formation was independent of the methylation status of the CpG site. These findings are discussed in relation to other studies that have shown that cytosine methylation has an inhibiting effect, an enhancing effect, or no effect on adduct formation with nearby guanine nucleotides.

Several reports have described how the presence of 5-methylcytosine (5mC*) modulates the reactivity of guanines in CpG sites toward various carcinogens (1–9). Sendowski and Rajewsky (1) described inhibitory effects of 5mC on O^6-guanine alkylation by alkylnitrosoureas in oligonucleotides. Methylation of cytosine decreased the level of ultraviolet light-induced pyrimidine (4–6) pyrimidone photoproducts at CpG sites (2) while sunlight preferentially induced pyrimidine cyclobutane dimers at 5-methylcytosine bases (3). We have shown inhibition of methylation of *N7*-guanine by *N*-methyl-*N*-nitrosourea (MNU) when 5mC was a 5′ neighbor, and even greater inhibition when 5mC was both a 5′ and 3′ neighbor to the target guanine (4). Recently Denissenko and co-workers (5, 6) showed enhanced binding of benzo[*a*]pyrene diol epoxide (BPDE) to the *p53* gene when CpG sites were methylated. Chen and co-workers (7) found that cytosine methylation increased adduct formation in guanine neighbors by four bulky carcinogens. Two other studies indicated that methylation of cytosine enhanced binding of select antitumor drugs to CpG guanine (8, 9). Therefore, the presence of 5mC in CpG sites may significantly modulate the distribution of damage within the genome, and in cases where enhanced binding is observed may help to explain the existence of several mutational hot-spots found at CpG sites (5) that cannot be explained by endogenous mechanisms of mutagenesis, e.g., spontaneous deamination of 5mC.

In the present investigation an oligonucleotide containing the sequence 5′-CCG$_1$G$_2$AG$_3$G$_4$CCCA-3′ (with or without 5mC in the CpG site), which encompasses codons 248 (CG$_1$G$_2$) and 249 (AG$_3$G$_4$) of the *p53* gene, was reacted with two DNA damaging agents, dimethyl sulfate (DMS) or aflatoxin B$_1$-*8,9*-epoxide (AFB$_1$-*8,9*-epoxide). The third base of codon 249 (G$_4$) has a high frequency of mutation in hepatocellular carcinomas associated with AFB$_1$ exposure (10–12), and is also known to be frequently targeted by AFB$_1$-8,9-epoxide (13). The effect of CpG methylation status on the reactivity of the adjacent guanine toward the genotoxins was of interest, particularly since the CpG site in codon 248 has been shown to be methylated in every human tissue examined (14), and since bases within codon 248 are found to be mutated in a variety of human cancers (15). Additionally, unmethylated or methylated 0.5 kb fragments of the human *HPRT* gene were exposed to

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Abbreviations used: DMS, dimethyl sulfate; AFB$_1$-*8,9*-epoxide, aflatoxin B$_1$-*8,9*-epoxide; AFB$_1$, aflatoxin B$_1$; BPDE, benzo[*a*]pyrene diol epoxide; 5mC, 5-methylcytosine; MNU, *N*-methyl-*N*-nitrosourea; HPRT, hydroxyphosphoribosyl transferase; SAM, S-adenosylmethionine; PAGE, polyacrylamide gel electrophoresis.

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UV-activated aflatoxin B_1 to determine whether 5mC modulated adduct formation.

MATERIALS AND METHODS

Chemicals and reagents. 11-mer oligonucleotides containing sequences identical to hot-spot codons 248 and 249 in the *p53* gene were purchased along with their complementary strands from Genosys (The Lakes, TX), and were used without further purification. The oligomers consisted of the following sequences: 5′-C $\underline{CG_1G_2}$ $\underline{AG_3G_4}$ CCCA-3′ and 5′-C $^{Me}\underline{CG_1G_2}$ $\underline{AG_3G_4}$ CCCA-3′, where the underlined sequences represent codons 248 and 249, respectively.

