Site-specifically modified oligodeoxynucleotides were used to investigate the mutagenic properties of a major cooked food mutagen-derived DNA adduct, N-(deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (dG-C8-PhIP). dG-C8-PhIP-modified oligodeoxynucleotides were prepared by reacting an oligodeoxynucleotide containing a single dG (5′-CTCTCCTGCTCTTC, where X = C, A, G, or T) with N-acetoxy-PhIP. The unmodified and dG-C8-PhIP-modified oligomers were inserted into single-stranded phagemid vectors. These single-stranded vectors were transfected into simian kidney (COS-7) cells. The progeny plasmid obtained was used to transform Escherichia coli DH10B. When dC was at the 5′-flanking position to dG-C8-PhIP, preferential incorporation of dCMP, the correct base, was observed opposite the dG-C8-PhIP. Targeted G → T transversions were detected, along with lesser amounts of G → A transitions and G → C transversions. No mutations were detected for the unmodified vector. The influence of sequence context on the dG-C8-PhIP mutation frequency and spectrum was also explored. When the dC 5′-flanking base was replaced by dT, dA, or dG, the mutational spectra were similar to that observed with dC-flanking base. Higher mutational frequencies (28–30%) were observed when dC or dG was 5′ to dG-C8-PhIP. A lower mutational frequency (13%) was observed when dA was at the 5′ to the lesion. Single-base deletions were detected only when dG or dT flanked the adduct. We conclude that dG-C8-PhIP is mutagenic, generating primarily G → T transversions in mammalian cells. The mutational frequency and specificity of dG-C8-PhIP vary depending on the neighboring sequence context.

Cooked meat contains potent mutagens and carcinogens, many of which are heterocyclic amines (1–6). 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is the most abundant heterocyclic amine formed during the cooking process (7–9) by the reaction of an amino acid with creatine (3, 4). PhIP was detected in the urine of human volunteers eating a normal diet (10–12). The average American public is exposed to 16–200 ng/kg/day of PhIP (6, 12). PhIP also is found in cigarette smoke condensate (13) and is a major mutagenic component recovered from the urine of cigarette smokers (14). This mutagen induces lymphomas in mice (15) and mammary, prostate, and colon carcinomas in rats (16, 17). Epidemiological studies indicate that heterocyclic amines, including PhIP, are linked to human colon cancer (18–20).

Treatment by PhIP produces several DNA adducts in tissues of animals (21, 22). To bind to DNA, PhIP is metabolically N-hydroxylated by hepatic microsomes, forming N-hydroxy-PhIP (23–26). Cytochrome P-450 1A1, 1A2, and 1B1 are involved in the N-hydroxylation (27–30). N-Hydroxy-PhIP is further converted to its ultimate carcinogen via O-sulfation or O-acetylation and binds covalently to DNA (14, 22, 31–34). N-Acetoxy-PhIP reacts only with 2′-deoxyguanosine (dG) residues in DNA, forming N′-(deoxyguanosin-8-yl)-PhIP (dg-C8-PhIP) (35, 36). Using 32P-postlabeling analysis, dG-C8-PhIP has been confirmed as a major DNA adduct in tissues of rats treated with PhIP (36).

Mutational events generated by PhIP have been observed primarily on GC pairs in the dhfr (37) and aprt (38) genes in Chinese hamster ovary cells, in the hgppt gene in Chinese hamster V79 cells (39), and in human lymphoblastoid cells (40). GC → TA transversions predominated, followed by GC → AT, GC → CG, and frameshift (deletion) mutations. Similar mutational spectra were detected in the lacI gene of the Big Blue mouse treated with PhIP (41) and in the APC gene in rat colon tumors induced by PhIP (42).

In this study, oligodeoxynucleotides containing a single dG-C8-PhIP were prepared post-synthetically and inserted into a single-stranded shuttle vector to minimize adduct repair (43). This vector was used to establish the mutagenic specificity and frequency of dG-C8-PhIP in simian kidney (COS-7) cells. In addition, to explore the effect of neighboring bases, mutational specificities were determined with dG-C8-PhIP embedded in different sequence contexts. Our results show that the dG-C8-PhIP-DNA adduct has significant mutagenic potential, generating primarily G → T transversions in mammalian cells. The mutational spectra and frequencies vary depending on the neighboring sequence context.

