

Fig. 1. Structures of the (-)- and (+)-BPDE-dG adducts.

κ , a homologue of *Escherichia coli* DinB (DNA Pol IV), appears to be involved in error-free TLS across N²-deoxyguanine adducts induced by BPDE, specifically (+)- and (-)-trans-anti-BPDE-N²-dG (Fig. 1)(hereafter, we call them (+)-BPDE-dG and (-)-BPDE-dG, respectively). Pol κ strongly binds to DNA containing (-)-BPDE-dG [12], and preferentially incorporates dCMP opposite both (+) and (-) lesions [12–17]. Pol κ has been implicated in error-free TLS past (+)-BPDE-dG in mouse embryonic fibroblasts transfected with a gapped plasmid containing a site-specific lesion [18]. Embryonic fibroblasts from Pol $\kappa^{-/-}$ mice display sensitivity to the killing effects of racemic (\pm)-anti-BPDE, as well as the mutagenic and killing effects of BP plus rat liver homogenates (S9 enzymes) in the presence of caffeine [19,20]. However, the catalytic mechanism by which Pol κ bypasses across (+)- and (-)-BPDE-dG is not fully understood.

Fortunately, X-ray structures of the ternary complex of Pol κ with DNA and incoming dNTPs have been resolved [21]. Based on these structures, it has been suggested that N²-dG adducts, for example trans-4-hydroxy-2-nonenal-N²-dG, are positioned at the minor groove in the active site. The predicted amino acid residues proximal to the adducts are phenylalanine 171 (F171), arginine 175 (R175), and leucine 197 (L197). Molecular dynamics studies also suggest that F171, which is conserved between Pol κ and *E. coli* DinB, stacks with the pyrene ring of (+)-BPDE-dG [22]. The goal of the current study is to elucidate the relationship between these Pol κ structural features and the TLS process. We have changed F171, R175, or L197 to alanine, and examined the TLS activities and DNA binding properties of the resulting mutant Pols. Remarkably, the substitution of F171 to alanine increased the TLS activities approximately 18-fold, and significantly enhanced the ability to bind DNA with (-)-BPDE-dG. These results suggest that the conserved F171 acts a molecular brake for Pol κ -mediated TLS across the BP adducts; additional space to accommodate the bulky lesions in the active site may be required for active TLS. Because of the presence of the molecular brake in the active site, we speculate that Pol κ may not have evolved to bypass the BPDE adducts or perhaps other adducts induced by polycyclic aromatic hydrocarbons. Possible candidates for DNA lesions suitable for TLS by Pol κ are discussed.

2. Materials and methods

2.1. Proteins

Human wild-type Pol κ and variant proteins with alanine substitutions (F171A, R175A, L197A) were prepared as C-terminal truncations with 10 His-tags, including 559 N-terminal residues as previously described [12]. Briefly, overexpression plasmids for F171A, R175A, and L197A were constructed from pYG8591 by site-directed mutagenesis using PCR. pYG8591 was also used for overexpression of wild-type Pol κ . The resulting plasmids were transformed into Rosetta (DE3) pLysS (Novagen), and protein expression was induced with isopropyl β -D-1-thiogalactopyranoside. Wild-type Pol κ , F171A, R175A and L197A were purified to near homogeneity using BD TALON Metal Affinity Resins (BD Biosciences), gel filtration (16/60 Superdex 200 pg, GE Healthcare), and ion-exchange chromatography (HiTrap Heparin HP columns, GE Healthcare) with an FPLC system (AKTAexplorer 10s, GE Healthcare) (Fig. 1 of the Supporting Information).

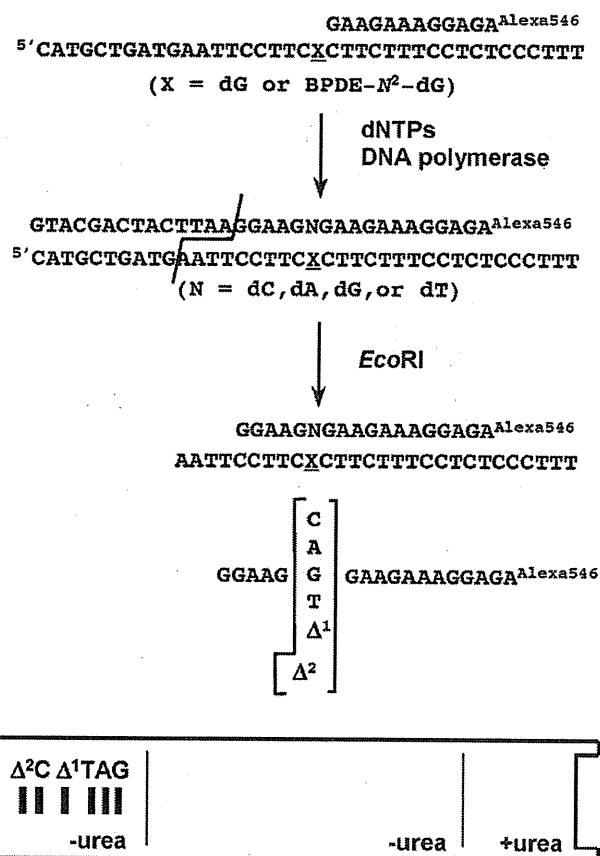


Fig. 2. Fluorescent two-phase PAGE determines miscoding specificity of DNA adducts. Unmodified and (-)/(+)-BPDE-dG-modified 38-mer templates were annealed to Alexa546-labelled 12-mer primers. Primer extension reactions catalyzed by wild-type or F171A Pol κ were conducted in the presence of all four dNTPs at concentration of 100 μ M each. Fully-extended products formed during DNA synthesis were recovered from the 20% polyacrylamide gel, annealed with a complementary 38-mer, cleaved with *EcoRI*, and subjected to two-phase PAGE (Section 2). To determine miscoding specificities, mobilities of the reaction products were compared with those of 18-mer standards containing dC, dA, dG, or dT opposite the lesion, and one-base (Δ^1) or two-base (Δ^2) deletions.

2.2. Oligonucleotide preparation

5'-Cy3-labelled oligonucleotides used for primers were purchased from BEX Co., Ltd. (Tokyo, Japan). Oligonucleotides labelled with biotin or Alexa546 at the 5' end, unmodified template oligonucleotides, and oligonucleotides with thymine glycol were purchased from Japan Bio Services Co., Ltd. (Saitama, Japan). Oligonucleotides containing (-) or (+)-BPDE-dG were synthesized as previously reported [23].

2.3. TLS assays

The Cy3-labelled primer DNA (5'-CTTCCCTAGGAAT-3') was annealed to the template DNA (5'-GCGCGCTTCTGGCAATXGAGAAITCTAGGGAAG-3', where X represents dG, (-)-BPDE-dG, or (+)-BPDE-dG) at a molar ratio of 1:1.2. 100 nM primer/template DNA hybrid was incubated with 1, 5, or 20 nM wild-type or mutant Pol κ in 10 μ L reaction mixtures containing 40 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 10 mM DTT, 0.1 mg/mL BSA, 60 mM KCl, 2.5% glycerol, and 100 μ M each of the four dNTPs. The reactions were carried out for 1 min or 10 min at 37 °C, and were terminated by addition of termination buffer (98% formamide, 10 mM EDTA, 10 mg/mL blue dextran). After heat denaturation at 95 °C for 5 min, the products were separated by 15% polyacrylamide gel electrophoresis (PAGE) on gels containing 8 M urea. The separated products were visualized with the Molecular Imager FX Pro System (Bio-Rad Laboratories) and quantified with Quantity One software version 4.5 (Bio-Rad Laboratories).

2.4. Steady-state kinetics analyses

When measuring the kinetic parameters for nucleotide insertion opposite dG, (-)-BPDE-dG, (+)-BPDE-dG, or thymine glycol, the reaction mixtures were identical

to the TLS assay mixtures except for the addition of 3–5 nM wild-type or variant Pol κ and varying amounts of single dNTPs. The 36-mer template DNA, containing thymine glycol and annealed to the 18-mer primer DNA, was also used for the steady-state kinetic assay. For incorporation kinetics opposite dG, reaction mixtures containing dCTP (0.2–20 μ M) and 3 nM wild-type or variant Pol κ were incubated for 30 s. For incorporation kinetics opposite (-)-BPDE-dG, reaction mixtures containing dCTP (50–4000 μ M) and 3 nM wild-type or variant Pol κ were incubated for 3 min (wild-type or R175A), 30 s (F171A) or 5 min (L197A). For incorporation kinetics opposite (+)-BPDE-dG, reaction mixtures containing dCTP (20–1000 μ M) and 5 nM wild-type or variant Pol κ were incubated for 20 min (wild-type, R175A or L197A) or 3 min (F171A). For incorporation kinetics opposite thymine glycol, reaction mixtures containing dATP (20–1000 μ M) and 3 nM wild-type or variant Pol κ were incubated for 30 s. To measure the extension parameters, the reaction mixtures were identical to the nucleotide insertion reactions, except that 120 nM Cy3-labelled 19-mer primer (5'-CTTCCCTAGGAATTCGCC-3') replaced the 18-mer primer. The concentrations of dNTPs and the incubation times were varied according to the activity of given Pols. For extension kinetics from dG/dC, reaction mixtures containing dATP (0.2–20 μ M) and 3 nM wild-type or variant Pol κ were incubated for 30 s. For extension kinetics from (-)-BPDE-dG/dC, reaction mixtures containing dATP (2–200 μ M) and 3 nM wild-type or variant Pol κ were incubated for 30 s (wild-type, R175A and L197A) or 20 s (F171A). For extension kinetics from (+)-BPDE-dG/dC, reaction mixtures containing dATP (2–500 μ M) and 3 nM wild-type or variant Pol κ were incubated for 10 min (wild-type, R175A and L197A) or 3 min (F171A). For extension kinetics from thymine glycol/dA, reaction mixtures containing dATP (20–500 μ M) and 3 nM wild-type or variant Pol κ were incubated for 30 s. The products were analyzed as described for TLS assays. The rate of incorporation was plotted against dNTP concentrations, and the apparent Michaelis–Menten constants, K_m , and V_{max} values were determined by the Enzyme Kinetics Module 1.1 of SigmaPlot 2001 software version 7.101 (SPSS, Inc). k_{cat} was calculated by dividing the V_{max} by the enzyme concentration. All values represent means plus standard errors from three independent experiments.