Dimethyl sulfate (DMS) was obtained from Eastman Kodak Chemicals (Rochester, NY). Aflatoxin B_1-*8,9*-epoxide (AFB_1-*8,9*-epoxide) was synthesized using the method of Iyer and Harris (16); the final product was dissolved in acetone. Piperidine was obtained from Sigma and [γ-^{32}P]ATP (4500 Ci/mmol) was purchased from ICN Chemicals (Costa Mesa, CA). T4 DNA kinase was from GIBCO-BRL (Bethesda, MD). All other chemicals and reagents not explicitly stated were reagent grade or better.

5′-End labeling and duplex DNA formation. Approximately 500 ng each of the oligonucleotides defined above were 5′-end labeled with [γ-^{32}P]ATP and T4 kinase. Unincorporated [γ-^{32}P]ATP was removed by two successive ethanol precipitations [glycogen (150 μg/ml final concentration) was added as a carrier] and the labeled DNA washed twice with 70% cold ethanol followed by two washes of 100% ice-cold ethanol and dried in a vacuum centrifuge.

The two complementary oligonucleotides in a total volume of 20 μl of annealing buffer (40 mM Tris–HCl, pH 7.5; 20 mM $MgCl_2$; 50 mM NaCl) were annealed by heating at 70°C followed by cooling over 1 h to ~35°C. The annealed DNA was placed on ice (0°C) for ~1 h prior to carcinogen treatment.

Reaction of double-stranded DNA oligonucleotides with DMS and AFB_1-8,9-epoxide. 5′-End-labeled double-stranded oligonucleotides (~60 ng of DNA) were reacted with DMS (50 mM) or AFB_1-*8,9*-epoxide (100 μM) in separate microfuge tubes, in 10 mM sodium citrate buffer, pH 7.2 (final volume of 200 μl per reaction). Values in parentheses represent the final concentrations of each genotoxin per reaction. The final concentration of the solvents, acetone or dimethyl sulfoxide, did not exceed 10% (v/v). Reactions were conducted at 37°C for 1 h and were terminated by ethanol precipitation of DNA in the presence of glycogen. The oligonucleotides were incubated in 1 M piperidine (100–200 μl total volumes) at 90°C for 8 h to determine the extent of covalent binding by the genotoxins to target guanines. Following incubation, piperidine and salts were removed by two successive ethanol precipitations, and the final precipitate was washed twice with 70% ethanol followed by two washes with 100% ice-cold ethanol and dried *in vacuo.* The precipitate was resuspended in 20 μl of loading buffer [95%(v/v) formamide, 0.05%(w/v) xylene cyanol FF, 0.05%(w/v) bromophenol blue, 20 mM EDTA] and heated at 70–80°C for 2 min, then placed on ice. Aliquots (containing equal amounts of radioactivity) were loaded onto denaturing 20% polyacrylamide gels and fractionated. Autoradiographs of gels were prepared with intensifying screens overnight at room temperature. The extent of reaction at the target guanines (G_1, G_2, G_3, and G_4) in the oligonucleotides was determined by densitometric analysis of autoradiograms using the freeware program NIH Image version 1.60. Three separate experiments were done with similar results.

Reaction of EcoRI–PstI restriction fragment with photoactivated AFB_1. A 0.5-kb 5′-*Eco*RI–*Pst*I-3′ fragment derived from the 5′ untranscribed region of the *HPRT* gene was prepared and 3′-end labeled on the anti-sense strand, as previously described (Ref. 4, Fig. 1). A portion of the labeled fragment was treated with S-adenosylmethionine (SAM) and the enzyme *Sss*I to induce methylation of cytosines found at CpG sites, while another portion was treated similarly but without *S*-adenosylmethionine. Methylation of cytosines in CpG sites was demonstrated by the resistance of the restriction fragment to cleavage by methyl-sensitive restriction endonucleases, as described previously (4). Following precipitation of DNA, both SAM-treated and non-treated ^{32}P-labeled fragments were washed twice with 70% ethanol and resuspended in water at 25,000–50,000 cpm/μl prior to reaction with the indicated amounts of ultraviolet light-activated AFB_1. Reaction of the DNA fragments with photoactivated AFB_1 (7.5 and 37.5 μM) was conducted as described previously (17–19). Concentrations refer to amounts of parental AFB_1 that were activated by UV light. Reactions were terminated and subjected to piperidine hydrolysis, electrophoresis, autoradiography, and quantified as previously described (4).