**EXPERIMENTAL PROCEDURES**

Materials—[γ-32P]ATP (specific activity, >6000 Ci/mmol) was obtained from Amersham Pharmacia Biotech. Escherichia coli DH10B was purchased from Life Technologies, Inc. COS-7 cell line was obtained from the tissue culture facility at SUNY at Stony Brook. EcoRI restriction endonuclease (100 units/μl) and T4 DNA ligase (400 units/μl) were obtained from New England Bio Labs.

Preparation of dG-C8-PhIP—N-Hydroxy-PhIP was synthesized by an established method (36). 1 mg of N-hydroxy-PhIP was dissolved in 420 μl of Me2SO/ethanol (v/v 4:1) and incubated for 5 min at 0 °C with 25 μl of acetic anhydride for the preparation of N-acetoxy-PhIP. 4G (1.5 mg) was reacted at 37 °C for 1 h with 30 μl of the preparation of N-acetoxy-PhIP in 500 μl of 10 mM potassium citrate buffer, pH 6.8. The

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reaction mixture was evaporated to dryness and subjected to HPLC. A Supelcosil LC-18S column (0.46 × 25 cm, Supelco, Inc.) was eluted with 50 mM ammonium formate, pH 3.5, containing 20% acetonitrile over 3 min, 20–50% over 5 min, 50% over 5 min, and subsequently 50–100% over 7 min at a flow rate of 0.8 ml/min. A Waters 990 HPLC instrument equipped with a photodiode array detector was used for isolation of dG-C8-PhIP.

Synthesis of Oligodeoxynucleotides—Unmodified 15-mer oligodeoxynucleotides (5'-TCCTCTXGGTGCTC, where X = C, A, T, or G) were prepared by solid-state synthesis, using an automated DNA synthesizer (44). A 15-mer oligodeoxynucleotide (150 μg) was incubated for 1 h at 37 °C with 50 μl of N-acetoxy-PhIP dissolved in 500 μl of 10 mM potassium citrate buffer, pH 6.8, concentrated on Centricon 3, and evaporated to dryness. The dG-C8-PhIP-modified oligomer was isolated from the unmodified oligomer on a Waters reverse-phase μBondapak C18 column (0.39 × 30 cm), using a linear gradient of 0.05 M triethylammonium acetate, pH 7.0, containing 10–20% or 10–30% acetonitrile with an elution time of 60 min and a flow rate of 1.0 ml/min, as described elsewhere (45). These oligomers were further purified by electrophoresis on 20% polyacrylamide gel in the presence of 7 M urea (35 × 42 × 0.04 cm) (46). The oligomers recovered from polyacrylamide...
gel electrophoresis were again subjected to HPLC to remove urea. **Enzymatic Digestion**—A 15-mer oligodeoxynucleotide (2.0 μg) was digested with nuclease P1 (2 units) and alkaline phosphatase (3 units) as described previously (46). Methanol extract obtained from the digested sample was evaporated to dryness and analyzed by HPLC as described under "Experimental Procedures." **RESULTS**

**Preparation of Oligodeoxynucleotides Containing a Single dG-C8-PhIP—** A 15-mer oligodeoxynucleotide (5'-TCCTCCTGCGCTCTTC, where X = C, A, T, or G) containing a single dG was reacted with a preparation of N-acetoxy-PhIP. HPLC systems were used to isolate the dG-C8-PhIP-modified 15-mer. For example, when dG was positioned at the 5'-flanking base (N), two modified 15-mers containing a single dG were isolated at 42.8 min (5'-G[PhIP]-G) and 46.4 min (5'-GG[PhIP]-) from the corresponding oligomer (tR = 26.3 min), as shown in Fig. 3A. Following treatment of 32P-labeled dG-C8-PhIP-modified oligomers by venom phosphodiesterase, the position modified by dG-C8-PhIP can be determined by blockage of the enzyme digestion (52). The first and second products represent oligomer containing 5'-G[PhIP]-G and 5'-GG[PhIP]-, respectively. The yield of dG-C8-PhIP-modified 15-mer was 1.0–4.5%. The dG-C8-PhIP-modified oligomers have UV absorbance at 300–420 nm. The UV maximum was 368 nm (Fig. 3B), similar to that of dG-C8-PhIP (data not shown). The modified oligomer was further purified by HPLC and gel electrophoresis. The migration of 32P-labeled dG-C8-PhIP-modified 15-mers was slower than that of the unmodified oligomer (data not shown). When the dG-C8-PhIP-modified 15-mer (5'-TCCTCCTGCG[PhIP]CCTCTTC) was digested enzymatically (46) and subjected to HPLC, dG-C8-PhIP was detected (Fig. 4). The molar ratio of dC and dT (9.1:5.0) was consistent with the theoretical value (dC:dT = 9:5).

