Translesional DNA Synthesis through a C8-Guanyl Adduct of 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in Vitro

REV1 INSERTS dC OPPOSITE THE LESION, AND DNA POLYMERASE κ POTENTIALLY CATALYZES EXTENSION REACTION FROM THE 3’-dC TERMINUS

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2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is the most abundant heterocyclic amine in cooked foods, and is both mutagenic and carcinogenic. It has been suspected that the carcinogenicity of PhIP is derived from its ability to form DNA adducts, principally dG-C8-PhIP. To shed further light on the molecular mechanisms underlying the induction of mutations by PhIP, in vitro DNA synthesis analyses were carried out using a dG-C8-PhIP-modified oligonucleotide template. In this template, the dG-C8-PhIP adduct was introduced into the second G of the TCC GGG AAC sequence located in the 5′ region. This represents one of the mutation hot spots in the rat Apc gene that is targeted by PhIP. Guanine deletions at this site in the Apc gene have been found to be preferentially induced by PhIP in rat colon tumors. DNA synthesis with A- or B-family DNA polymerases, such as Escherichia coli polymerase (pol) I and human pol δ, was completely blocked at the adducted guanine base. Translesional synthesis polymerases of the Y-family, pol η, pol ι, pol κ, and REV1, were also used for in vitro DNA synthesis analyses with the same templates. REV1, pol η, and pol κ were able to insert dCTP opposite dG-C8-PhIP, although the efficiencies for pol η and pol κ were low. pol κ was also able to catalyze the extension reaction from the dC opposite dG-C8-PhIP, during which it often skipped over one dG of the triple dG sequence on the template. This slippage probably leads to the single dG base deletion in colon tumors.

Heterocyclic amines (HCAs) are naturally occurring genotoxic carcinogens produced from cooking meat (1). The initial carcinogenic event induced by HCAs is metabolic activation and subsequent covalent bond formation with DNA (1, 2). 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is the most abundant heterocyclic amine in cooked foods, and was isolated from fried ground beef (3, 4). PhIP possesses both mutagenic and carcinogenic properties (5–8). Epidemiological studies have revealed that a positive correlation exists between PhIP exposure and mammary cancer incidence (9). PhIP induces colon and prostate cancers in male rats and breast cancer in female rats (8, 10).

The incidences of colon, prostate, and breast cancers are steadily increasing in Japan and other countries and this has been found to correlate with a more Westernized lifestyle. Elucidating the molecular mechanisms underlying PhIP-induced mutations is therefore of considerable interest. It is suspected that the carcinogenicity of PhIP is derived from the formation of DNA adducts, principally dG-C8-PhIP (11–14) (see Fig. 1). Studies of the mutation spectrum of PhIP in mammalian cultured cells and transgenic animals have revealed that G to T transversions are predominant and that guanine deletions from G stretches, especially from the 5′-GGGA-3′ sequence, are significant (15–20). Five mutations in the Apc gene were detected in four of eight PhIP-induced rat colon tumors, and all of these mutations involved a single base deletion of guanine from 5′-GGGA-3′ (21). These mutation spectra are thought to be influenced by various factors, including the primary structure of the target gene itself, the capacity of translesional DNA polymerases, and the activity level of repair enzymes (1). However, the molecular mechanisms underlying the formation of PhIP-induced mutations are largely unknown.

To shed further light on the molecular processes that underpin the mutations induced by PhIP, we performed in vitro DNA synthesis analyses using a dG-C8-PhIP-modified oligonucleotide template. We have recently reported the successful synthesis of oligonucleotides harboring a site-specific PhIP adduct.

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‡ The abbreviations used are: HCA, heterocyclic amines; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; TLS, translesional DNA synthesis; IQ, 2-amino-3-methylimidazo[4,5-f]quinoline; pol, DNA polymerase; DTT, dithiothreitol; PCNA, proliferating cell nuclear antigen; PIPES, 1,4-piperazinediethanesulfonic acid.
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(22). In our current study, we used this synthesis method to construct a 32-mer oligonucleotide template containing a 5’-TTCGGGAAC-3’ sequence with different site-specific PhIP adducts. We then utilized the resulting constructs in DNA synthesis analyses to reconstitute the PhIP-induced mutagenesis of the rat APC gene. DNA synthesis reactions with A- or B-family DNA polymerases, such as Escherichia coli pol I and human pol δ, or translesional synthesis (TLS) polymerases of the Y-family, pol η, pol ι, pol κ, and REV1, were carried out. Kinetic analyses of pol κ and REV1, for which TLS activities at the PhIP adduct were detected, were also performed.