RESULTS

The influence of 5mC on the reactivity of guanines in *p53* oligonucleotides toward DMS and AFB_1-*8,9*-epoxide was investigated. There appeared no significant change ($p \leq 0.05$) in the reactivity of G_1 toward AFB_1-*8,9*-epoxide when found in the context 5′-^{5m}CpG-3′, compared to the non-methylated oligomer (Fig. 1A, lane 3 versus lane 6; Fig. 1B). Additionally, adduction to other target guanines in the oligomer did not appear to be altered by the presence of the 5′-^{5m}CpG-3′ sequence. Similar results were obtained using 10 μM final concentration of AFB_1-epoxide instead of 100 μM (data not shown). The presence of 5mC in the oligomer also did not detectably alter the reaction of DMS with guanine (Fig. 1A, compare lane 2 with lane 5), consistent with what was previously observed (4).

In either oligonucleotide, G_2 reacted with AFB_1-epoxide to the greatest degree; in both cases the reactivity was approximately twofold greater than the reactivity of G_1 (Fig. 1B). However, from the empirical rules of the reactivity of AFB_1-*8,9*-epoxide with DNA (20), G_1 would be predicted to be more highly modified than G_2. Denissenko and co-workers (6) also observed an unexplained enhanced reactivity of the third guanine in codon 248 of *p53* toward BPDE that may be unique to that particular nucleotide and sequence context.

A sequencing gel of DNA fragments following reaction of photoactivated aflatoxin B_1 with 3′-end labeled fragments of the *HPRT* gene (containing either fully methylated CpG sites or nonmethylated CpG sites) indicated that aflatoxin B_1 binding to the poly-CpG region of the fragment (region between positions −360 and −405 of the fragment) was similar for both forms of DNA and that the presence of 5mC had little effect on guanine adduction (Fig. 2A). Three of the four most reactive guanines in the examined sequence were found in the sequence context 5′-C$\underline{G}$G-3′ (guanine positions: −366, −386, and −397). Visual inspection (lane 3 versus lane 4; lane 5 versus lane 6) and densitometric analysis of the autoradiograph (Fig. 2B) indicated that methylation of cytosine did not lead to an enhanced (or decreased) binding of AFB_1 to the nearest neighbor guanines. A similar lack of adduction modulation caused by 5mC was observed at less reactive