**Preparation of Vectors Containing a dG-C8-PhIP—** Unmodified and dG-C8-PhIP-modified oligodeoxynucleotides were ligated into a gapped single-strand vector (Fig. 2). When a part of the ligation mixture was cleaved with Ban I and Hae III restriction enzymes and labeled with 32P, a 40-mer product was detected on 12% denaturing polyacrylamide gel electrophoresis (Fig. 5). The migration of 40-mers containing a dG-C8-PhIP was slower than that of the unmodified oligomer, as similarly observed for the dG-C8-PhIP-modified 15-mers. The final concentration of ss DNA vector was quantified by Southern blot hybridization. The S13 probe was hybridized to the ligation site of the ss vector (Fig. 2). Using a β-phosphorimager, the net radioactivity in the sample with that in known amounts of ss DNA was dissolved in distilled water. A portion of the ligation mixture and known amounts of ss pMS2 were subjected to electrophoresis on a 0.9% agarose gel to separate closed circular and linear ss DNA. DNA was transferred to a nylon membrane and hybridized to a 32P-labeled S13 probe complementary to DNA containing the 15-mer insert. The absolute amount of closed circular ss DNA was established by comparing the radioactivity in the sample with that in known amounts of ss DNA.

**COS-7 Cells**

COS-7 cells were transfected with ss DNA (100 fmol) over 18 h using Lipofectin (47), after which the cells were grown for 2 days in Dulbecco's modified Eagle's medium/10% fetal calf serum. Progeny plasmids were recovered by the method described by Hirt (48), treated with S1 nuclease to digest input ss DNA, and used to transform E. coli DH10B. Transformants were analyzed for mutations by oligodeoxynucleotide hybridization (49, 50). The oligodeoxynucleotide probes used to identify progeny phagemids are shown in Fig. 2. Probes L13 and R13 were used to select phagemids containing the correct insert. Transformants that failed to react with L13 and R13 were omitted from the analysis. When L13/R13-positive transformants failed to hybridize to the probes designed to detect events targeted to the lesion site, double-strand DNA was prepared and subjected to dideoxynucleotide sequencing analysis (51). **Mutational Specificity of dG-C8-PhIP—** When C was posi-
tioned 5' to dG-C8-PhIP, dG-C8-PhIP promoted preferential incorporation of dCMP (72.5%), the correct base, opposite the lesion (Table I). Targeted G → T transversions (24.4%) were detected, along with small number of G → A transitions (2.6%) and G → C transversions (0.5%). In addition, significant numbers of nontargeted mutations representing C → T transitions were observed opposite dC 5' to the dG-C8-PhIP lesion (Table I). No mutations were observed with the unmodified vector.

The influence of sequence context on the mutational specificity and frequency of dG-C8-PhIP was explored. When the 5'-flanking base was dT, targeted G → T base substitutions (14.4%) were observed along with G → A transitions (1.3%) and G → C (1.4%) mutations (Table I). In addition, a single-base deletion was detected. The mutational specificity was similar, and the mutational frequency was slightly less under these conditions than when dC flanked dG-C8-PhIP.

When dA was the 5'-flanking base, the mutational frequency was 2.2 times less than that observed when dC flanked dG-C8-PhIP (Table I). Preferential G → T mutations (11.4%) were observed along with G → A transitions (1.3%). No G → C mutations were detected.

Oligomers containing a dG-C8-PhIP lesion positioned at codon 60 or 61 of the noncoding strand of the human c-Ha-ras 1 gene was inserted into single-stranded vectors. When dG 5'

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**TABLE I**

*Mutational specificity of dG-PhIP adduct in mammalian cell*

Adducted ss DNA (100 fmol) was used to transfect COS-7 cells. Progeny phagemid was recovered and used to transform E. coli DH10B for mutation analysis.

