*Proc. Natl. Acad. Sci. USA*
Vol. 91, pp. 1261–1264, February 1994
Biochemistry

# Free radical adducts induce alterations in DNA cytosine methylation

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*Communicated by Robert L. Letsinger, October 4, 1993 (received for review May 14, 1993)*

**ABSTRACT** **Methylation of cytosines in DNA is important for the regulation of expression of many genes. During carcinogenesis, normal patterns of gene methylation can be altered. Oxygen radical injury, shown to damage DNA in a variety of ways associated with cancer development and other conditions, has been suggested to affect DNA methylation, but a mechanism has not been demonstrated. Using oligonucleotides containing the common oxygen radical adduct 8-hydroxyguanine to replace guanine, we found that the enzymatic methylation of adjacent cytosines is profoundly altered. Furthermore, there is a high degree of positional specificity with respect to this effect. Thus, free radical injury may explain some of the altered methylation observed during carcinogenesis.**

Endogenously generated reactive oxygen species, such as peroxides and oxygen free radicals, may play an important role in carcinogenesis (reviewed in refs. 1–3). These substances induce DNA strand breaks (4) and a number of specific types of adducts to DNA bases (1, 5, 6). These oxidants also produce a variety of other effects characteristic of carcinogens, including induction of malignant transformation in tissue culture (7, 8), chromosomal changes, mutations, and gene amplification. In addition, we recently observed alterations in specific DNA sequences in oxidant-transformed cells suggestive of altered cytosine methylation (9, 10). Cytosine methylation is a covalent modification of DNA that is important in gene regulation; it may have other functions as well (11–14). Most 5-methylcytosines occur at CpG sites. There is evidence that tissue-specific patterns of methylation are established during embryonic development and are faithfully maintained from cell generation to generation. In recent years, alterations in cytosine methylation, most commonly hypomethylation, have been associated with the development of cancer (15, 16). With the recognition that 8-hydroxyguanine is a common oxygen radical adduct of DNA (5, 6, 17–22), we constructed a model system to investigate the possible interrelationship between oxidants and DNA methylation. This model system consisted of a DNA methylase (*Hpa* II methylase), *S*-adenosylmethionine (as a source of methyl groups), and a series of synthetic deoxynucleotide oligomers containing complementary CCGG sites (the *Hpa* II methylase recognition site). We found that substitution of either of the guanines of the CCGG recognition site with 8-hydroxyguanine, a common oxygen radical-induced guanine derivative, dramatically altered binding of the methylase to the oligomer and could dramatically inhibit methylation. Thus, oxygen radical injury to DNA may influence gene expression by affecting DNA methylation.

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## MATERIALS AND METHODS

Four synthetic oligonucleotides containing 8-hydroxyguanine residues were kindly provided by Francis Johnson (23). Two of the four 8-hydroxyguanine-containing oligomers also contained 5-methylcytosines at the CpG site. Sequences of the oligomers were as follows:

A, 5′-GTACCCGGTGACACACC-3′;

B, 5′-GTACCCGGTGACACACC-3′;

C, 5′-GTACCCGGTGACACACC-3′;

D, 5′-GTACCCGGTGACACACC-3′

Complementary strands were synthesized, one unmethylated and one with the CpG cytosine methylated (designated U and M, respectively), in the Northwestern University Biotechnology Facility. Oligonucleotides A, B, C, and D were annealed independently with oligonucleotide U; oligonucleotides C and D were also independently annealed to oligonucleotide M (see Table 1).

For the kinetic experiments, methylation of cytosines was measured by quantifying incorporation of tritiated methyl groups into the oligonucleotide pairs. Various oligonucleotide pairs (1.0 μM) and 4.0 μM *S*-adenosyl-L-[*methyl*-$^3$H]methionine (Amersham; specific activity, 15 Ci/mmol; 1 Ci = 37 GBq) were preincubated at 37°C for 15 min in 50 mM Tris·HCl, pH 7.5/10 mM EDTA/5 mM 2-mercaptoethanol/200 μg of bovine serum albumin per ml, after which time *Hpa* II methylase (New England Biolabs; specific activity, $7.69 \times 10^{11}$ units/mol) was added to a final concentration of 52 nM. Final reaction volumes were 200 μl. Triplicate aliquots were removed from the incubation mixtures at various times and the incorporation of tritiated methyl groups into the oligonucleotides was determined by scintillation counting of trichloroacetic acid precipitates. Each experiment included an oligonucleotide pair without the *Hpa* II methylation site as a negative control, and the negative control values were subtracted from data, which was normalized with respect to a set of standard oligonucleotide values. Assays were performed within 4 days of annealing the oligonucleotides, and annealing was confirmed by autoradiography of $^{32}$P-end-labeled oligonucleotide pairs after gel electrophoresis.