EXPERIMENTAL PROCEDURES

Enzymes and Materials—T4 polynucleotide kinase and T4 DNA ligase were purchased from Toyobo Biochem (Osaka, Japan) and Takara Biotech (Tokyo, Japan), respectively. Other materials were obtained from Sigma or Wako (Osaka, Japan).

DNA Polymerases and PCNA—Human recombinant DNA polymerases, pol δ, pol η, pol κ, and REV1, and PCNA were expressed and purified as described previously (23–27). Human DNA polymerase α and DNA polymerase ι were purchased from Chimerx. E. coli DNA polymerases I (Takara Biotech) and Klenow Fragment (Takara Biotech), and thermophilic bacterial DNA polymerases, Taq (Toyobo Biochem) and Thd (Toyobo Biochem) were used.

Oligonucleotides—The method used to chemically synthesize three 9-mer oligonucleotides, 5’-TTCGGGAAC-3’, containing a PhIP adduct on either the first, second, or third G (p9B, p9C, and p9D, respectively) has been described previously (22). All other synthetic oligonucleotides were synthesized and purified using a reverse-phase cartridge (Operon Biotech Japan (Tokyo, Japan). The 23-mer oligonucleotides: p23a, 5’-TGAC-TCGTCTGACTGGGAAC-3’, and p23b, 5’-GTACCGACTGGGAAC-3’, were used for constructing the template oligonucleotides as described below. A 32-mer oligonucleotide without the PhIP adduct, p32A, was used as a control template (see Table 1). Its 3’ complementary 29-, 28-, 27-, 26-, 22-, and 17-mer sequences (p29, p28, p27, p26, p22, and p17) were used as extension primers (see Table 1).

Construction of Template-Primer Complexes Containing the PhIP Adduct—A 32-mer template oligonucleotide p32C (see Table 1) was constructed by ligation of p9C with p23a as follows. The 5’-end of p23a was phosphorylated by T4 polynucleotide kinase and ATP. A mixture of p9C, p23a, and p23b (3 nmol each) in 250 μl of a buffer containing 5 mm Tris-HCl, 0.5 mm EDTA, 50 mm NaCl, pH 8.0, was denatured for 5 min at 95 °C, incubated for 10 min at 60 °C, and then cooled slowly to form the partial duplex structure of these three oligonucleotides (supplemental Fig. S1). The sample of the duplex oligonucleotide was mixed with 190 μl of Milli-Q water and 50 μl of ×10 ligation buffer (500 mm Tris-HCl (pH 7.5), 100 mm MgCl₂, 100 mm DTT, 10 mm ATP). Ligation was initiated by adding 10 μl of T4 DNA ligase (4.000 units), and the mixture was then incubated for 20 h at 16 °C. An additional incubation at 37 °C for 60 min was carried out after the addition of 1 μl of T4 DNA ligase, and the reaction was stopped by further incubation at 68 °C for 10 min. The p32C was separated by 18% PAGE containing 8 m urea, and excised and eluted as described previously (28). p32B and p32D were constructed using a similar method as for p9B and p9D, respectively (see Table 1). The purities of these oligonucleotides, p32B, p32C, and p32D, were determined by denatured PAGE after 5’-end labeling and UV absorbance at 260 and 370 nm.

Primer oligonucleotides were labeled with 32P at the 5’-end as described previously (29), and then purified by MicroSpin™ G-25 or G-50 columns (GE Healthcare) as recommended by the supplier. The mixture of template and labeled primer (50 pmol each) in 400 μl of a buffer containing 8 mm Tris-HCl, 0.8 mm EDTA, 150 mm KCl (pH 8.0) was heated at 70 °C for 7 min, and then cooled slowly to room temperature. In the case of the substrates for TLS polymerases, pol η, pol ι, pol κ, and REV1, the final concentrations of template-primer and the constituents of the annealing buffers were changed to 500 nm and 10 mm Tris-HCl, 1 mm EDTA, and 50 mm NaCl (pH 8.0), respectively.