2.5. Quantitative assays for miscoding specificity

The method used to determine miscoding specificities has been previously described [24–26] and is presented in Fig. 2. Briefly, 5'-Alexa546-labelled 12-mer primer (5'-AGAGGAAAGAAG-3') was annealed to 38-mer template DNA (5'-CATGCTGATGAATTCCTTCTCTTCTTCTCTCTTT-3'), where X represents dG, (-)-BPDE-dG, or (+)-BPDE-dG at a 1:1.5 ratio. 50 nM primer/template DNA was incubated with 2.5 nM wild-type Pol κ or F171A for 30 min at 25 °C in the presence of all four dNTPs (100 μ M).

Approximately 80% of primer DNA was converted to full length to ensure that the assay has an ability to detect infrequent occurrences. Namely, 81.2%, 76.8% and 80.9% of the starting primer DNA was fully extended when the wild-type Pol κ continued DNA synthesis across dG, (-)-BPDE-dG and (+)-BPDE-dG, respectively. Similarly, 83.8%, 78.5% and 79.5% of the starting primer DNA was converted to the fully-extended products when F171A continued DNA synthesis across dG, (-)-BPDE-dG and (+)-BPDE-dG, respectively. The extended products were separated by 20% denaturing PAGE and extracted from the gel. The extracted DNA was annealed to the unmodified 38-mer template having dG at the position X, cleaved with EcoRI, and subjected to two-phase PAGE with 7 M urea in the upper phase and no urea in the lower phase. To determine base substitutions and deletions, we used Alexa546-labelled 18-mer standard DNA containing dC, dA, dG, or dT at the 13th position (18C, 18A, 18G or 18T, respectively, shown in Fig. 4), 17-mer DNA lacking one base at the 13th position (17 Δ 1), or 16-mer DNA lacking two bases at positions 13 and 14 (16 Δ 2) as authentic samples for comparison with reaction product mobility [25,26]. Exact sequences of the authentic samples are shown in Fig. 2. The separated products were visualized with the Molecular Imager FX Pro System (Bio-Rad Laboratories) and quantified with Quantity One software version 4.5 (Bio-Rad Laboratories).

2.6. DNA binding BIAcore assay

The DNA binding assay was performed using the BIAcore3000 instrument (GE Healthcare) as previously described [12]. Oligonucleotides were similar to those used in the primer extension assay, except for 5'-biotinylation of the primers for immobilisation to the Sensor Chip SA (GE Healthcare). The equilibrium dissociation constants (K_D) were calculated from the kinetic traces using BIAevaluation version 4.0, employing local fitting according to the "1:1 binding with drifting baseline" predefined model (GE Healthcare).

2.7. Modeling of (-) and (+)-BPDE-adducts in the Pol κ active site

To visualize the relative positions of F171/A171 and (-)/(+)-BPDE-dG following the incorporation of dCMP opposite (-)/(+)-BPDE-dG, the conformation of (-)/(+)-BPDE-dG with dCTP in the active site of Pol κ was drawn based on the reported crystallographic structure of Pol κ with DNA and incoming dTTP [PDB ID: 2OH2 [21]], using the 2007.09 version of the Molecular Operating Environment (MOE) (Chemical Computing Group Inc., Montreal, Canada) and MOE-ASEDock 2005 [27]. We replaced the original dTTP with dCTP, and substituted the original template dA with (-)-BPDE-dG [PDB ID: 1AXL [28]] or (+)-BPDE-dG [PDB ID: 1AXO [29]].

The resulting template and primer sequences were 5'-CCXGGGTCCTCCCC-3' and 5'-GGGGAAGGACCC-3', respectively, where X represents (-)-BPDE-dG or (+)-BPDE-dG. After all replacements, we minimised the potential energies of F171 and the replaced residues. The F171A mutant was visualized by substituting alanine for F171 and repeating the above processes.

3. Results

3.1. Enhanced incorporation of dCMP opposite (-) and (+)-BPDE-adducts by F171A

Primer extension reactions were performed in order to examine the effects of alanine substitutions of residues F171, R175, and L197 on the translesion activities of Pol κ variants (Fig. 3). Interestingly, TLS by F171A past (-) and (+)-BPDE-dGs was much more active than by wild-type Pol κ . DNA synthesis by F171A past unmodified dG appeared similar to that of the wild-type enzyme (Fig. 3A). DNA synthesis by R175A and L197A across unmodified dG, the (-) or (+)-BP lesions was lower than that of the wild-type Pol κ .

As a quantitative analysis, we carried out steady-state kinetic analyses to determine the catalytic efficiencies (k_{cat}/K_m) of dCMP incorporation opposite unmodified dG, dCMP opposite the BP lesions, and dAMP opposite thymine glycol (Fig. 2 of the Supporting Information and Table 1). Remarkably, the efficiency of dCMP incorporation opposite (-) and (+)-BPDE-dGs by F171A was 18-fold higher than by the wild-type Pol κ , while the k_{cat}/K_m values for dCMP incorporation opposite normal dG were almost identical. Both Pols bypassed the (-)-adduct approximately four times more actively than the (+)-adduct. In contrast, the efficiency of dATP incorporation opposite thymine glycol by F171A was less than two-fold higher than by the wild-type Pol κ . Therefore, the marked increase in catalytic efficiency by the amino acid substitution appeared specific to TLS across the BPDE-adducts. The incorporation efficiencies displayed by the R175A variant were the same as the wild-type Pol κ . Finally, the substitution of L197 to alanine reduced efficiency by 30% for incorporation opposite the BPDE-adducts, and by 50% for incorporation opposite thymine glycol.

We also determined the kinetic parameters for extension reactions; the k_{cat} and K_m values for dAMP incorporation opposite template dT next to unmodified dG or the lesions appear in Table 1. Extension of 3'-dC primers was enhanced 1.6-fold and 3.7-fold opposite template (-) and (+)-BPDE-dGs, respectively, by F171A Pol κ relative to wild type. The extension efficiency of F171A from 3'-dA opposite thymine glycol was 1.3-fold higher than by the wild type. Thus it would appear that the F171A substitution affects the extension reactions less than the insertion reactions. The extension efficiencies of R175A and L197A from dCs opposite (-) and (+)-BPDE-dGs and from dA opposite thymine glycol were the same or lower in comparison to the wild-type enzyme. We also prepared and purified a catalytically "dead" variant of Pol κ , in which the active-site residues aspartic acid 198 (D198) and glutamic acid 199 (E199) were substituted with alanine (D198A/E199A), by the same method as for F171A, R195A and L197A. D198A/E199A Pol κ completely lost polymerase activity (data not shown). Therefore, the possibility that bacterial polymerases, such as DNA polymerase IV (DinB), may have contaminated the preparations of F171A, R195A and L197A and affected the results can be ruled out.

3.2. Comparison of DNA synthesis fidelities of wild-type and F171A Pol κ across (-) and (+)-BPDE-adducts

We wished to determine whether the TLS enhancement displayed by the F171A variant was accompanied by an increased mutation (substitutions and deletions) rate of sequences containing BP lesions. Examination of the molecules produced by wild-type

Table 1

Kinetic parameters for nucleotide insertion and extension reactions catalyzed by wild-type, F171A, R175A, and L197A Pol κ .

Template/dNTP	Pol κ	k_{cat} , min ⁻¹	K_m , μ M	k_{cat}/K_m , μ M ⁻¹ min ⁻¹	Related efficiency
dNTP incorporation dG/dCTP	WT ^a	28 \pm 2.3 ^b	7.4 \pm 1.8	3.8	1.0
	F171A	27 \pm 3.1	6.8 \pm 1.9	4.0	1.0
	R175A	32 \pm 4.4	9.7 \pm 3.1	3.3	0.87
	L197A	20 \pm 1.7	23 \pm 3.3	0.87	0.23
	WT	1.9 \pm 0.2	1200 \pm 310	0.0016	1.0
(-)-BPDE-dG ^c /dCTP	F171A	15 \pm 0.56	520 \pm 69	0.029	18
	R175A	1.8 \pm 0.17	1100 \pm 300	0.0016	1.0
	L197A	0.55 \pm 0.045	1100 \pm 250	0.00050	0.31
	WT	0.26 \pm 0.019	700 \pm 95	0.00037	1.0
	F171A	1.9 \pm 0.18	290 \pm 70	0.0066	18
(+)BPDE-dG ^d /dCTP	R175A	0.24 \pm 0.021	650 \pm 98	0.00037	1.0
	L197A	0.087 \pm 0.010	680 \pm 89	0.00013	0.32
	WT	15 \pm 0.95	220 \pm 37	0.068	1.0
	F171A	18 \pm 1.0	140 \pm 24	0.13	1.9
	R175A	14 \pm 1.4	200 \pm 60	0.070	1.0
Thymine glycol/dATP	L197A	9.1 \pm 1.2	270 \pm 95	0.034	0.50
	WT	48 \pm 1.8	1.5 \pm 0.19	32	1.0
	F171A	28 \pm 0.96	0.88 \pm 0.12	32	1.0
	R175A	59 \pm 1.7	2.4 \pm 0.22	25	0.77
	L197A	23 \pm 1.6	5.3 \pm 1.1	4.3	0.14
(-)-BPDE-dG/dC	WT	16 \pm 0.70	36 \pm 4.7	0.44	1.0
	F171A	26 \pm 1.5	37 \pm 6.6	0.70	1.6
	R175A	20 \pm 1.2	42 \pm 6.9	0.47	1.0
	L197A	1.2 \pm 0.15	53 \pm 18	0.023	0.052
	WT	1.2 \pm 0.33	260 \pm 110	0.0046	1.0
(+)BPDE-dG/dC	F171A	3.9 \pm 0.74	230 \pm 72	0.017	3.7
	R175A	1.1 \pm 0.18	260 \pm 68	0.0042	0.91
	L197A	0.57 \pm 0.10	210 \pm 65	0.0027	0.59
	WT	8.6 \pm 0.37	32 \pm 5.7	0.27	1.0
	F171A	14 \pm 0.66	41 \pm 7.3	0.34	1.3
Thymine glycol/dA	R175A	11 \pm 0.62	57 \pm 11	0.19	0.71
	L197A	7.9 \pm 0.61	69 \pm 17	0.11	0.41

^a WT: wild-type Pol κ .

^b Data are expressed as mean \pm SE obtained from three independent experiments.