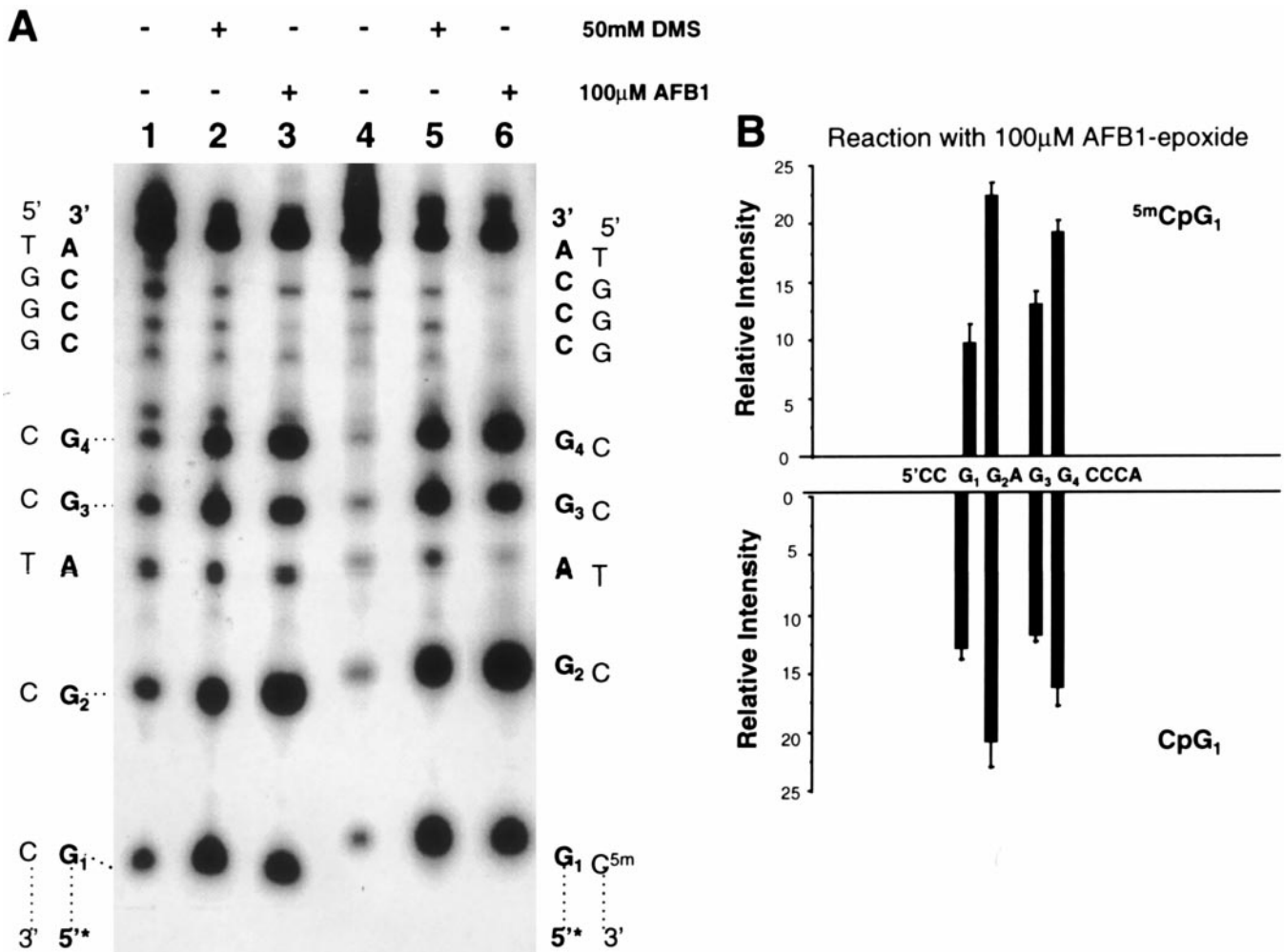

FIG. 1. Effect of 5-methylcytosine on the reactivity of *p53* oligonucleotides with AFB_1-*8,9*-epoxide and DMS. (A) Denaturing 20% PAGE of cleavage products following exposure of double-stranded 5′-end-labeled unmethylated and methylated oligomer to either DMS or AFB_1-*8,9*-epoxide and subsequent 1M piperidine incubation (8 h). Details of reaction conditions are given under Materials and Methods. Lanes 1–3: unmethylated oligomer, lane 1: control (no genotoxin treatment); lane 2: DMS (50 mM); lane 3: AFB_1-*8,9*-epoxide (100 μM). Lanes 4-6: methylated oligomer, lane 4: control (no carcinogen treatment); lane 5: DMS (50 mM); lane 6: AFB_1-*8,9*-epoxide (100 μM). The sequences of the oligonucleotides are indicated on the left and right side of the autoradiogram. The bold faced type refers to the oligonucleotide which is 5′-end labeled. The methylated cytosine is indicated by 5m in the unlabeled strand. Position of 5mC in labeled strand is not shown. Band intensity represents the extent of chemical modification at a particular sequence site. (B) Relative intensities of reaction at the indicated guanines toward AFB_1-*8,9*-epoxide in the two oligonucleotides (top graph refers to methylated oligomer, bottom graph refers to unmethylated oligomer), as determined by the normalized band density in each lane. The total amounts of radioactivity in each lane (as determined by band density on the autoradiograph) were summed and the total considered 100% of the radioactivity in each lane. The relative intensity of an individual band refers to the amount of radioactivity in that band divided by the total amount in the lane multiplied by 100. Error bars refer to the SEM of three independent experiments. No statistically significant differences in reaction intensity were seen at $p \leq 0.05$.