In Vitro DNA Synthesis Assay—A primer extension reaction was performed as described previously (30) with some modifications. Briefly, an aliquot of 0.75 μl of this primer-annealed template (final concentration, 12.5 nm) was mixed with 0.75 μl of ×10 Klenow buffer (100 mm Tris-HCl (pH 7.5), 70 mm MgCl₂, 1 mm DTT), 0.5 μl of 500 mm KCl, 0.5 μl of dNTP mixture (50 μM each), and 4.5 μl of Milli-Q water. After addition of 0.5 μl of Klenow fragment, the mixture was incubated at 37 °C for 10 min. The reaction was terminated by adding 1.5 μl of stop solution (160 mm EDTA, 0.7% SDS, 6 mg/ml proteinase K), and the samples were incubated at 37 °C for 30 min. Subsequently, 5.5 μl of the gel loading solution (30 mm EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol, 97% formamide) was added to the samples. For pol δ, a ×10 reaction buffer containing 200 mm PIPES (pH 6.8), 20 mm MgCl₂, 10 mm 2-mercaptoethanol, 200 μg/ml bovine serum albumin, and 50% glycerol was used instead of the buffer described above, and the reaction was carried out at 37 °C for 10 min. For other DNA polymerases, pol α, pol ι, rTaq, and Thd, the constituent of each ×10 reaction buffer was altered as recommended by the suppliers.

The reaction using pol κ was performed as described above with some modifications. Briefly, an aliquot of 0.5 μl of this primer-annealed template (final 50 nm) was mixed with 0.5 μl of 10 × TLS buffer (250 mm Tris-HCl (pH 7.0), 50 mm MgCl₂, 50 mm DTT, 1 mg/ml bovine serum albumin), 0.5 μl of dNTP solution, and 3.0 μl of Milli-Q water. After addition of 0.5 μl of pol κ, the mixture was incubated at 30 °C for 20 min. The reac-
tion was terminated by adding 8.8 μl of the gel loading solution and a further incubation at 95 °C for 3 min. The reaction of REV1 was performed in the same manner as the reaction of pol κ with the exception that the standard reaction time was 5 min. For pol η, a × 10 reaction buffer containing 400 mM Tris-HCl (pH 8.0), 10 mM MgCl2, 100 mM DTT, 1 mg/ml bovine serum albumin, and 450 mM KCl was used instead of the ×10 TLS buffer. The 32P-labeled fragments were denatured and electrophoresed in a 9.5% polyacrylamide gel containing 8 M urea. The radioactivity of the fragments was determined using a Bio-Imaging Analyzer (BAS2500, Fuji Photo Film, Kanagawa, Japan). Kinetic parameters were determined by steady-state gel kinetic assays under similar conditions as described above. The incubation time for pol κ was changed to 10 min. K_m and k_cat were evaluated from the plot of the initial velocity versus the dCTP or dGTP concentration using a hyperbolic curve-fitting program in SigmaPlot 11 (Systat Software, Inc.). Data from two or three independent experiments were plotted together.

RESULTS

Construction of Template Oligonucleotides Containing a PhIP Adduct—We designed oligonucleotides containing a dG-C8-PhIP adduct at specific sites for use as templates in in vitro DNA synthesis analyses. For this purpose, we selected the 5’-TCCGGGAAC-3’ sequence as: 1) it corresponds to codon 868–870 of the rat Apc gene, one of three mutation hot spots (a single base deletion of G) in PhIP-induced colon tumors (21), and could thus be used as a model template that would reconstitute mutations of this gene; 2) two other mutation hot spots in the rat Apc gene and many mutated sites induced by PhIP in cultured cells and animal models contain 5’-GGGA to GGA mutations to some extent; and 3) some mutagenic compounds forming dG adducts, including PhIP, are expected to react preferentially with the 5’-G of a GG dinucleotide site when compared with a single G residue (31). We thus selected a sequence containing GGG as a template for our initial analysis.

We have recently synthesized three 9-mer oligonucleotides separately harboring a PhIP adduct on each G within the sequence 5’-TCCGGGAAC-3’ (22). Three 32-mer template oligonucleotides, p32A, p32B, and p32D, were constructed in our present study by ligation of these 9-mer oligonucleotides containing the dG-PhIP adduct with a 23-mer oligonucleotide, p23a, (Table 1 and supplemental Fig. S1). The purities of these oligonucleotides were tested after resolution by electrophoresis. In our present study, we principally describe the results of our in vitro DNA synthesis analysis using p32C as the template to avoid complexity.