| Plasmid | Number of targeted mutations (dG or dG-PhIP → X) | G | T | A | C | Δ | Othersa |
|---------|--------------------------------------------------|---|---|---|---|---|---------|
| 5'-CG-  | Exp. 1<sup>b</sup> | 161 | 0 | 0 | 0 | 0 | 0 |
|         | Exp. 2 | 159 | 0 | 0 | 0 | 0 | 0 |
|         | Total | 320 (100%) | 0 (<0.3%) | 0 (<0.3%) | 0 (<0.3%) | 0 (<0.3%) | 0 |
| 5'-CGPhIP- | Exp. 1<sup>b</sup> | 57 | 24 | 1 | 0 | 0 | 0 |
|         | Exp. 2 | 83 | 23 | 4 | 1 | 0 | 0 |
|         | Total | 140 (72.5%) | 47 (24.4%) | 5 (2.6%) | 1 (0.5%) | 0 (<0.5%) | 12 |
| 5'-TG-  | Exp. 1<sup>b</sup> | 74 | 0 | 0 | 0 | 0 | 0 |
|         | Exp. 2 | 80 | 0 | 0 | 0 | 0 | 0 |
|         | Total | 154 (100%) | 0 (<0.6%) | 0 (<0.6%) | 0 (<0.6%) | 0 (<0.6%) | 0 |
| 5'-TGPhIP- | Exp. 1<sup>b</sup> | 46 | 10 | 0 | 2 | 0 | 0 |
|         | Exp. 2 | 64 | 10 | 4 | 2 | 1 | 3 |
|         | Total | 110 (79.1%) | 20 (14.4%) | 6 (4.3%) | 2 (1.4%) | 1 (0.7%) | 10 |
| 5'-ΔG-  | Exp. 1<sup>b</sup> | 66 | 0 | 0 | 0 | 0 | 0 |
|         | Exp. 2 | 53 | 0 | 0 | 0 | 0 | 0 |
|         | Total | 119 (100%) | 0 (<0.8%) | 0 (<0.8%) | 0 (<0.8%) | 0 (<0.8%) | 2 |
| 5'-ΔGPhIP- | Exp. 1<sup>b</sup> | 72 | 8 | 2 | 0 | 0 | 0 |
|         | Exp. 2 | 66 | 10 | 0 | 0 | 0 | 9 |
|         | Total | 138 (87.3%) | 18 (11.4%) | 2 (1.3%) | 0 (<0.6%) | 0 (<0.6%) | 18 |

<sup>a</sup> Nontargeted mutations were: 5'-TCC(→3T/TC(→T)CTC(→9T,2A,GPhIP) CCTCTC 5'-TC(→A/C(→T/TCC(→A,T,Δ)/T→A,Δ)/T→2A,2Δ/G-<br/>

<sup>b</sup> Data of experiments (Exp.) 1 and 2 were obtained using independently prepared progeny phagemid.

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**FIG. 5. Construction and analysis of shuttle vector.** A portion of the vector annealed to the 61-mer scaffold was digested with BanI and HaeIII followed by exchange of the terminal phosphate residue using [γ-<sup>32</sup>P]ATP and T4 polynucleotide kinase and subjected to 12% dehydrating polyacrylamide gel electrophoresis as described under “Experimental Procedures.”
Mutagenesis of dG-C8-PhIP DNA Adduct

Mutational specificity of dG-PhIP adduct positioned at human C-Ha-ras 1 gene

Adducted as DNA (100 fmol) was used to transfect COS-7 cells. Progeny phagemid was recovered and used to transform E. coli DH10B for mutation analysis.

| Plasmid | Number of targeted mutations (dG or dG-PhIP → X) |
|---------|--------------------------------------------------|
|         | G | T | A | C | Δ¹ | Others* |
| 5'-TGG- | Exp. 1b | 139 | 0 | 0 | 0 | 0 | 0 |
|         | Exp. 2 | 62 | 0 | 0 | 0 | 0 | 0 |
|         | Exp. 3 | 92 | 0 | 0 | 0 | 0 | 0 |
|         | Total | 293 (100%) | 0 (<0.3%) | 0 (<0.3%) | 0 (<0.3%) | 0 (<0.3%) | 0 |
| 5'-TGPhIPG- | Exp. 1b | 57 | 3 | 0 | 2 | 0 | 2 |
|         | Exp. 2 | 58 | 7 | 0 | 0 | 1 | 1 |
|         | Total | 115 (89.8%) | 10 (7.8%) | 0 (<0.8%) | 2 (1.6%) | 1 (0.8%) | 3 |
| 5'-TGG | Exp. 1b | 139 | 0 | 0 | 0 | 0 | 0 |
|         | Exp. 2 | 62 | 0 | 0 | 0 | 0 | 0 |
|         | Exp. 3 | 92 | 0 | 0 | 0 | 0 | 0 |
|         | Total | 293 (100%) | 0 (<0.3%) | 0 (<0.3%) | 0 (<0.3%) | 0 (<0.3%) | 0 |
| 5'-TGPhIP- | Exp. 1b | 76 | 19 | 6 | 3 | 4 | 3 |
|         | Exp. 2 | 88 | 22 | 8 | 5 | 4 | 3 |
|         | Total | 164 (69.8%) | 41 (17.4%) | 14 (6.0%) | 8 (3.4%) | 8 (3.4%) | 6 |