guanines found in the sequence context 5′-CGC-3′ (e.g., positions −369 and −371).

DISCUSSION

The mechanisms by which 5mC may alter the reactivity of its base paired guanine or its 3′ neighbor guanine, are unknown. One possibility suggested by Denissenko and co-workers (6) is that the presence of the methyl group at the C-5 position of cytosine increases the hydrophobicity of intercalation sites enhancing the non-covalent binding of agents that intercalate into DNA (e.g., BPDE). A second possibility is that the methyl group on 5mC (due to its polarizability) induces field effects that increases the *N2* nucleophilicity of its base paired guanine partner (9). This potential mechanism is consistent with the observed enhanced modification of guanines by both BPDE and mitomycin C, two agents that covalently react with the *N2* position of guanine (6, 9).

It was of interest to determine whether AFB_1-*8,9*-epoxide, another carcinogen well known to intercalate

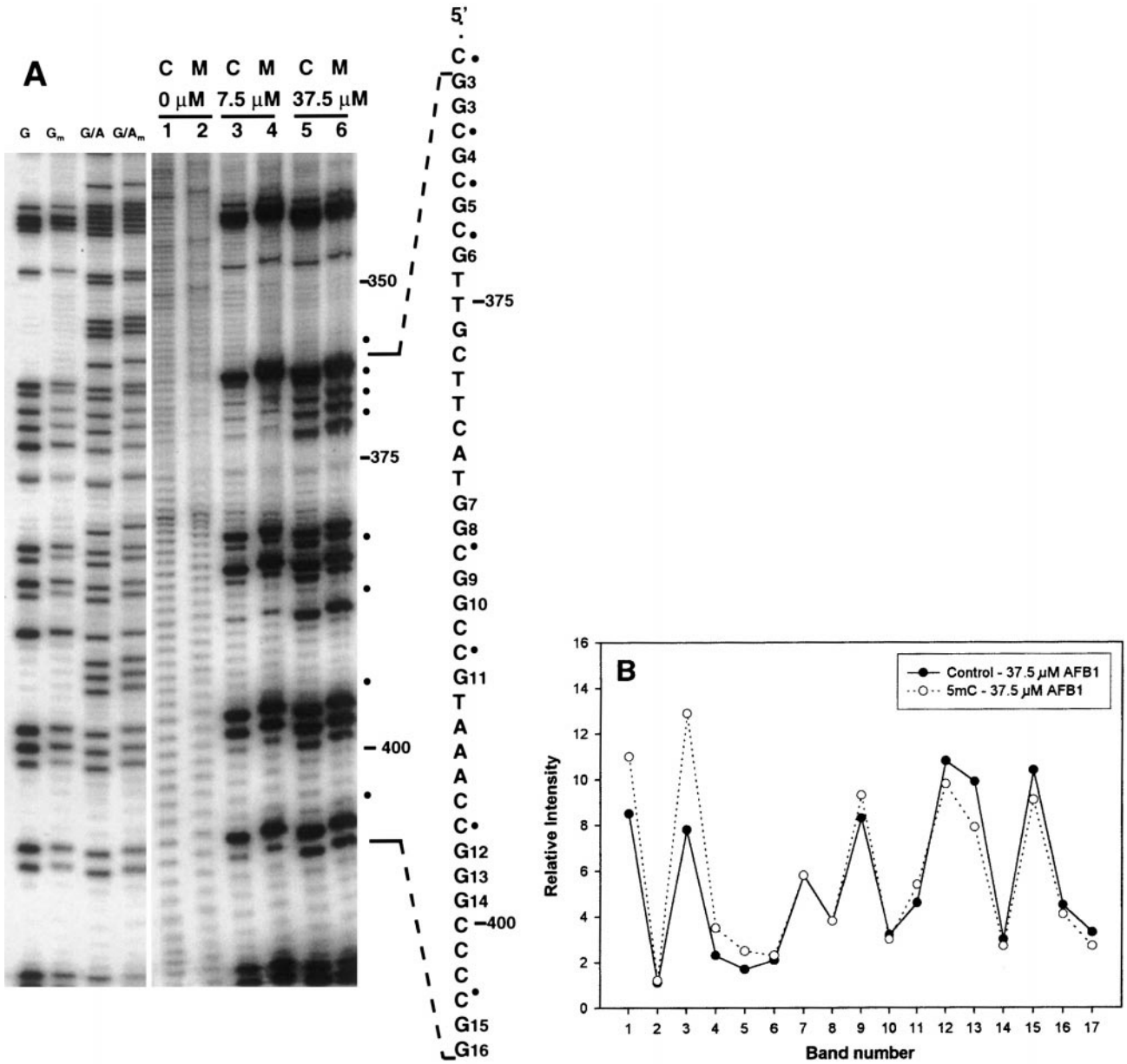

FIG. 2. (A) Sequencing gels for nonmethylated and methylated *HPRT* restriction fragments following reaction with photoactivated aflatoxin B_1. Chemical sequencing lane identification: G, DMS reaction for guanines in unmethylated fragment; G_m, DMS reaction for guanines in methylated fragment; G/A, formic acid reaction for adenine and guanine in unmethylated fragment, G/A_m formic acid reaction for adenine and guanine in methylated fragment. Lanes 1, 3, and 5: C, unmethylated control fragment; lanes 2, 4, and 6: M, methylated fragment. Lanes 1, 2: control (no AFB_1 treatment); lanes 3 and 4: 7.5 μM AFB_1; lanes 5 and 6: 37.5 μM AFB_1. DNA sequence examined is shown on the right side of the autoradiograph. Closed circles (●) indicate position of 5mC in methylated DNA. Numbers next to