In Vitro DNA Synthesis by A- and B-family DNA Polymerase—Many of the chemical compounds that can form DNA adducts in vivo and that show mutagenicity have been reported to impede the progress of DNA synthesis to different extents. The molecular size of PhIP is greater than most other mutagenic chemicals that form adducts. Hence, dG-PhIP was expected to block DNA synthesis to a considerable extent. To examine the effects of the dG-C8-PhIP adduct upon DNA synthesis, primer extension experiments using p32B, p32C, and p32D as templates were carried out (see Table 1). The length of each produced fragment was precisely determined using ladders of oligonucleotide fragments as markers (data not shown). The Klenow fragment of E. coli DNA polymerase I, a member of the A-family DNA polymerases, was first used in this analysis. The production of a 28-, 27-, and 26-mer from these primer-template complexes was 12.5 min. Concentrations of Klenow fragment were 0 (lane 1), 7.8 (lane 2), 23 (lane 3), and 78 units/ml (lane 4).
Finally, DNA synthesis analyses with human DNA polymerase δ (pol δ), a member of the B-family DNA polymerases and a truly replicative polymerase, were carried out. In the case of using p32C and p17 (C-p17) as a template-primer complex, the production of 27-mer fragments indicated the stalling of pol δ just before the PhIP adduct (Fig. 3, lane 11). From a control reaction using A-p17, a template-primer complex without the PhIP adduct, a full-length product of 32-mer was generated (Fig. 3, lane 8). In addition to these major products, minor products extended one nucleotide further (28- and 33-mer) and ladders of bands indicating degradation of primer (<17-mer) were observed (Fig. 3), corresponding with previous results reporting terminal dA transferase and exonuclease activities of pol δ (32).

PCNA, an accessory protein acting as a sliding clamp for pol δ, was previously reported to promote DNA synthesis by pol δ past several template lesions, including abasic sites, 8-oxo-dG, and aminofluorene-dG (32). In the case of dG-C8-PhIP, however, PCNA was unable to promote the bypass synthesis of pol δ beyond the lesion (Fig. 3, lane 12). Extension reaction from the longer 22-mer primer, p22, also paused completely just before the PhIP adduct in the presence or absence of PCNA (Fig. 3, lanes 5 and 6). These results strongly suggest that the dG-C8-PhIP adduct on genome DNA in the living cells induces the complete block of replication forks including pol δ, PCNA, and pol α.

**Translesional DNA Synthesis by Y-family DNA Polymerases**—Translesional DNA synthesis at the dG-C8-PhIP adduct by the Y-family DNA polymerases, pol η, pol κ, pol λ, and REV1 was next examined. Two substrates, C-p27 and C-p28, and their counterparts without a PhIP adduct, A-p27 and A-p28, were used in these experiments (Fig. 4). Substrate C-p27 was prepared by annealing the p32C template (see Table 1) to its 3’-complimentary 27-mer sequence, p27, and was used to identify the nucleotides that are inserted opposite the dG-C8-PhIP adduct (Fig. 4). Similarly, substrate C-p28 was used to analyze the extension reaction from the 3’-end of the dC bases opposite the dG-C8-PhIP adduct (Fig. 4). We found that recombinant human DNA polymerase η (pol η) could insert a dC opposite the dG-C8-PhIP adduct, although at low efficiency compared with control experiments without the PhIP adduct (Fig. 5, A and B). Extension reactions catalyzed by pol η from the 3’-end of dC opposite the adduct were barely detectable (Fig. 5D), although an excessive amount of pol η produced byproducts that incorporated a mismatch nucleotide, dG, dA, or dT (supplemental Fig. S4). In the case of dG, incorporation of one to three dG nucleotides was observed (supplemental Fig. S4). In control experiments without the PhIP adduct, minor products were produced that incorporated mismatch nucleotides, in addition to a major product that incorporated a dC (Fig. 5C).
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FIGURE 6. Translesional DNA synthesis by pol κ using substrates C-p27 and C-p28. Control reactions were performed using substrates without the PhIP adduct, A-p27 (A) and A-p28 (C). An insertion reaction was performed with substrate C-p27 (B) and an extension reaction with substrate C-p28 (D). A single dNTP (G, A, T, or C) was added into the reaction mixture as indicated by G, A, T, and C above each lane. The lanes indicated by − are controls without any nucleotides. The concentrations of pol κ were 250 (A and C), 500 (B), and 1000 nm (D), respectively. The concentration of each dNTP was 100 μM.