^c (-)-BPDE-dG: (-)-trans-anti-BPDE-N²-dG.

^d (+)-BPDE-dG: (+)-trans-anti-BPDE-N²-dG.

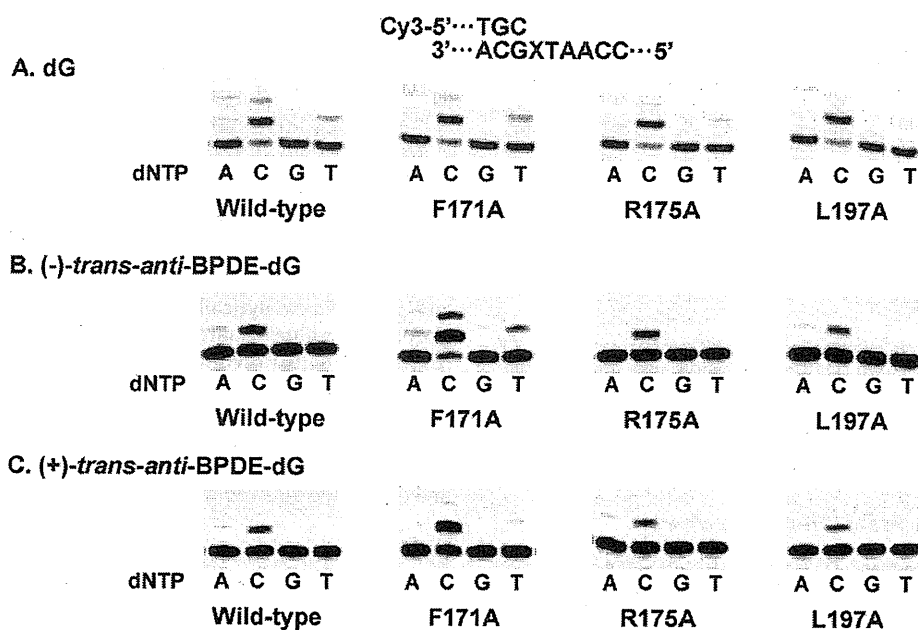


Fig. 3. Incorporation of dNMPs opposite dG, (-)-BPDE-dG, or (+)-BPDE-dG by wild-type, F171A, R175A, or L197A Pol κ . The template strands contained dG (A), (-)-BPDE-dG (B), or (+)-BPDE-dG (C) at position X. 100 nM template/primer DNA was incubated with 10 nM wild-type, F171A, R175A, or L197A Pol κ in the presence of 100 μ M dATP, dCTP, dGTP, or dTTP for 1 min (A) or 10 min (B and C) at 37 $^{\circ}$ C. Extended primers were separated by denaturing PAGE.

Table 2
Miscoding properties of (–)– and (+)– trans-anti-BPDE-*N*²-dG by wild-type and F171A Pol κ ^{a,b,c}.

Pol κ	DNA adduct	dC ^b [%]	dA [%]	dG [%]	dT [%]	$\Delta 1$ [%]	$\Delta 2$ [%]
WT ^d	dG	79.9 \pm 3.2					
	(–)-BPDE-dG ^e	74.8 \pm 0.1	ND	ND	ND	2.1 \pm 0.4	ND
	(+)-BPDE-dG ^f	70.7 \pm 1.9	ND	ND	ND	10.1 \pm 1.8	ND
F171A	dG	81.3 \pm 2.5					
	(–)-BPDE-dG	74.6 \pm 2.0	ND	ND	ND	2.5 \pm 0.3	ND
	(+)-BPDE-dG	67.5 \pm 1.7	ND	ND	ND	12.0 \pm 1.7	ND

^a Primer extension reactions were conducted for 30 min at 25 °C as described in the legend of Fig. 3.

^b Expressed as percentage of starting primer converted to fully extended product.

^c ND indicates not detectable.

^d WT: wild-type.

^e (–)-BPDE-dG: (–)-trans-anti-BPDE-*N*²-dG.

^f (+)-BPDE-dG: (+)-trans-anti-BPDE-*N*²-dG. Data are expressed as mean \pm SE.

and F171A Pol κ is detailed in Fig. 4 and Table 2. dCMP was exclusively incorporated opposite (–)-BPDE-dG by both wild-type and F171A Pol κ . Small amounts of one-base deletions were also identified in the products generated by the enzymes. However, there were no obvious differences in the deletion frequencies. When confronted with template (+)-BPDE-dG, both polymerases preferentially incorporated dCMP and generated one-base deletions; the percentage of one-base deletions was approximately five-fold higher than with TLS across (–)-BPDE-dG. There were no differences in the one-base-deletion percentages of wild-type and F171A Pol κ .

3.3. Binding of the wild-type and F171A Pol κ with (–)- or (+)-BPDE-dG template/primer DNA

To determine whether the F171A substitution modulates the DNA-binding properties of Pol κ , we examined the physical interactions between Pol κ and the template/primer DNA with or without the BP lesions in the absence of dNTPs using surface plasmon resonance (Fig. 5, Table S1). Adducts were positioned 19 nucleotides from the 3'-terminus on the 36-mer template strand. Primers of varying lengths (18–22 nucleotides) were separately annealed to the template. These primers contained dC at the 19th position from the 5'-terminus. As has been previously reported [12], wild-type Pol κ bound to primer/template DNA with (–)-BPDE-dG approximately 3-fold more strongly than unmodified DNA when the primer length was between 19 and 21 nucleotides. Interestingly, the F171A substitution resulted in a two-fold increase in the binding affinity of Pol κ to template/primer DNA with (–)-BPDE-dG, when the primers were 19-mers or 20-mers. These increases were not observed with

18-mer, 21-mer, or 22-mer primers. However, this substitution did not affect the binding affinity to template/primer DNA containing unmodified dG or (+)-BPDE-dG. In fact, no binding strength increase was observed between the wild-type Pol κ and (+)-BPDE-dG template/primer DNA even when the primers were 19-, 20-, or 21-mers. Thus, the strong binding of Pol κ is specific to DNA containing (–)-BPDE-dG.

We also examined the binding properties of the R175A and L197A variants of Pol κ (Table S1). Both R175A and L197A displayed reduced affinities to modified and unmodified DNA compared with the wild-type Pol κ . However, binding to (–)- and (+)-BPDE-adducts was not altered by the R175A and L197A substitutions; both proteins preferred to bind to the (–)-adduct over the (+)-adduct.

4. Discussion

TLS mediated by specialized Pols is an important process for protecting human cells from genotoxic insults [3]. BP is a common environmental pollutant and has been recognized as a class 1 human carcinogen by the IARC [30]. (+)- and (–)-BPDE-dGs are major and minor DNA products, respectively, generated by BP upon metabolic activation [11,31], which strongly block DNA replication by replicative Pols [32,33]. The specialized Pols bypass the lesions, thereby contributing to chromosome replication continuity [7]. Among the specialized human Pols, Pol κ seems to be an error-free mediator of TLS across the BPDE lesions [18,19]. If Pol κ is inactivated in the cells, other specialized Pols, such as Pol η and Pol ζ , may provide error-prone TLS past the lesions, resulting in mutations [18,34]. The TLS activity by Pol κ past BPDE-dG is thus a critical step in avoiding the mutagenic and carcinogenic effects of BP. In the current study, we investigated the possible mechanisms underlying the TLS across BP adducts by changing three amino acids located in the Pol κ catalytic center (F171, R175, and L197) to alanine, and examining the resulting catalytic and DNA binding properties.

Molecular modeling predicted the stacking of F171 with the pyrene rings of (+)-BPDE-adduct in the active site of Pol κ , when the adduct was placed in the anti-conformation opposite the incoming dCTP [22]. The stacking interactions were suggested to stabilize Watson-Crick base pairing between the adduct and dCTP. We expected, therefore, that the alanine substitution would decrease the efficiency and fidelity of bypass synthesis across the lesions. Contrary to our hypothesis, however, the F171A substitution increases dCMP insertion efficiency opposite (–)- and (+)-BPDE-adducts approximately 18-fold (Fig. 3 and Table 1). This increased efficiency results from a reduction in K_m for the incoming dCTP and an elevation in k_{cat} of the enzyme. The F171A variant was not substantially more proficient at extending primers after dC incorporation opposite (–)- or (+)-BPDE-dG compared to wild-type Pol κ . Thus, we conclude that F171, which is conserved between human Pol κ and *E. coli* DinB, suppresses the incorporation step of TLS across (–)- and (+)-BPDE-adducts.

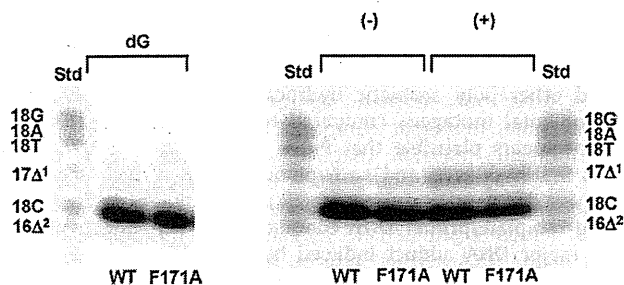


Fig. 4. Miscoding specificities in reactions catalyzed by wild-type and F171A Pol κ . Alexa546-labelled 12-mer primer DNA was annealed to 38-mer template DNA containing dG, (–), or (+)-BPDE-dG at the 19th position from the 3'-terminus. (–) and (+) denote (–)- and (+)-BPDE-dGs, respectively. 50 nM primer/template DNA was incubated with 2.5 nM wild-type or F171A Pol κ for 30 min at 25 °C in the presence of all four dNTPs (100 μ M each). The reaction products were separated by PAGE and extracted from the gel. The recovered extended primer DNA was annealed to unmodified 38-mer DNA and cleaved with *Eco*RI, followed by two-phase PAGE with 7 M urea in the upper phase and no urea in the lower phase. Product mobilities were compared with standard DNA containing dC (18C), dA (18A), dG (18G), dT (18T), one-base (17 Δ ¹), or two-base (16 Δ ²) deletions opposite the adducts.