guanines in sequence refer to band number in densitometry graph. (B) Densitometry results of the relative intensities of each band in lanes 5 and 6 of the autoradiograph. Band intensities as a result of adduction by AFB_1 appear to coincide with one another. The relative intensity of band 3 is the contribution of two contiguous guanines that were not adequately resolved on the sequencing gel. This may account for the slight quantitative differences between control and methylated fragments at this position. Similar densitometry results were observed when lanes 3 and 4 were compared (data not shown).

into DNA (21), preferentially modifies guanines within methylated CpG sites. The lack of enhanced binding of AFB_1-*8,9*-epoxide to a fully methylated CpG site (observed in this study) in a sequence of DNA resembling the hot-spot codons 248 and 249 of the *p53* gene was not consistent with the hypothesis that the 5-methyl group of cytosine increases the hydrophobicity of an intercalation site and thereby enhances covalent binding of agents that intercalate into DNA. In addition to the work with *p53* oligonucleotides, previous work in

our laboratory had investigated the role of cytosine methylation on the reactivity of nearest neighboring guanines toward various genotoxic agents, e.g., MNU (4) and AFB_1-*8,9*-epoxide (this study), in a 0.5-kb fragment derived from the *HPRT* gene. Guanines located in specific ^{5m}CpG sites were found to be less reactive toward MNU with subsequent decreases in the amounts of *N7* methylation of guanine (4). Here it is shown that cytosine methylation did not alter the reactivity of guanine with aflatoxin B_1 in the same sequence. These data are consistent with those obtained in the study of the *p53* oligonucleotides that contained only a single CpG site.