FIGURE 7. Translesional DNA synthesis by REV1 using substrates C-p27 and C-p28. Control reactions were performed using substrates without the PhIP adduct, A-p27 (A) and A-p28 (D). Insertion reactions were performed with substrate C-p27 (B and C) and an extension reaction with substrate C-p28 (E). A single dNTP (G, A, T, and C) or a mixture of each was added into the reaction mixture as indicated by G, A, T, and C above each lane. The lanes indicated by − are controls without any nucleotides. The concentrations of each dNTP were 100 μM (A, B, D, and E) and 320 μM (C), respectively. The N mixture contained each dNTP at a concentration of 25 μM.

We next examined translesional DNA synthesis beyond the PhIP adduct using a truncated form of human DNA polymerase κ containing the N-terminal 559 amino acids. One or two dCs were inserted opposite the dG-C8-PhIP adduct by this polymerase, and misinsertions of three other nucleotides were also observed to a certain extent (Fig. 6B). pol κ incorporated two dCs and misincorporated dG, dA, and dT into the A-p27 substrate without the PhIP adduct at a low efficiency (Fig. 6A). Misincorporations of dG, dA, and dT into the A-p28 substrate without the adduct were also observed (Fig. 6C). In the case of the extension reaction from 3′-dC opposite the dG-PhIP adduct, pol κ also incorporated dC and misincorporated dT into the C-p28 substrate at low efficiency (Fig. 6D). Interestingly, one- and two-base incorporations of dG into the substrate C-p28 by pol κ dominated the incorporation of a dC (Fig. 6D). In the extension reaction with pol κ in the presence of all four dNTPs, fragments of 29 and 30 nucleotides were observed as major products, and a small amount of the 31-nucleotide fragment was observed (see supplemental Fig. S5, lane 6). Full-length products of 32 nucleotides were observed only when an excess amount of pol κ was present (data not shown). This poor extension activity of pol κ after adding two nucleotides was probably caused by the shortness (~4 nucleotides) of the 5′ region to the lesion in the template oligonucleotide. Extension with pol κ, pol η, and pol δ from the mismatched primers, where the 3′-terminal nucleotide of the p28 primer, dC, was substituted with another nucleotide, could not be observed (data not shown). REV1 inserted a dC opposite the PhIP adduct dC insertion into C-p28 (0.039 min⁻¹ mm⁻¹) was found to be 4-fold greater than that into C-p27 (0.011 min⁻¹ mm⁻¹). These results indicate that pol κ catalyzes the extension reaction from the 3′-terminal of dC opposite the dG-C8-PhIP with a higher efficiency than the insertion reaction opposite the adduct. The k_cat/K_m values of the dC insertion opposite the adduct were roughly 4 orders of magnitude less than those into counterparts without the adduct (see Table 2). The k_cat/K_m value of the dG incorporation into C-p28 was slightly higher than that of dC, and more than 8-fold higher than that of dG into C-p27 (see Table 2). This result indicates that pol κ skipped over the dG site just 5′ of dG-C8-PhIP on the template and incorporated dG opposite dC on the template strand of C-p28 with a high efficiency. The k_cat/K_m values of the dC incorporation into D-p27 (0.19 min⁻¹ mm⁻¹) were over 4-fold greater than into C-p28 (0.039 min⁻¹ mm⁻¹) and over 8-fold higher than that of dG into B-p29 (0.023) (see supplemental Table S1). These data indicate that the efficiencies of the extension reaction by pol κ are the highest for template p32D containing the PhIP adduct in the third G of the triple G run, next for template p32C containing the PhIP/adduct in the second G, and lowest for template p32B containing the PhIP adduct in the first G.