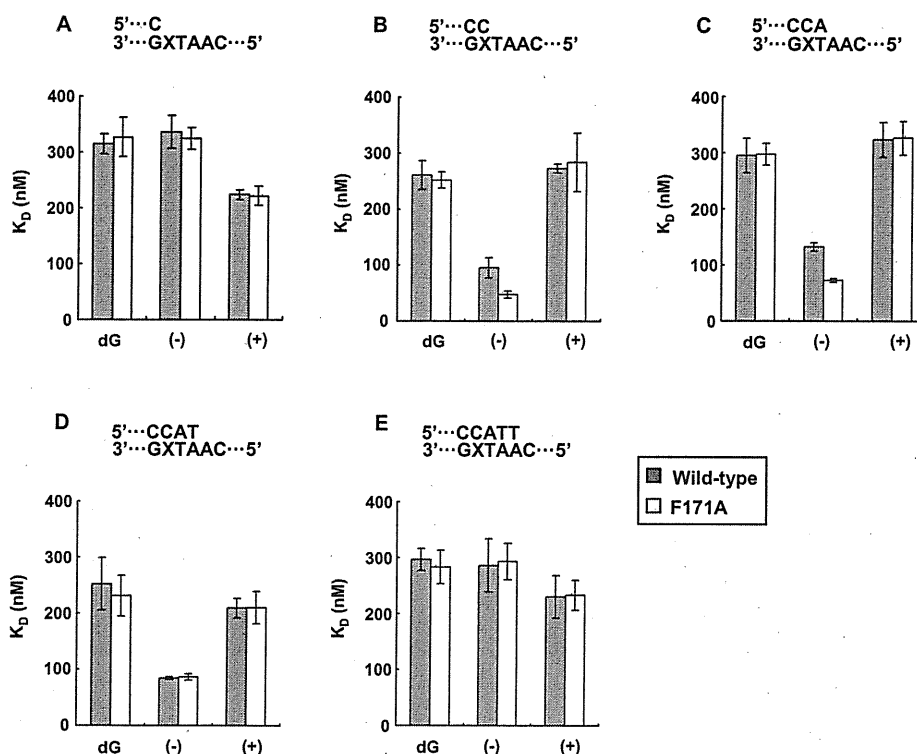


Fig. 5. Comparison of DNA-binding affinities of wild-type and F171A Pol κ for template/primer DNA with (-) or (+)-BPDE-adduct. (-) and (+) denote (-) and (+)-BPDE-dGs, respectively. Primers of varying lengths were annealed to 36-mer template DNA containing (-) or (+)-BPDE-dG at position 19 from the 3'-terminus (X). Primers were 18-mers (A), 19-mers (B), 20-mers (C), 21-mers (D), or 22-mers (E). As a control, template DNA with an unmodified dG was annealed to the primers. The DNA-protein affinity was measured with the BIAcore 3000 system (GE Healthcare), and the K_D value was calculated using BIAevaluation version 4.0 (GE Healthcare). The bar heights represent the average K_D values of three experiments. Error bars indicate the standard deviation.

Based on the results shown above, along with the molecular dynamics studies [22] and the X-ray structures of the ternary complex of Pol κ [21], we speculate that the aromatic ring of residue F171 may rotate flexibly; this rotation enables F171 to interact with both (-) and (+)-BPDE-adducts, which exhibit different configurations in DNA (Fig. 6). We hypothesize that residue F171 interacts with both (-) and (+)-BPDE-adducts, because the F171A substitution enhanced the efficiency of TLS across both adducts (Table 1). As the K_m for incoming dCTP was reduced by the amino acid substitution, the interactions may interfere with correct Watson-Crick base pairing between the modified dG and incoming dCTP. F171 may also hinder the progress of Pol κ along the template. The substitution of F171 with alanine may eliminate such detrimental effects, thereby enhancing the dCMP incorporation efficiency opposite the adducts. The amino acid change affected neither the fidelity across the lesions nor the efficiency of dCMP incorporation opposite normal template dG (Fig. 4, Tables 1 and 2). We suggest, therefore, that the F171A substitution does not induce substantial conformational changes in the overall structure of Pol κ . This substitution may generate space in the active site that is necessary to accommodate the bulky lesions for active TLS. To our knowledge, this study presents the first instance of a variant specialized Pol exhibiting a marked increase in TLS activity past DNA lesions without altering synthesis fidelity.

Other alanine substitutions displayed very limited or detrimental effects on the catalytic activity of Pol κ (Fig. 3, Table 1). The substitution of R175 to alanine did not affect the efficiency of TLS across (-) and (+)-BPDE-adducts, nor across thymine glycol. The mutation reduced the efficiency of dCMP incorporation opposite normal template dG by only 10%, and only mildly affected the extension reactions. Thus, this amino acid appears to play a very

limited role in Pol κ DNA synthesis. The L197A substitution reduced the efficiency of Pol κ dCMP incorporation opposite normal template dG by approximately 80%, and reduced TLS activities past (-) or (+)-BPDE-dG or thymine glycol by 69, 68 to 50%, respectively. In extension reactions, L197A reduced Pol κ activity, particularly from 3'-dC paired with (-)-BPDE-dG (a 95% reduction). We conclude, therefore, that L197 plays some role in the catalytic activity of Pol κ . Due to the long side chain of L197, this amino acid may maintain proper space in the active site where template DNA passes through during DNA synthesis [21].

Why does Pol κ possess the molecular brake for bypass beyond BPDE adducts in the active site? It seems counterintuitive that the polymerase has such a brake to continue DNA synthesis across the adducts in an almost error-free manner. However, BP and other poly aromatic hydrocarbons are relatively new environmental mutagens compared to UV or radiation. Therefore, it appears plausible that Pol κ may have evolved not to bypass BPDE adducts (and perhaps other DNA adducts induced by polycyclic aromatic hydrocarbons). Actually, Pol κ does not bind to template/primer DNA containing the (+)-adduct, which is the major DNA adduct induced by BPDE (Fig. 5, Table S1). Rather, it binds strongly to template/primer DNA containing the (-)-adduct, which is a minor adduct upon metabolic activation of BP. In addition, Pol κ more effectively bypasses the (-)-adduct than the (+)-adduct in DNA (Fig. 3, Table 1). These results raise the possibility that Pol κ is not necessarily well-tuned to bypass BPDE adducts. We speculate that Pol κ may be specialized to protect cells from the genotoxic effects of endogenous DNA adduct(s) such as those induced by methylglyoxal [35], estrogen [36] and reactive oxygen species [37]. The roles of Pol κ in protection from various exogenous and endogenous geno-

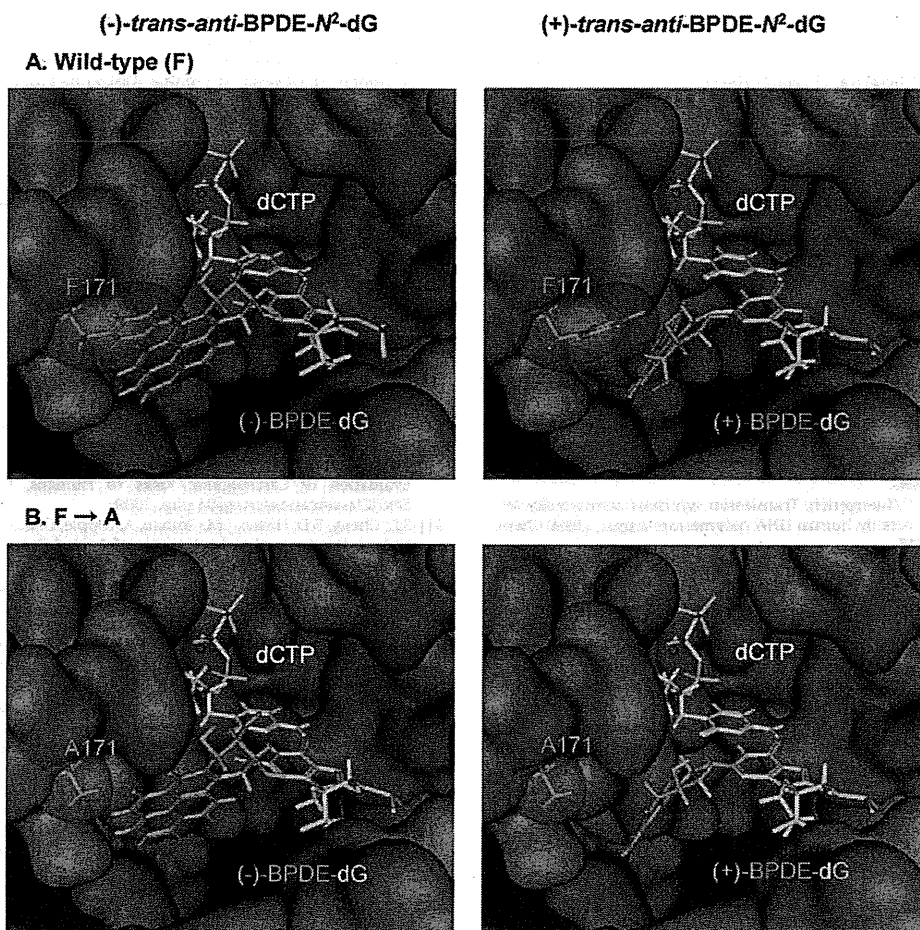


Fig. 6. Models of (-) or (+)-BPDE-dG in the active site of wild-type or F171A Pol κ . (A) (-) or (+)-BPDE-dG in the active site of wild-type Pol κ . The aromatic ring of F171 (cyan) may interfere with the pyrene ring of (-) or (+)-BPDE-dG (red). (B) (-) or (+)-BPDE-dG in the active site of F171A Pol κ . When F171 was replaced with alanine (cyan), no steric constraints appear between the pyrene ring and A171. dCTP is highlighted in light blue, the guanine base of (-) or (+)-BPDE-dG is colored orange, and the active site of Pol κ is displayed as a Gaussian surface (gray). Template/primer DNA other than (-) or (+)-BPDE-dG is not depicted.

toxic insults should be more thoroughly investigated *in vitro* and *in vivo*.

In summary, we have demonstrated that residue F171 acts as a molecular brake for Pol κ TLS past BPDE-*N*²-dG adducts in DNA, and suggest that this residue may slow down the TLS process via stacking interactions with the adducts. Our results also suggest that variant F171A is a more active TLS polymerase. In this regard, it may be interesting to examine the killing and the mutagen sensitivity of human cells expressing F171A instead of the wild-type Pol κ to various environmental mutagens. These experiments may shed light on the roles of Pol κ in suppression of genotoxicity of environmental mutagens. These studies are currently in progress in our laboratory.

Conflicts of interest

The authors have no conflicts of interest to declare.