These results suggested that AFB_1-*8,9*-epoxide does not preferentially modify guanines in methylated CpG sites. Enhanced hydrophobic effects caused by methylated CpG sequences is not likely to be a mechanism that can singly account for preferential targeting of methylated CpG sites by intercalative agents. Other factors may play a part in the previously described binding modulations (1–9) caused by 5mC. These include the sequence context around the CpG site; the chemical nature of the ultimate electrophile and its mechanism of reaction with DNA; and, as already described above, field effects that could alter the nucleophilicity of reactive atoms such as Gua-*N2* (9). Recently, it was purported that alkylation of the *N2* position of guanine found in CpG sites by mitomycin C was enhanced by cytosine methylation and diminished by 5-fluoro substitution of the cytosine (22). The C-5 substitutions apparently only influenced the reactivity of the base paired guanine in the opposing strand. These initial results support the argument that inductive effects (due to the electron-donating property of the 5-methyl group) enhances the nucleophilicity of the base paired *N2* atom of guanine (9). This may be a general mechanism for enhanced binding of drugs and carcinogens that react at the Gua-*N2* position. Our results describing a lack of enhanced binding of aflatoxin B_1 to the *N7* atom of guanine in a methylated CpG site is also consistent with such a mechanism, since inductive effects caused by the 5-methyl group of cytosine will not influence the ring *N7* position of guanine (9). In addition to aflatoxin B_1, DMS (which forms *N7*-methylguanine adducts) was also found to modify guanines in a methylated and unmethylated CpG site to the same extent (4, this study). The difference in reactivity of the S_N1-like MNU and the S_N2-like DMS at Gua-*N7* may be related to their different mechanisms of reaction.

It was reported recently that four bulky carcinogens, including aflatoxin B_1, preferentially bound to methylated CpG in *p53* mutational hot spots, including the CpG site found in codon 248, using a UvrABC incision method (7). One explanation for the apparent lack of enhancement found in our study may be the type of DNA target utilized. In one of our experiments a fragment of the regulatory region of the *HPRT* gene was utilized and the lack of enhanced AFB_1 binding to methylated CpG may reflect gene specific differences between *p53* and *HPRT*. Further, differences in local sequence context surrounding CpG sites may result in subtle differences in local structure. Indeed, in the reported study (7), not all CpG dinucleotides within *p53* showed the same enhancement for guanine alkylation. The regulatory region of the *HPRT* gene is CpG-rich and full methylation of such sites could potentially induce a B to Z transition as has been previously observed with poly(^{5m}CpG) DNA (23). Any potential positive influence on guanine alkylation that arises due to the increased hydrophobicity of methylated CpG (6, 24) may be offset by the induced Z-like local structure of the DNA which acts to reduce efficient non-covalent intercalation of AFB_1-*8,9*-epoxide (25). These self-canceling mechanisms may account for the observed lack of enhanced alkylation of guanine by AFB_1 in the current study. However, such an explanation falls short in explaining the similar lack of enhanced alkylation seen at the single CpG site in codon 248 within the 11-bp duplex, in contrast to the enhanced binding seen at the same site in the *p53* gene fragment of the Chen study (7). These apparently conflicting results may be related to the complex structures of the longer DNA fragment (secondary structures) as opposed to the simpler linear structure of the 11-bp duplex. Alternatively, end effects due to the proximity of G_1 to the 5′ end of the duplex may account for the observed differences. Additionally, while both methods of inducing strand breaks in DNA at the site of an *N7*-Gua bulky adduct (chemical treatment with piperidine vs UvrABC nucleases) are quantitative (26, 27), it cannot be ruled out that there may also exist assay-specific differences that account for these dissimilar results.