$V_{max}$ and $k_{cat}$ values were obtained under conditions of substrate excess, as described above, as determined in preliminary experiments using a wide range of substrate oligonucleotide concentrations. *S*-Adenosyl-L-methionine was present at a concentration at which <5% of this substrate was consumed. Oligonucleotide concentrations were either 1.0 or 1.67 μM (both concentrations gave comparable results). The maximum initial velocity ($V_{max}$) values were derived from least-squares linear regression analysis (INPLOT 4.0; Graph-

Pad Software, San Diego) of the raw data collected over times when the curves were approximately linear. Catalytic rate constant ($k_{cat}$) values were calculated from the equation $V_{max} = k_{cat}[E]_0$, where $[E]_0$ is the initial enzyme concentration. The $K_m$ values reported were calculated by using standard Lineweaver–Burk plots.

For the experiments in which incorporation of methyl groups as a function of oligonucleotide concentration was studied, conditions were as follows: various concentrations of oligonucleotide pairs were incubated with 2 μM *S*-adenosyl-L-[*methyl*-$^3$H]methionine as described above, after which *Hpa* II methylase was added to a final concentration of 101 nM. Final reaction volume was 50 μl. Incubations continued for 3 hr, and incorporation of $^3$H was measured as described above.

Annealing temperatures were determined by combining equimolar amounts of paired oligonucleotides in 0.9 M sodium chloride/0.09 M sodium citrate, pH 7.0. The mixtures were placed in a Cary 219 spectrophotometer connected to a circulating water bath. The mixtures were incubated at 90°C for 5 min, after which they were allowed to cool to 20°C at a rate of ≈1°C/min. $A_{260}$ was recorded at every 1–3°C change in temperature. The midpoint of the declining curve of absorbance was designated as the annealing temperature. Melting curves were obtained by allowing the annealed mixtures to cool down and then the mixtures were heated back up at approximately the same rate as described above. The increase in $A_{260}$ was followed and the midpoint of the melting curve was designated the $t_m$.

Gel-retardation experiments were performed as follows: $1 \times 10^4$ cpm of [$\alpha$-$^{32}$P]dCTP-labeled oligonucleotides (specific activity, $7.1 \times 10^7$ cpm/μg) were incubated with increasing molar amounts of *Hpa* II methylase for 30 min at 37°C in a total vol of 30 μl containing 0.1 M Tris (pH 8.0), 10 mM EDTA, 10 mM dithiothreitol, 2 μg of poly(dI-dC)·poly(dI-dC) (Sigma), and 6 μg of bovine serum albumin. Assay mixtures were loaded onto 12% polyacrylamide gels and subjected to electrophoresis as described (24). DNA–protein complexes were localized by autoradiography.

## RESULTS AND DISCUSSION

Analysis of incorporation of tritiated methyl groups into annealed pairs of these oligonucleotides demonstrates that methylation of cytosines in DNA can be greatly influenced by hydroxyl free radical adducts on adjacent guanine residues. In addition, there is a large amount of positional specificity with respect to the magnitude of this effect. Fig. 1 illustrates that two of the constructs containing 8-hydroxyguanine, AU and DM (see Table 1), methylated reasonably well, while the four other experimental constructs BU, CU, DU, and CM, did not. Table 1 shows the structures of the respective oligonucleotide pairs, as well as the results of calculations of kinetic parameters of methylation obtained under conditions in which enzyme concentrations were fixed, and substrate oligonucleotide and *S*-adenosylmethionine concentrations were very high.