Acknowledgements

This work was supported by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan [MEXT, 18201010]; the Ministry of Health, Labor, and Welfare, Japan [H21-Food-General-009]; and the Japan Health Science Foundation [KHB1007]. We thank Dr. Takayoshi Suzuki

(National Institute of Health Sciences) for allowing us to use the BIAcore3000. We acknowledge Dr. Masami Yamada (National Institute of Health Sciences) for critical reading of this manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mrgentox.2010.11.002.

References

- [1] T. Nohmi, Environmental stress and lesion-bypass DNA polymerases, *Annu. Rev. Microbiol.* 60 (2006) 231–253.
- [2] S. Prakash, R.E. Johnson, L. Prakash, Eukaryotic translesion synthesis DNA polymerases: specificity of structure and function, *Annu. Rev. Biochem.* 74 (2005) 317–353.
- [3] A.R. Lehmann, Translesion synthesis in mammalian cells, *Exp. Cell Res.* 312 (2006) 2673–2676.
- [4] R.E. Johnson, M.T. Washington, S. Prakash, L. Prakash, Fidelity of human DNA polymerase η , *J. Biol. Chem.* 275 (2000) 7447–7450.
- [5] C. Masutani, R. Kusumoto, A. Yamada, N. Dohmae, M. Yokoi, M. Yuasa, M. Araki, S. Iwai, K. Takio, F. Hanaoka, The XPV (xeroderma pigmentosum variant) gene encodes human DNA polymerase η , *Nature* 399 (1999) 700–704.
- [6] J.Y. Choi, J.S. Stover, K.C. Angel, G. Chowdhury, C.J. Rizzo, F.P. Guengerich, Biochemical basis of genotoxicity of heterocyclic arylamine food mutagens: Human DNA polymerase η selectively produces a two-base deletion in copying the *N*²-guanyl adduct of 2-amino-3-methylimidazo[4,5-f]quinoline but not the C8 adduct at the NarI G3 site, *J. Biol. Chem.* 281 (2006) 25297–25306.
- [7] E.C. Friedberg, R. Wagner, M. Radman, Specialized DNA polymerases, cellular survival, and the genesis of mutations, *Science* 296 (2002) 1627–1630.

- [8] M.F. Goodman, Error-prone repair DNA polymerases in prokaryotes and eukaryotes, *Annu. Rev. Biochem.* 71 (2002) 17–50.
- [9] D.F. Jarosz, P.J. Beuning, S.E. Cohen, G.C. Walker, Y-family DNA polymerases in *Escherichia coli*, *Trends Microbiol.* 15 (2007) 70–77.
- [10] D.H. Phillips, P.L. Grover, Polycyclic hydrocarbon activation: bay regions and beyond, *Drug Metab. Rev.* 26 (1994) 443–467.
- [11] K. Peltonen, A. Dipple, Polycyclic aromatic hydrocarbons: chemistry of DNA adduct formation, *J. Occup. Environ. Med.* 37 (1995) 52–58.
- [12] N. Niimi, A. Sassa, A. Katafuchi, P. Gruz, H. Fujimoto, R.R. Bonala, F. Johnson, T. Ohta, T. Nohmi, The steric gate amino acid tyrosine 112 is required for efficient mismatched-primer extension by human DNA polymerase kappa, *Biochemistry* 48 (2009) 4239–4246.
- [13] N. Suzuki, E. Ohashi, A. Kolbanovskiy, N.E. Geacintov, A.P. Grollman, H. Ohmori, S. Shibutani, Translesion synthesis by human DNA polymerase kappa on a DNA template containing a single stereoisomer of dG(+) or dG(–)-anti-N(2)-BPDE (7,8-dihydroxy-anti-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene), *Biochemistry* 41 (2002) 6100–6106.
- [14] Y. Zhang, F. Yuan, X. Wu, M. Wang, O. Rechkoblit, J.S. Taylor, N.E. Geacintov, Z. Wang, Error-free and error-prone lesion bypass by human DNA polymerase kappa *in vitro*, *Nucleic Acids Res.* 28 (2000) 4138–4146.
- [15] O. Rechkoblit, Y. Zhang, D. Guo, Z. Wang, S. Amin, J. Krzeminsky, N. Louneva, N.E. Geacintov, trans-Lesion synthesis past bulky benzo[a]pyrene diol epoxide N²-dG and N⁶-dA lesions catalyzed by DNA bypass polymerases, *J. Biol. Chem.* 277 (2002) 30488–30494.
- [16] J.Y. Choi, K.C. Angel, F.P. Guengerich, Translesion synthesis across bulky N²-alkyl guanine DNA adducts by human DNA polymerase kappa, *J. Biol. Chem.* 281 (2006) 21062–21072.
- [17] X. Huang, A. Kolbanovskiy, X. Wu, Y. Zhang, Z. Wang, P. Zhuang, S. Amin, N.E. Geacintov, Effects of base sequence context on translesion synthesis past a bulky (+)-trans-anti-B[a]P-N²-dG lesion catalyzed by the Y-family polymerase Pol kappa, *Biochemistry* 42 (2003) 2456–2466.
- [18] S. Avkin, M. Goldsmith, S. Velasco-Miguel, N. Geacintov, E.C. Friedberg, Z. Livneh, Quantitative analysis of translesion DNA synthesis across a benzo[a]pyrene-guanine adduct in mammalian cells: the role of DNA polymerase kappa, *J. Biol. Chem.* 279 (2004) 53298–53305.
- [19] T. Ogi, Y. Shinkai, K. Tanaka, H. Ohmori, Polkappa protects mammalian cells against the lethal and mutagenic effects of benzo[a]pyrene, *Proc. Natl. Acad. Sci. U.S.A.* 99 (2002) 15548–15553.
- [20] J.N. Stancel, L.D. McDaniel, S. Velasco, J. Richardson, C. Guo, E.C. Friedberg, Polk mutant mice have a spontaneous mutator phenotype, *DNA Repair (Amst.)* 8 (2009) 1355–1362.
- [21] S. Lone, S.A. Townson, S.N. Uljon, R.E. Johnson, A. Brahma, D.T. Nair, S. Prakash, L. Prakash, A.K. Aggarwal, Human DNA polymerase kappa encircles DNA: implications for mismatch extension and lesion bypass, *Mol. Cell* 25 (2007) 601–614.
- [22] L. Jia, N.E. Geacintov, S. Broyde, The N-clasp of human DNA polymerase kappa promotes blockage or error-free bypass of adenine- or guanine-benzo[a]pyrenyl lesions, *Nucleic Acids Res.* 36 (2008) 6571–6584.
- [23] F. Johnson, R. Bonala, D. Tawde, M.C. Torres, C.R. Iden, Efficient synthesis of the benzo[a]pyrene metabolic adducts of 2'-deoxyguanosine and 2'-deoxyadenosine and their direct incorporation into DNA, *Chem. Res. Toxicol.* 15 (2002) 1489–1494.
- [24] M. Yasui, E. Suenaga, N. Koyama, C. Masutani, F. Hanaoka, P. Gruz, S. Shibutani, T. Nohmi, M. Hayashi, M. Honma, Miscoding properties of 2'-deoxyinosine, a nitric oxide-derived DNA Adduct, during translesion synthesis catalyzed by human DNA polymerases, *J. Mol. Biol.* 377 (2008) 1015–1023.
- [25] S. Shibutani, Quantitation of base substitutions and deletions induced by chemical mutagens during DNA synthesis *in vitro*, *Chem. Res. Toxicol.* 6 (1993) 625–629.
- [26] S. Shibutani, N. Suzuki, Y. Matsumoto, A.P. Grollman, Miscoding properties of 3,N⁴-etheno-2'-deoxycytidine in reactions catalyzed by mammalian DNA polymerases, *Biochemistry* 35 (1996) 14992–14998.
- [27] J. Goto, R. Kataoka, H. Muta, N. Hirayama, ASEDock-docking based on alpha spheres and excluded volumes, *J. Chem. Inf. Model.* 48 (2008) 583–590.
- [28] B. Feng, A. Gorin, A. Kolbanovskiy, B.E. Hingerty, N.E. Geacintov, S. Broyde, D.J. Patel, Solution conformation of the (–)-trans-anti-[BP]dG adduct opposite a deletion site in a DNA duplex: intercalation of the covalently attached benzo[a]pyrene into the helix with base displacement of the modified deoxyguanosine into the minor groove, *Biochemistry* 36 (1997) 13780–13790.
- [29] B. Feng, A. Gorin, B.E. Hingerty, N.E. Geacintov, S. Broyde, D.J. Patel, Structural alignment of the (+)-trans-anti-benzo[a]pyrene-dG adduct positioned opposite dC at a DNA template-primer junction, *Biochemistry* 36 (1997) 13769–13779.
- [30] International Agency for Research on Cancer, IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, <http://monographs.iarc.fr/ENG/Classification/crthgr01.php>, 2009.
- [31] S.C. Cheng, B.D. Hilton, J.M. Roman, A. Dipple, DNA adducts from carcinogenic and noncarcinogenic enantiomers of benzo[a]pyrene dihydrodiol epoxide, *Chem. Res. Toxicol.* 2 (1989) 334–340.
- [32] A.M. Hruszkewycz, K.A. Canella, K. Peltonen, L. Kotrappa, A. Dipple, DNA polymerase action on benzo[a]pyrene-DNA adducts, *Carcinogenesis* 13 (1992) 2347–2352.
- [33] L.J. Lipinski, H.L. Ross, B. Zajc, J.M. Sayer, D.M. Jerina, A. Dipple, Effect of single benzo[a]pyrene diol epoxide-deoxyguanosine adducts on the action of DNA polymerases *in vitro*, *Int. J. Oncol.* 13 (1998) 269–273.
- [34] B. Zhao, J. Wang, N.E. Geacintov, Z. Wang, Poleta, Polzeta and Rev1 together are required for G to T transversion mutations induced by the (+)- and (–)-trans-anti-BPDE-N²-dG DNA adducts in yeast cells, *Nucleic Acids Res.* 34 (2006) 417–425.
- [35] B. Yuan, H. Cao, Y. Jiang, H. Hong, Y. Wang, Efficient and accurate bypass of N²-(1-carboxyethyl)-2'-deoxyguanosine by DinB DNA polymerase *in vitro* and *in vivo*, *Proc. Natl. Acad. Sci. U.S.A.* 105 (2008) 8679–8684.
- [36] A. Mizutani, T. Okada, S. Shibutani, E. Sonoda, H. Hochegger, C. Nishigori, Y. Miyachi, S. Takeda, M. Yamazoe, Extensive chromosomal breaks are induced by tamoxifen and estrogen in DNA repair-deficient cells, *Cancer Res.* 64 (2004) 3144–3147.
- [37] J.H. Yoon, G. Bhatia, S. Prakash, L. Prakash, Error-free replicative bypass of thymine glycol by the combined action of DNA polymerases kappa and zeta in human cells, *Proc. Natl. Acad. Sci. U.S.A.* 107 (2010) 14116–14121.