Even at higher concentrations of dNTPs, extension reactions catalyzed by REV1 for substrate C-p28 could not be monitored (Table 3, Fig. 7E). The k_cat/K_m value of the dC incorporation by REV1 into substrate C-p27 was more than 2,000 times greater than that by pol κ, and 1/44 of the values for counterparts with-
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**TABLE 2**

$k_{cat}/K_m$ values for pol κ

| Substrate | $K_m$ (μM) | $k_{cat}$ (×10^3 min⁻¹) | $k_{cat}/K_m$ (min⁻¹ μM⁻¹) |
|-----------|------------|--------------------------|-----------------------------|
| C-p27     | dCTP       | 70                       | 0.76                        | 0.011                       |
|           | dGTP       | 47                       | 0.24                        | 0.0050                      |
| C-p28     | dCTP       | 8.0                      | 0.32                        | 0.039                       |
|           | dGTP       | 11                       | 0.48                        | 0.042                       |
| A-p27     | dCTP       | 0.035                    | 4.4                         | 130                         |
|           | dGTP       | 0.26                     | 1.3                         | 5.0                         |
| A-p28     | dCTP       | 0.027                    | 3.7                         | 140                         |
|           | dGTP       | 2.1                      | 8.8                         | 4.1                         |

**TABLE 3**

$k_{cat}/K_m$ values for dCTP-insertion by REV1

| Substrate | $K_m$ (μM) | $k_{cat}$ (×10^3 min⁻¹) | $k_{cat}/K_m$ (min⁻¹ μM⁻¹) |
|-----------|------------|--------------------------|-----------------------------|
| C-p27     | 12         | 320                      | 27                          |
| C-p28     | ND*        | ND                       | ND                          |
| A-p27     | 0.36       | 390                      | 1100                        |

* ND, not detectable.

FIGURE 8. Translesional DNA synthesis by pol κ. Nucleotide incorporation by pol κ for substrates C-p27 (A) and C-p28 (B). Either dCTP (lanes 2-5) or dGTP (lanes 6-9) was added into the reaction mixture. Lane 1 indicates a control without any nucleotides. The concentration of pol κ was 910 nM. The concentrations of dCTP or dGTP, respectively, were 25 (lanes 2 and 6), 12.5 (lanes 3 and 7), 6.25 (lanes 4 and 8), and 3.13 μM (lanes 5 and 9). C, incorporation efficiencies of dCTP and dGTP into substrate C-p27 and C-p28. Incorporations of dCTP into C-p27, dGTP into C-p27, dCTP into C-p28, and dGTP into C-p28 are indicated by open diamonds, open triangles, closed diamonds, and closed triangles, respectively. Each data point represents the mean of two separate experiments. The error bars represent residuals.

**DISCUSSION**

In Vitro TLS Analysis Reconstituting PhIP-induced Mutations—HCAs are food-borne carcinogens produced when cooking meat (1, 9, 33). The most significant aspect of these molecules is that they exist normally in cooked food and are thus ubiquitous carcinogens (32). The mutagenicity and carcinogenicity of HCAs are mainly attributed to C8- and N2-dG adducts (9). Both excision repair and translesional DNA synthesis play critical roles in the mutagenesis steps induced by HCAs. However, despite the importance of HCAs as common environmental mutagens, there have been very few previous reports regarding the stalling of DNA polymerases and TLS caused by the DNA adducts they form. This is mainly because of the difficulty in preparing template DNA with introduced HCA adducts at specific sites. Choi et al. have recently undertaken a biochemical study of TLS at adducts of the HCA 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) using purified human polymerases. In our current study of TLS, we describe our findings for adducts of PhIP, the most abundant HCA in cooked foods (4).

A rat colon cancer model induced by PhIP shows profiles of cancer development similar to the multistep model of colon carcinogenesis in humans (35). In this rat model, p53 and K-ras mutations are rarely observed, whereas mutations in Apc and its downstream gene β-catenin have been frequently observed (21, 36–38). Hence, mutations in Apc or β-catenin have been speculated to play a critical role in PhIP-induced colon carcinogenesis. Five mutations in the Apc gene were previously detected in four of eight PhIP-induced rat colon tumors, and all of these mutations involved a single guanine deletion in the 5′-GGGA-3′ sequence (21). This characteristic mutation induced by PhIP, 5′-GGGA-3′ to 5′-GGA-3′, was also observed in other in vivo mutation analyses using transgenic animals harboring introduced reporter genes, such as lacI (18–20). Hence, the 5′-TCCGGAAC-3′ sequence corresponding to a mutation hot spot within the rat Apc gene, which we utilized to introduce the PhIP adduct and employed as the template for in vitro DNA synthesis analyses, could be a suitable model for revealing the molecular mechanisms associated with PhIP-induced mutations.
As discussed later, our results indicate a possible molecular mechanism for the 5′-GGGA-3′ to 5′-GGA-3′ mutation induced by PhIP.