Additional methylation experiments were performed by using the four oligonucleotide pairs that methylated well under the conditions used in Fig. 1. Fig. 2 shows results of methylation measurements with oligonucleotide concentrations less than the $K_m$ where the velocity is very sensitive to changes in the substrate concentration and reflects differences in substrate affinity for the enzyme. These incubations were for 3 hr to achieve maximum methylation and assume no product inhibition (<5% of the substrate is consumed during the reaction). Under these conditions, as shown, the two control oligonucleotides (UU and UM) methylated much more efficiently than the hydroxyl-modified oligonucleotides. ($K_m$ with UU = 55 nM, DM = 242 nM, AU ≈ 96 nM.)

Fig. 3 shows results of gel-retardation assays using the four oligonucleotide pairs analyzed in Fig. 2. Interestingly, the relative intensity of the shifted bands roughly corresponds to the methylating activity shown in Fig. 2. Neither BU nor CU demonstrated gel shifts (data not shown). The compound sinefungin, an *S*-adenosylmethionine analog previously shown to stabilize binding of the *Eco*RI adenine methylase to DNA in mobility shifts (24), had no apparent effect on binding of the *Hpa* II methylase to DNA under our experimental conditions (data not shown).

In these studies, we chose to use the prokaryotic enzyme rather than the mammalian DNA methyltransferase for two reasons. First, the *Hpa* II methyltransferase is able to methylate efficiently short unmethylated oligonucleotides while the mammalian enzyme is not (25–27). Second, mammalian methyltransferase activity is highly sensitive to secondary structures induced in DNA by supercoiling or other changes (for example, left-handed Z-form DNA is not a substrate for the methyltransferase) (28). We were therefore concerned that concatamerization of the oligonucleotides required for their use as substrates for the mammalian methylase could have dramatically amplified any minor effects on secondary structure, since hydroxylated bases would occur at a frequency of >1/20 instead of the much rarer frequency observed in whole cells or tissue *in vivo* (17). Thus, we might have difficulty discriminating between the effects of overall changes in secondary structure and the effects of guanine hydroxylation on methylation at specific sites.

Having made the quantitative and kinetic observations reported here, we are now interested in pursuing more qualitative investigations of the methylation of specific sites in constructs where 8-hydroxyguanine moieties are inserted at single specific sequences in viral or plasmid genomes (29–31). Using such methods, Wood *et al.* (29) have shown protection from restriction enzyme cleavage by substitution of 8-hydroxyguanine instead of guanine at such a site.

DNA is subjected to a broad range of free radical and oxidative injuries *in vivo* (2, 5, 6, 19, 21). Despite the existence of specific glycosylases to remove bases injured by oxidative damage (32, 33), high levels of adducts such as 8-hydroxyguanine can persist in tumors or in tissues exposed to carcinogens or oxidative stress (17–21). These adducts have been shown to predispose to base mispairing and mutation (29, 31).

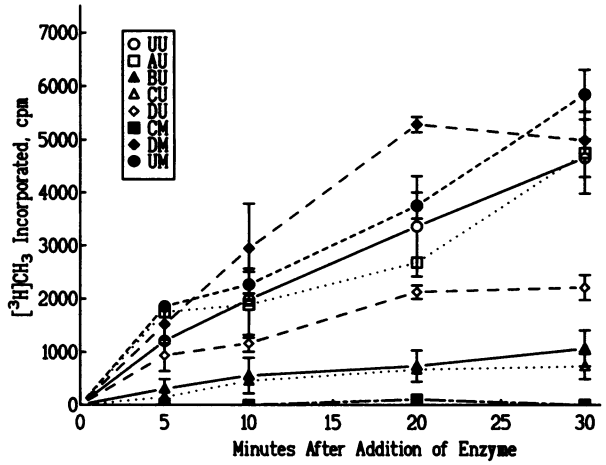


FIG. 1. Incorporation of methyl groups into oligonucleotide pairs as a function of time. Each point represents the mean ± SD of three to six determinations. The respective oligonucleotides are labeled as in Table 1.