Research Article

Error-Prone Translesion DNA Synthesis by *Escherichia coli* DNA Polymerase IV (DinB) on Templates Containing 1,2-dihydro-2-oxoadenine

Masaki Hori,¹ Shin-Ichiro Yonekura,^{1,2} Takehiko Nohmi,³ Petr Gruz,³ Hiroshi Sugiyama,⁴ Shuji Yonei,¹ and Qiu-Mei Zhang-Akiyama¹

¹Laboratory of Stress Response Biology, Graduate School of Science, Kyoto University, Kitashirakawa-Oiwakecho, Sakyo-ku, Kyoto 606-8502, Japan

²Institute of Molecular and Cellular Biosciences, The University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan

³Division of Genetics and Mutagenesis, National Institute of Health Science, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

⁴Laboratory of Biological Chemistry, Graduate School of Science, Kyoto University, Kitashirakawa-Oiwakecho, Sakyo-ku, Kyoto 606-8502, Japan

Correspondence should be addressed to Qiu-Mei Zhang-Akiyama, qmzhang@kingyo.zool.kyoto-u.ac.jp

Received 13 May 2010; Revised 14 July 2010; Accepted 5 August 2010

Academic Editor: Ashis Basu

Copyright © 2010 Masaki Hori et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Escherichia coli DNA polymerase IV (Pol IV) is involved in bypass replication of damaged bases in DNA. Reactive oxygen species (ROS) are generated continuously during normal metabolism and as a result of exogenous stress such as ionizing radiation. ROS induce various kinds of base damage in DNA. It is important to examine whether Pol IV is able to bypass oxidatively damaged bases. In this study, recombinant Pol IV was incubated with oligonucleotides containing thymine glycol (dTg), 5-formyluracil (5-fodU), 5-hydroxymethyluracil (5-hmdU), 7,8-dihydro-8-oxoguanine (8-oxodG) and 1,2-dihydro-2-oxoadenine (2-oxodA). Primer extension assays revealed that Pol IV preferred to insert dATP opposite 5-fodU and 5-hmdU, while it inefficiently inserted nucleotides opposite dTg. Pol IV inserted dCTP and dATP opposite 8-oxodG, while the ability was low. It inserted dCTP more effectively than dTTP opposite 2-oxodA. Pol IV's ability to bypass these lesions decreased in the order: 2-oxodA > 5-fodU ~ 5-hmdU > 8-oxodG > dTg. The fact that Pol IV preferred to insert dCTP opposite 2-oxodA suggests the mutagenic potential of 2-oxodA leading to A:T → G:C transitions. Hydrogen peroxide caused an ~2-fold increase in A:T → G:C mutations in *E. coli*, while the increase was significantly greater in *E. coli* overexpressing Pol IV. These results indicate that Pol IV may be involved in ROS-enhanced A:T → G:C mutations.

1. Introduction

In recent years, novel types of DNA polymerase have been characterized in prokaryotes and eukaryotes. They share significant amino acid sequence identity and are characterized by their low fidelity and low processivity of DNA synthesis [1–3] and are classified as Y-family DNA polymerases. These DNA polymerases have the ability to catalyze synthesis past DNA lesions that otherwise block replication [1–3]. This process is termed translesion DNA synthesis (TLS). Y-family DNA polymerases have been identified in nearly all organisms. In *Escherichia coli*, two DNA polymerases, Pol IV and Pol V, have been classified into the Y-family polymerases

[1–4]. They are induced in the SOS response when *E. coli* cells encounter environmental stresses such as UV light and are involved in induction of mutations [4–8].

The *dinB* gene product has been shown to possess DNA synthesizing activity and is named DNA polymerase IV (Pol IV) [4]. Recent studies revealed an important role of the Y family of DNA polymerases in tolerance mechanisms towards various types of DNA damage [1–3, 9–11]. Napolitano et al. [7] found that Pol IV is able to bypass benzo(a)pyrene-adducts in DNA via both error-free and error-prone pathways. Jarosz et al. [9] reported that Pol IV is responsible for TLS over potentially lethal nitrofurazone-induced DNA adducts. In addition, Pol IV can synthesize

accurately across N2-furfuryl-guanine lesions. Recently, Yuan et al. [10] found that Pol IV efficiently and accurately bypassed N2-(1-carboxyethyl)-2'-deoxyguanosine, one of the major byproducts of the glycolysis pathway. Furthermore, Pol IV has the ability to bypass acrolein-mediated guanine DNA-peptide crosslinks [11]. These findings indicate that Pol IV contributes to the replicative bypass of lesions that block synthesis by replicative DNA polymerases and thereby helps to minimize the generation of DNA strand breaks, chromosome aberrations, and cell death.

In aerobic organisms, reactive oxygen species (ROS) are continuously produced during normal metabolism and by exogenous agents such as ionizing radiation. ROS react with DNA, proteins, and lipids and thereby cause harmful effects on cells. When cellular DNA is attacked by ROS, various types of DNA damage are generated [12–15] and might be involved in aging and many diseases including cancer [14, 15]. Oxidatively damaged bases produced by ROS have abnormal structures that induce several kinds of biological consequences. Bacterial and eukaryotic cells have DNA repair systems to remove damaged bases and restore DNA to its normal sequence [12, 16, 17]. If unrepaired, damaged bases would block DNA replication or cause the insertion of “incorrect” nucleotides opposite the lesion to form mismatches.

In this study, we examined whether Pol IV can bypass oxidatively damaged bases and is involved in mutation induction at the damaged sites. The primer extension assay revealed that Pol IV did not bypass the thymine glycol-(dTg-) containing DNA, while Pol IV preferred to insert dATP opposite 5-formyluracil (5-fodU) and 5-hydroxymethyluracil (5-hmdU), major oxidative products of thymine. dCTP and dATP were inserted opposite 7,8-dihydro-8-oxoguanine (8-oxodG) but the insertion ability was low. Pol IV more efficiently inserted dCTP than dTTP opposite 1,2-dihydro-2-oxoadenine (2-oxodA) in the template DNA, suggesting the mutagenic potential of 2-oxodA leading to A:T → G:C mutations. Pol IV's ability to handle these lesions decreased in the order: 2-oxodA > 5-fodU~5-hmdU > 8-oxodG > dTg. It was also found that hydrogen peroxide treatment caused an increase in A:T → G:C mutations in *E. coli*, while the increase was significantly greater in *E. coli* overexpressing Pol IV. These results indicate that Pol IV may be involved in these ROS-enhanced A:T → G:C mutations.

2. Materials and Methods

2.1. Chemicals and Enzymes. Ampicillin and isopropyl- β -D-thiogalactopyranoside (IPTG) were purchased from Wako Pure Chemicals (Osaka, Japan). T4 polynucleotide kinase, Taq DNA polymerase, and restriction enzymes were obtained from Takara Shuzo (Kyoto, Japan) and Toyobo (Osaka, Japan). [γ -³²P]ATP (>148 TBq/mmol) was obtained from ICN Biomedicals Inc. (Costa Mesa, CA). Columns used for column chromatography and high performance liquid chromatography (HPLC) were purchased from Pharmacia (Uppsala, Sweden).

2.2. Synthesis of Substrate Oligonucleotides Containing Oxidatively Damaged Bases. 24-mer oligonucleotide containing 2-oxodA was synthesized as previously described by Sugiyama et al. [18]. 24-mer oligonucleotide containing dTg was synthesized as described by Dianov et al. [17]. 22-mer oligonucleotides containing 5-fodU and 5-hmdU were synthesized and purified as previously described [19]. 24-mer oligonucleotides containing 8-oxodG was obtained from Trevigen (Gaithersburg, MD). The structures of the studied oxidatively damaged bases are illustrated in Figure 1. Primers were synthesized and purified by Takara Shuzo (Kyoto, Japan). The nucleotide sequences of template and primer oligonucleotides used in this study are shown in Table 1.

2.3. Expression and Purification of Pol IV with Histidine Tag. Pol IV was overproduced and purified from *E. coli* BL21(DE3)/pLysS carrying pET16B-DinB, as described by Wagner et al. [4]. The cells were grown in M9 minimal medium containing 50 μ g/mL ampicillin and 30 μ g/mL chloramphenicol. Overnight cultures (5 mL) were inoculated into 500 mL of prewarmed LB medium containing 50 μ g/mL ampicillin. The culture was incubated with shaking at 37°C until the optical density at 600 nm reached about 0.9, and expression of the proteins was induced by adding IPTG to a final concentration of 1 mM. After 30 min of incubation at 30°C, rifampicin was added to a final concentration of 100 μ g/mL, and incubation was continued for an additional 3 hr at 30°C. *E. coli* cells were then harvested, washed once in ice-cold buffer A (50 mM Tris-HCl (pH 8.0), 300 mM NaCl and 20 mM imidazole), resuspended in a total volume of 8 mL of the same buffer, and frozen in dry ice/ethanol bath. Frozen cells were thawed and supplemented with 1 mg of chicken egg lysozyme, 0.4 mg of pefabloc SC, and β -mercaptoethanol (EtSH) to a final concentration of 20 mM. Chromosomal DNA was sheared by sonication, and lysates were treated with DNase I at 40 mg/mL and RNase TI at 130 U/mL for 10 min at room temperature. The final volume was then adjusted to 15 mL with buffer A supplemented with 20 mM EtSH, and the cell lysate was cleared by centrifugation at 13,000 \times g. The supernatant was applied to a 2-mL column connected to a FPLC system. The column was washed with 20 mL of NaCl and then developed with a linear gradient of imidazole up to 1 M. DinB protein started to elute at approximately 300 mM imidazole. Fractions containing His-tag DinB were combined and concentrated to about 2.6 mL containing 5.6 mg/mL His-tag DinB (fraction 2). Fraction 2 was then applied to a Superdex 75 XK 16/60 column connected to an FPLC system equilibrated with 20 mM HEPES (pH 7.4), 150 mM NaCl, 0.1 mM EDTA, 10% w/v glycerol, and 1 mM dithiothreitol (DTT). Fractions corresponding to the DinB peak, which eluted at an approximate molecular weight of 32 kDa, were combined together to give a total volume of 3 mL containing 8 mg of pure HT-dinB. The purified protein was stored at -80°C.