Regardless, the influence of 5mC in modulating the distribution of DNA damage by genotoxic agents will continue to be an active area of future investigations and the gene target and specific experimental assay utilized will be important considerations in such studies.

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REFERENCES

1. Sendowski, K., and Rajewsky, M. F. (1991) *Mutat. Res.* **250,** 153–160.
2. Pfeifer, G. P., Drouin, R., Riggs, A. D., and Holmquist, G. P. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 1374–1378.
3. Tommasi, S., Denissenko, M. F., and Pfeifer, G. P. (1997) *Cancer Res.* **57,** 4727–4730.
4. Mathison, B. H., Said, B., and Shank, R. C. (1993) *Carcinogenesis* **14,** 323–327.

5. Denissenko, M. F., Pao, A., Tang, M. S., and Pfeifer, G. P. (1996) *Science* **274,** 430–432.
6. Denissenko, M. F., Chen, J. X., Tang, M. S., and Pfeifer, G. P. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 3893–3898.
7. Chen, J. X., Zheng, Y., West, M., and Tang, M-S. (1998) *Cancer Res.* **58,** 2070–2075.
8. Mathur, P., Xu, J., and Dedon, P. C. (1997) *Biochemistry* **36,** 14868–14873.
9. Johnson, W. S., He, A. Y., and Tomasz, M. (1995) *Biorganic Med. Chem.* **3,** 851–860.
10. Ozturk, M. (1991) *Lancet* **338,** 1356–1359.
11. Aguilar, F., Hussain, S. P., and Cerutti, P. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 8586–8590.
12. Mace, K., Aguillar, F., Wang, J. S., Vautravers, P., Lechon, M. G., Gonzalez, F. J., Groopman, J., Harris, C. C., and Pfeifer, M. A. (1997) *Carcinogenesis* **18,** 1291–1297.
13. Puisieux, A., Lim, S., Groopman, J., and Ozturk, M. (1991) *Cancer Res.* **51,** 6185–6189.
14. Zingg, J.-M., and Jones, P. A. (1997) *Carcinogenesis* **18,** 869–882.
15. Hollstein, M., Sidransky, D., Vogelstein, B., and Harris,C. C. (1991) *Science* **253,** 49–53.
16. Iyer, R., and Harris, T. M. (1993) *Chem. Res. Toxicol.* **6,** 313–316.
17. Said, B., and Shank, R. C. (1991) *Nucleic Acids Res.* **19,** 1311–1316.
18. Shieh, J.-C., and Song, P.-S. (1980) *Cancer Res.* **40,** 689–695.
19. Shaulsky, G., Johnson, R. L., Shockor, J. P., Taylor, L. C. E., and Stark, A.-A. (1990) *Carcinogenesis* **11,** 519–527.
20. Benasutti, M., Ejadi, S., Whitlow, M. D., and Loechler, E. L. (1988) *Biochemistry* **27,** 472–481.
21. Iyer, R. S., Coles, B. F., Raney, K. D., Thier, R., Guengerich, F. P., and Harris, T. M. (1994) *J. Am. Chem. Soc.* **116,** 1603–1609.
22. Tang, K. S., Das, A., Waring, M. J., and Tomasz, M. (1998) *Proc. Am. Assoc. Cancer Res.* **39,** 1503.
23. Behe, M., and Felsenfeld, G. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 1619–1623.
24. Geacintov, N. E., Shahbaz, M., Ibanez, V., Moussaoui, K., and Harvey, R. G. (1988) *Biochemistry* **27,** 8380–8387.
25. Raney, V. M., Harris, T. M., and Stone, M. P. (1993) *Chem. Res. Toxicol.* 6, 64–68.
26. Kobertz, W. R., Wang, D., Wogan, G. N., and Essigmann, J. M. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 9579–9584.
27. Tang, M-s. (1996) *in* Technologies for Detection of DNA Damage and Mutation (Pfeifer, G., Ed.), pp. 139–153, Plenum Press, New York.