Table 1. Oligonucleotide structures and kinetic values of methylation

| Oligonucleotide | Structure | $k_{cat}$, $min^{-1}$ | $V_{max}$, mol·liter$^{-1}$·min$^{-1}$ per mg of enzyme | Annealing temperature/$t_m$, °C |
|---|---|---|---|---|
| UU | C C G G<br>G G C C | 1.065 | $5.5 \times 10^{-8}$ | 64.3/80.0 |
| UM | C M C G G<br>G G C C | 1.345 | $6.99 \times 10^{-8}$ | 63.3/78.5 |
| AU | C M OH C G G<br>G G C C | 1.425 | $7.4 \times 10^{-8}$ | 58.0/77.8 |
| BU | C M OH C G G<br>G G C C | 0.13 | $0.6 \times 10^{-8}$ | |
| CU | C C OH G G<br>G G C C | 0.165 | $0.8 \times 10^{-8}$ | |
| DU | C C OH G G<br>G G C C | 0.255 | $1.3 \times 10^{-8}$ | |
| CM | C C OH G G<br>G G C C<br>M | 0.015 | $0.075 \times 10^{-8}$ | 52.5/not done |
| DM | C C OH G G<br>G G C C<br>M | 1.335 | $6.93 \times 10^{-8}$ | 51.5/68.7 |

Methylation assays, $V_{max}$, $k_{cat}$, annealing temperatures, and $t_m$ determinations were performed as described. Arrows indicate target cytosines for methylation.

The appearance of altered DNA methylation or hypomethylation during cancer development has been recognized for several years but remains poorly understood (15, 16). What does seem clear is that the presence or absence of 5-methylcytosines at specific sites can regulate binding of specific proteins to DNA and may influence gene expression (12, 13). Thus, carcinogen-induced hypomethylation might lead to expression of certain genes that are ordinarily methylated and unexpressed in specific tissues (for example, see ref. 34). Holliday (35) has suggested that, in addition to causing mutations, chemical carcinogens might also "act by altering the normal epigenetic controls of gene activity in specialized cells, and thereby produce aberrant heritable phenotypes." Our data suggest that oxygen free radicals might also be carcinogenic by both of these mechanisms.

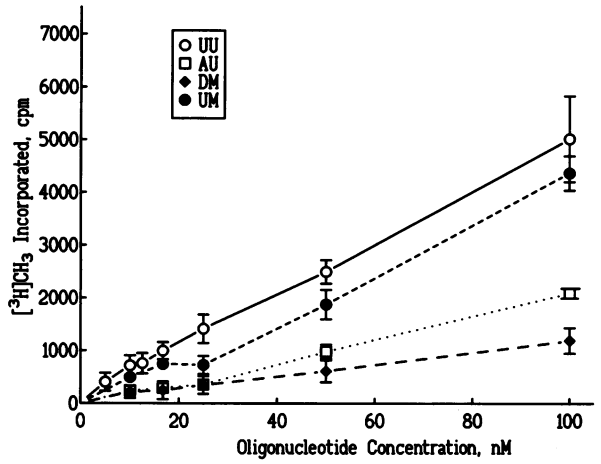


FIG. 2. Incorporation of methyl groups into oligonucleotide pairs as a function of oligonucleotide concentration. Each point represents the mean ± SD of three to six determinations. The respective oligonucleotides are labeled as in Table 1.

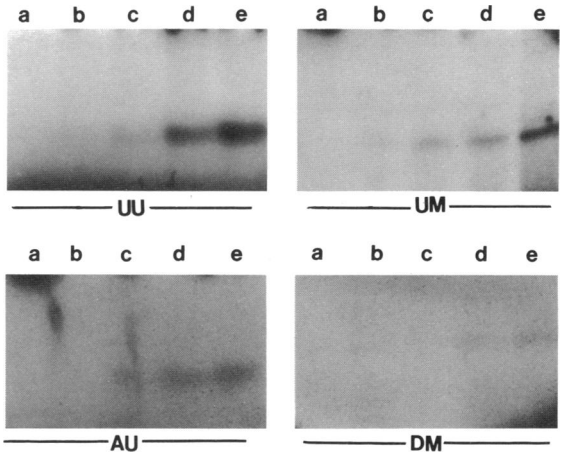


FIG. 3. Gel-retardation experiments: binding of methylase to radiolabeled oligonucleotides. Lanes a–e display reaction mixtures containing the following molar concentrations of *Hpa* II methylase: 0, $4.3 \times 10^{-8}$, $8.6 \times 10^{-8}$, $2.6 \times 10^{-7}$, and $4.3 \times 10^{-7}$, respectively. Exposure times of the autoradiographs for oligonucleotides AU and DM were four times longer than that required for UU and UM.

We thank Marie Lee for secretarial support. This work was supported by National Institutes of Health Grant RO1 AG11536.

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