2.4. In Vitro DNA Synthesis. Primers were labeled at the 5'-end with [γ -³²P]ATP by T4 polynucleotide kinase and annealed with the appropriate template oligonucleotides.

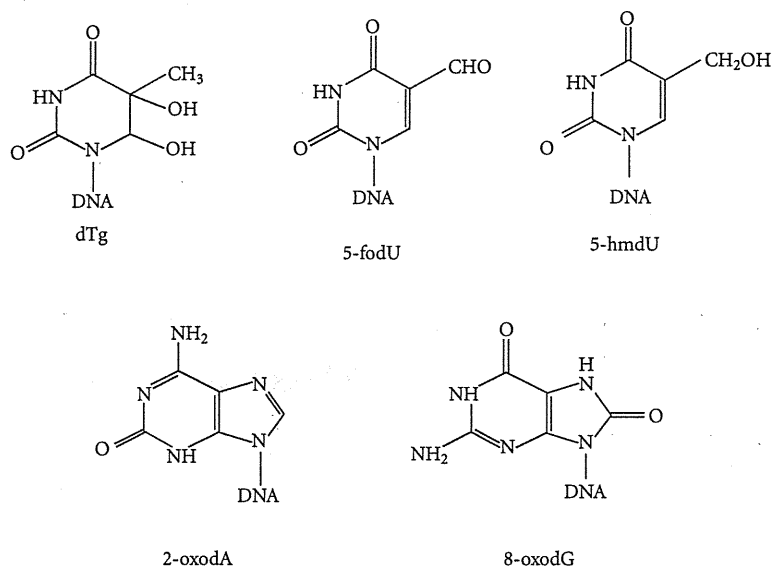


FIGURE 1: The structures of the studied oxidatively damaged bases, thymine glycol (dTg), 5-formyluracil (5-fodU), 5-hydroxymethyluracil (5-hmdU), 1,2-dihydro-2-oxoadenine (2-oxodA), and 7,8-dihydro-8-oxoguanine (8-oxodG).

TABLE 1: Nucleotide sequences of oligonucleotides used in this study.

Template 1	3'-CGTCGGGCCCCCTA A GTGATCAAG-5'
Template 2	3'-CGTCGGGCCCCCTA 2 GTGATCAAG-5'
Template 3	3'-CGTCGGGCCCCCTA G GTGATCAAG-5'
Template 4	3'-CGTCGGGCCCCCTA 8 GTGATCAAG-5'
Template 5	3'-ACGTCCAGCTCACATC T CCTAG-5'
Template 6	3'-ACGTCCAGCTCACATC F CCTAG-5'
Template 7	3'-ACGTCCAGCTCACATC H CCTAG-5'
Template 8	3'-CGACGGGCCCCCA A TGAGAACAAG-5'
Template 9	3'-CGACGGGCCCCCA X GAGAACAAG-5'
Primer 1	5'- ³² P-GCTGCCCGGGGGTT-3'
Primer 2	5'- ³² P-TGCAGGTCGACTCTAG-3'
Primer 3	5'- ³² P-GCAGCCCGGGGGAT-3'

2, F, H, 8, and X represent 1,2-dihydro-2-oxoadenine (2-oxodA), 5-formyluracil (5-fodU), 5-hydroxymethyluracil (5-hmdU), 7,8-dihydro-8-oxoguanine (8-oxodG), and thymine glycol (dTg), respectively.

The substrates thus prepared were incubated with purified Pol IV in a reaction mixture (10 μ l) containing 30 mM KH_2PO_4 (pH 7.4), 7.5 mM MgCl_2 , 1 mM DTT, 5 mM NaCl, 0.1 mg/mL BSA, 5% glycerol, and 150 mM of the specified deoxyribonucleotide(s). The amount of Pol IV and incubation time are described in the figure legends. After incubation at 20°C, the reaction was terminated by addition of stop solution (95% formamide, 20 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol). The samples were then heated at 95°C for 5 min, and immediately cooled on ice and then loaded onto 20% polyacrylamide gels in 90 mM Tris-borate (pH 8.3) containing 7 M urea and 2 mM EDTA. After electrophoresis at 1,300 V, the gels were dried

and autoradiographed using Fuji RX films at -80°C. The intensity of each band was determined using an imaging analyzer (Fuji BAS 1800II).

2.5. In Vivo Mutagenesis. Single colonies of *E. coli* CC101, CC105, and CC106 [20] transformed with a plasmid encoding wild-type pDinB or a mutant Pol IV (pDinB003) [4] were inoculated into a minimal glucose medium containing 50 μ g/mL of ampicillin and incubated at 37°C for 40 hr. The mutant Pol IV was generated by site-specific mutagenesis replacing aspartic acid in position 103 with asparagine [4]. The cultures were centrifuged, resuspended in the same medium with 1 mM of IPTG, and further incubated for 5 hr at 37°C. Hydrogen peroxide (H_2O_2) was added to the cultures at a final concentration of 10 mM, followed by incubation at 37°C for 1 hr. The cultures were centrifuged, washed, and resuspended in prewarmed LB medium. After incubation to stationary phase and appropriate dilution, the cell suspensions were spread on both duplicate minimal lactose plates and minimal glucose plates for the detection of lactose-fermenting (Lac^+) revertants and viable cells, respectively. Mutant colonies on the plate were counted after incubation overnight. The mutation frequency was expressed as number of mutants/ 10^8 viable cells.

3. Results

3.1. Replication of DNA Containing dTg by Pol IV. In this study, primer extension assays were performed with Pol IV purified by affinity chromatography to examine whether Pol IV could bypass oxidatively damaged bases. Oligonucleotides containing dTg, 5-fodU, 5-hmdU, 8-oxodG, or 2-oxodA (Table 1) were annealed to appropriate primers and

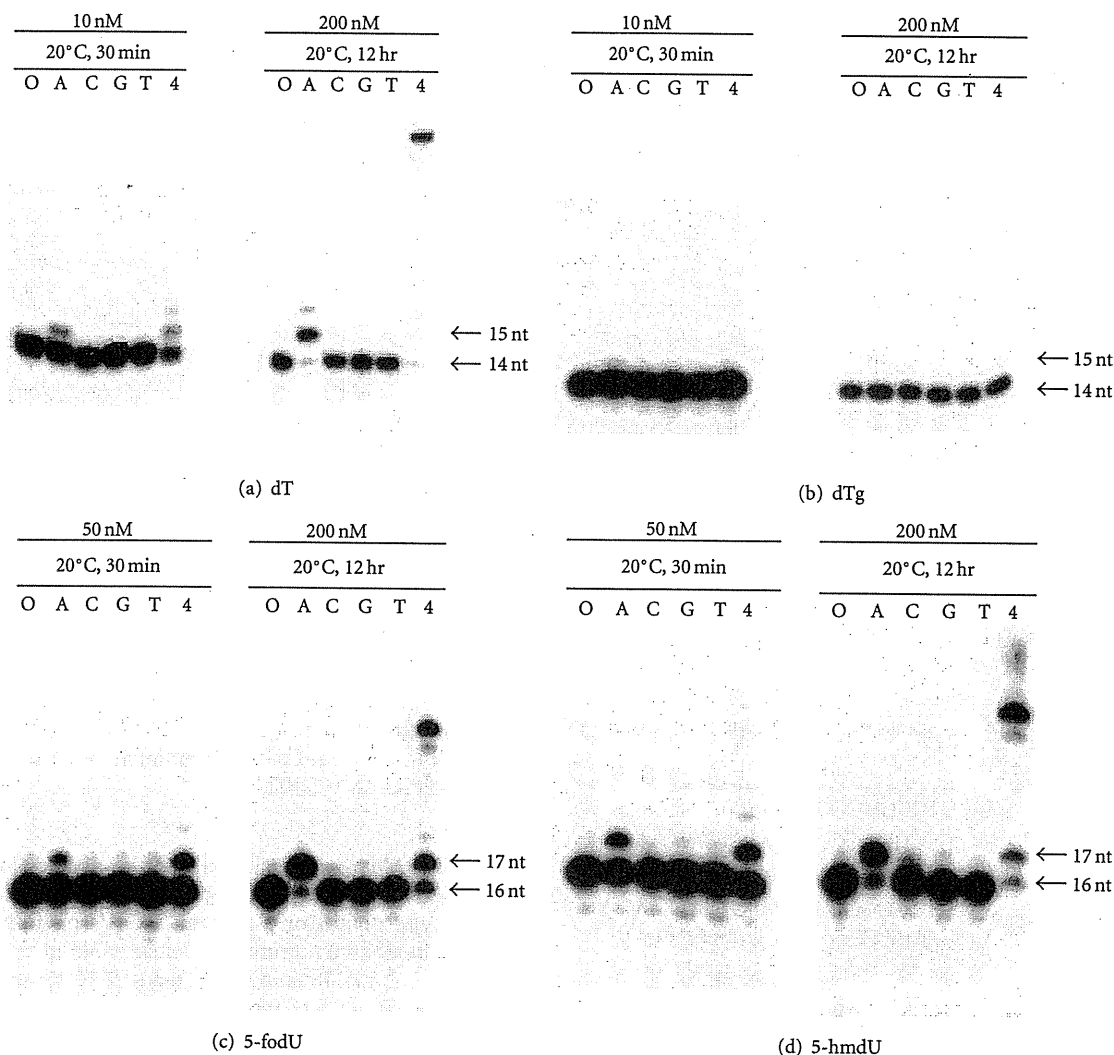


FIGURE 2: Primer extension assay for Pol IV to bypass dTg, 5-fodU, and 5-hmdU in the template oligonucleotides. Primers were labeled at the 5'-terminal by polynucleotide kinase and annealed with appropriate template oligonucleotide. Primer/templates (50 fmol) in a 10- μ l reaction mixture was incubated at 20°C with purified Pol IV at 50 nM for 30 min (left) and at 200 nM for 12 hr (right), followed by polyacrylamide gel electrophoresis. (a) dT (primer 1/template 8), (b) dTg (primer 1/template 9), (c) 5-fodU (primer 2/template 6), and (d) 5-hmdU (primer 2/template 7).

incubated with purified Pol IV. Pol IV was added to the reaction mixture at 50 and 200 nM.

dTg is a major oxidative product of thymine in DNA and blocks DNA synthesis by most DNA polymerases [12, 15]. However, certain TLS polymerases such as a human Pol Nu can occasionally bypass dTg and thereby continue DNA replication beyond the lesion [12, 21–23]. Hence, it was of interest to examine whether Pol IV has the ability to bypass dTg. In this study, we prepared oligonucleotide containing dTg by osmium tetroxide treatment [17]. Pol IV replicated the oligonucleotides containing undamaged thymine (Figure 2(a)). As shown in Figure 2(b), we could not detect the insertion of dNTPs opposite the lesion, while a faint band of dATP inserted was seen when the oligonucleotide was incubated with Pol IV reacted at 200 nM for 12 hr.