DNA Polymerases Involved in TLS through the dG-PhIP Adduct—TLS through many DNA lesions requires the action of two different polymerases, an “inserter” and an “extender,” the former to perform nucleotide insertions opposite the lesion site and the latter for subsequent extensions (39). The catalytic efficiency of the dCTP-insertion reaction opposite the dG-PhIP adduct by REV1 was found to be more than 2,000-fold greater than that by pol δ (see Tables 2 and 3). This result strongly suggests that REV1 functions in vivo as an inserter polymerase for TLS through the dG-PhIP adduct. This insertion step by REV1 is also error free. REV1 has been reported previously to insert dCTP opposite abasic sites and various N2-dG adducts (26, 39–41). However, our current study is the first to show that REV1 inserts dCTP opposite a large size C8-dG adduct. We used a shorter (C-terminal deleted) form of pol δ in our current experiments and an intact pol δ may be more effective for this insertion reaction. As for pol η, a detailed kinetic analysis was not performed. Hence, the possibility cannot be excluded that pol κ and pol η also function as inserter polymerases.

In addition to the Y-family DNA polymerases, DNA polymerase ζ (pol ζ), belonging to the B-family DNA polymerases, is considered to be involved in TLS through various lesions as an extender DNA polymerase (39, 42, 43). We have not carried out a primer extension assay with pol ζ and thus the possibility cannot be completely excluded by our current data that pol ζ functions in vivo as an extender polymerase for TLS through the dG-PhIP adduct. In our present study, we provide evidence that pol κ can extend from dC opposite the dG-C8-PhIP adduct in vitro. It is, therefore, possible that pol κ, at least partially, functions as an extender polymerase in vivo for TLS through the dG-PhIP adduct. Further study about cooperation between two or more DNA polymerases, including pol ζ, is necessary to verify which DNA polymerases are involved in the bypass synthesis through the PhIP lesion.

The catalytic efficiency of pol κ for a dGTP insertion into substrate C-pG was a little higher than that for dCTP insertions (see Table 2 and Fig. 6D). The former generates a single guanine deletion, and the latter is an error-free extension. Consequently, our data suggest that the extension reaction with pol κ from the nucleotide opposite the dG-C8-PhIP adduct causes frequent single-guanine deletions from the GGG stretch. It has been reported that one characteristic feature of pol κ homologs, from bacteria to humans, is their propensity to generate single-base deletions (44–47). The crystal structure of Dpo4, a thermophilic archaea homolog of pol κ, in ternary complexes with DNA and an incoming nucleotide supports the model that a single base deletion by pol κ is generated through a misaligned intermediate complex where the template dG forms an extra-helical looped out structure and the incoming dGTP skips this extrahelical base and pairs with the next template base dC (48) (see supplemental Fig. S6). It is reasonable to speculate therefore that, in the case of TLS through dG-C8-PhIP, mammalian pol κ generates the single guanine deletion via a similar intermediate where the PhIP-adducted dG is looped out and template-primer slippage occurs. However, further analyses for determining whether the one-base skipping of pol κ beyond the lesion observed by us is dependent on the nucleotide placed 5′ to the lesion or not, are necessary to clarify the detailed molecular mechanism underlying one base skipping of pol κ.

Molecular Mechanisms Underlying Mutation Induction by PhIP—We have demonstrated herein by in vitro DNA synthesis analyses using oligonucleotide templates containing dG-PhIP that: 1) replicative DNA polymerases stall at the PhIP adduct and cannot perform translesional DNA synthesis beyond this point; 2) REV1 inserts a dC opposite the dG-PhIP with a much higher efficiency than other TLS polymerases, including pol κ and pol η; and 3) pol κ has a potential ability to catalyze an extension reaction from the 5′-dC opposite the adduct and often skips over one dG in the template during this extension step. A working model for the induction of mutations at the PhIP adducts based on the results shown in the present study is illustrated in supplemental Fig. S6. This model could be adopted for other sequences containing a G repeat stretch longer than GGG.

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