* Nontargeted mutations were: 5'-TCCTCC(T→2AΔ¹)/TGPhIPG(-GCCTCTC) and 5'-TCCTCC(T→C,2T,2A)/GPhIPC(-ACCTCTC).

b Data of experiments (Exp.) 1 and 2 were obtained using independently prepared progeny phagemid.

A single-strand plasmid vector was used to explore the mutagenic property of dG-C8-PhIP replicating in COS-7 cells. Targeted G → T transversions were detected, along with a small number of G → A transitions and/or G → C transversion. Our mutational spectra are consistent with those observed in the Big Blue mouse treated with PhIP (41) and in the lacI gene of rat colon tumors induced by PhIP (42). Thus, the 5’ neighboring base influences the mutational frequency and specificity of dG-C8-PhIP.

We also determined the mutational properties of dG-C8-PhIP having the same 5’-flanking base but a different 3’-flanking base (5’-TGPhIPC- and 5’-TGPhIPG-). In both cases, G → T mutations predominated, along with a small number of G → C mutations and single-base deletions. However, no G → A transitions were produced when G 3’-flanking base was used. In addition, the mutational frequency of dG-C8-PhIP having C 3’ position was two times higher than that having G 3’ position. This indicates that the 3’-flanking sequence context also affects the mutational properties of dG-C8-PhIP.

A single-base frameshift (deletion) was observed in dGdC sequences, but not in dAdT sequences (37, 39, 40). Single-base deletions were frequently detected on the contiguous dGdC sequences of the APC gene (5’-GGA-) in rat colon tumors induced by PhIP (42) and in lacI gene (5’-GCCGC-) in colon of the

**Fig. 6.** Mutation spectra of dG-C8-PhIP embedded in different neighboring sequence context. Data are taken from Tables I and II.

**Fig. 7.** A proposed mechanism for one-base deletion.
Big Blue mouse treated with PhIP (41). These contiguous dGdC sequences were recognized as mutation hot spots for PhIP (41). In our in vivo studies using 5′-CGPhIP-C or 5′-dGPhIP-C sequence, no deletions were observed. However, when G was at the 5′-flanking base (5′-CGPhIP-C), significant amounts of single-base deletions were detected (Fig. 6). In addition, when T flanked 5′ to the dG-C8-PhIP (5′-TGPhIP-C or 5′-dGPhIP-C), a single-base deletion was also observed. Thus, the formation of single-base deletions may be influenced by the neighboring sequence context of dG-C8-PhIP. We have proposed a general framework deletion mechanism for chemical carcinogens, including dG-C8-AAF (53). Formation of single-base deletion induced by dG-C8-PhIP is consistent with this in vitro model. During DNA synthesis catalyzed by DNA polymerases on a dG-C8-PhIP-modified template, dCMP was preferentially inserted opposite dG-C8-PhIP, forming single-base deletions (Fig. 7, lowest energy structure, dG-C8-PhIP resides in the B-DNA model. During DNA synthesis catalyzed by DNA polymerases on a dG-C8-PhIP-modified template, dCMP was preferentially inserted opposite dG-C8-PhIP, forming single-base deletions (Fig. 7, lowest energy structure, dG-C8-PhIP resides in the B-DNA model). The mutagenic frequency varies depending on the neighboring sequence context to the dG-C8-PhIP position may influence the mutational frequency.

We conclude from this study that dG-C8-PhIP, a major PhIP-induced DNA adduct, is mutagenic lesion in mammalian cells. The mutagenic frequency varies depending on the neighboring sequence context to the lesion. This lesion may be involved in the development of human cancers including colon and breast cancers.

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