3.2. Insertion of dATP and dCTP opposite 8-oxodG in the Template DNA by Pol IV. 8-oxodG is a major oxidative product of guanine and has high miscoding potential [12–16, 24]. Replicative DNA polymerases insert dATP as frequently as dCTP opposite 8-oxodG in the template. Therefore, G:C \rightarrow T:A transversions occur at the site of 8-oxodG [12–16].

In this study, when Pol IV was incubated at 50 nM with the template oligonucleotide containing 8-oxodG for 30 min, no nucleotides were inserted opposite the lesion. However, when added at 200 nM and incubated with the substrate for 12 hr, Pol IV inserted both dCTP and dATP opposite 8-oxodG (Figure 3(a)). These results indicate that Pol IV could bypass over 8-oxodG through both error-free and error-prone processes. However, the Pol IV's insertion ability was very low.

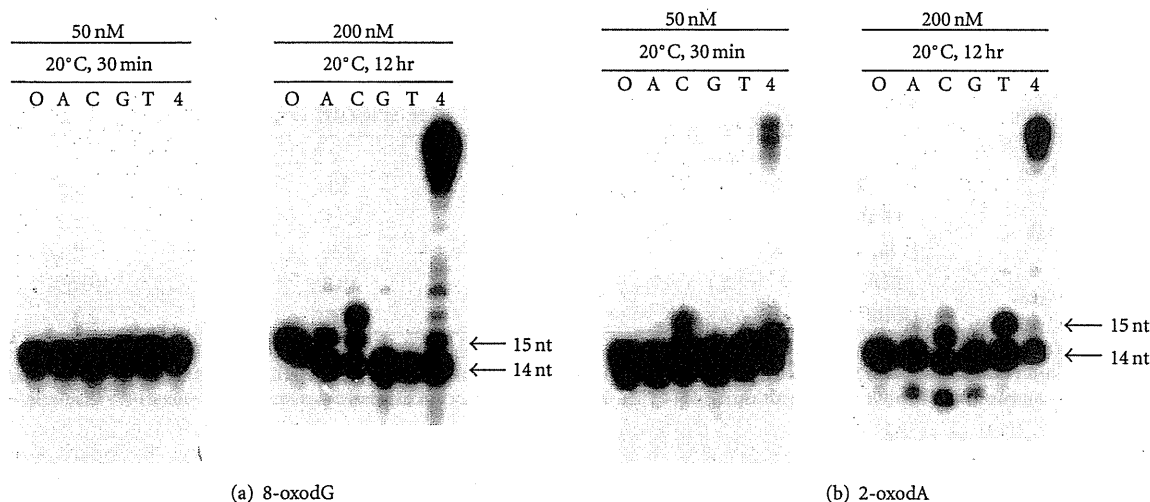


FIGURE 3: Primer extension assay for Pol IV to bypass 8-oxodG and 2-oxodA in the template oligonucleotide. Primer 3 was labeled at the 5'-terminal by polynucleotide kinase and annealed with appropriate template oligonucleotides. Primer 3/templates (50 fmol) in a 10- μ l reaction mixture were incubated at 20°C with purified Pol IV at 50 nM for 30 min (left) and at 200 nM for 12 hr (right), followed by polyacrylamide gel electrophoresis. (a) 8-oxodG (primer 3/template 4), (b) 2-oxodA (primer 3/template 2).

3.3. *Replication of DNA Containing 5-fodU and 5-hmdU by Pol IV.* 5-fodU and 5-hmdU are major products of oxidative damage of the methyl group of thymine [12, 19, 25]. Attack of hydroxyl radicals on the 5-methyl group generates 5-hydroperoxymethyluracil, the most stable thymine hydroperoxide [12]. It decomposes to the more stable products 5-fodU and 5-hmdU [12, 25]. Recent studies showed that 5-fodU is a potentially mutagenic lesion [25, 26]. It directs insertion of mismatched bases opposite the lesion during DNA synthesis *in vitro* [27]. We previously showed that Klenow fragment with and without 3' \rightarrow 5' exonuclease (KFexo⁺ and KFexo⁻, resp.), *Thermus thermophilus* (Tth) DNA polymerase (exonuclease-deficient) and *Pyrococcus furiosus* (Pfu) DNA polymerase (exonuclease-proficient) read through the site of 5-fodU in the template [27]. 5-fodU directs insertion of dCMP and dGMP in addition to dATP opposite the lesion by these DNA polymerases. Furthermore, KFexo⁻ and Tth can bypass the 5-hmdU template via the insertion of dAMP opposite the 5-hmdU [27].

The primer extension assays for the templates containing 5-fodU and 5-hmdU showed that Pol IV preferred to insert dATP opposite 5-fodU and 5-hmdU, while other dNTP insertions were <5% (Figures 2(c) and 2(d)). Pol IV synthesized full-length duplex 22-mer oligonucleotides when added at 200 nM and incubated for 12 hr (Figures 2(c) and 2(d)).

3.4. *Bypass of 2-oxodA by Pol IV.* 2-oxodA is a common product of adenine generated by ROS [12, 28]. Previous studies showed that replicative DNA polymerases and KFexo⁻ mainly insert dATP and dGTP opposite 2-oxodA during DNA synthesis *in vitro* [12, 29, 30]. It is important to elucidate the mechanism of bypass 2-oxodA by Pol IV.

TABLE 2: The insertion kinetics of dCTP and dTTP opposite 1,2-dihydro-2-oxoadenine (2-oxodA) by Pol IV.

Substrate	K _m (fmol/ mL /min)	V _{max} (min ⁻¹)	k _{cat}	k _{cat} /K _m
dCTP	8.5	217.4	4.4 × 10 ⁻³	5.2 × 10 ⁻⁴
dTTP	25.6	42.9	8.6 × 10 ⁻⁴	3.4 × 10 ⁻⁵

Primer 3 was labeled at the 5'-terminal by polynucleotide kinase and annealed with appropriate template oligonucleotides. Primer 3/template 2 (50 fmol) in a 10- μ l reaction mixture was incubated at 20°C for 60 min with purified Pol IV (50 nM) in the presence of dTTP or dCTP at various concentrations (0.02~500 μ M), followed by polyacrylamide gel electrophoresis.

2-oxodA significantly reduced the rate of DNA synthesis by KFexo⁻ (data not shown). When incubated with templates containing 2-oxodA, Pol IV inserted both dTTP and dCTP opposite 2-oxodA. It was evident that Pol IV inserted dCTP more efficiently than dTTP (Figure 3(b)). The results were obtained by a comparison of the full-length products obtained by *in vitro* DNA synthesis in the presence of the four nucleotides. We determined the insertion kinetics of Pol IV for dTTP and dCTP opposite 2-oxodA. Comparing the k_{cat}/K_m values revealed that Pol IV inserted dCTP opposite 2-oxodA with nearly 30 fold greater catalytic efficiency than dTTP (Table 2). It is suggested that 2-oxodA in DNA induces A:T \rightarrow G:C transitions.

3.5. *Overexpression of Pol IV Causes A:T \rightarrow G:C Transitions In Vivo.* To clarify the roles of Pol IV in mutation induction *in vivo*, we carried out the *in vivo* mutagenesis assay with *E. coli* strains bearing a F[']lac containing a lacZ allele, which codes for inactive β -galactosidase [20]. Unless base

TABLE 3: Frequencies of mutations to Lac⁺ in *E. coli* CC101~CC106 strains with overexpressed *dinB* gene after incubation with hydrogen peroxide.

Strain	Base substitution	Plasmid	Mutants/10 ⁸ viable cells		Increase in mutation frequency (b - a)	Fold increase in mutation frequency (b/a)
			No H ₂ O ₂ ^a	10 mM H ₂ O ₂ ^b		
CC101	A:T → C:G	pDinB	1.9	4.2	2.3	2.2
		pDinB003	1.5	1.8	0.3	1.2
CC105	A:T → T:A	pDinB	3.6	4.4	0.8	1.2
		pDinB003	1.5	3.4	1.9	2.3
CC106	A:T → G:C	pDinB	1.1	11.6	10.5	10.5
		pDinB003	0.8	1.8	1.0	2.3

E. coli CC101, CC105, and CC106 transformed with a plasmid wild-type plasmid (pDinB) and a mutant Pol IV (pDinB003) in stationary phase were incubated at 37°C with 1 mM of IPTG for 5 hr, followed by the treatment 10 mM H₂O₂ for 1 hr. The mutant Pol IV (DinB003) was generated by site-specific mutagenesis replacing aspartic acid in position 103 with asparagine [4]. The mutation frequency was expressed as number of mutants/10⁸ viable cells.

substitution mutations occur, these *E. coli* cells cannot grow on lactose minimal medium. To determine the type of base substitutions caused by overexpression of Pol IV, we constructed *E. coli* CC101, CC105, and CC106 strains [20] with pDinB or a mutant Pol IV [4], where these *dinB* genes were overexpressed. The mutant Pol IV was generated by site-specific mutagenesis replacing aspartic acid in position 103 with asparagine [4].

2-oxodA has been shown to occur in DNA by treatment of cells with hydrogen peroxide (H₂O₂) [12, 28]. Hence, the *E. coli* cells were exposed to H₂O₂ and the Lac⁺ reversion frequency was measured. Compared with CC101 and CC105, CC106 with the pDinB plasmid showed a significant increase in the frequency of Lac⁺ reversions when exposed to hydrogen peroxide (Table 3). Hydrogen peroxide treatment caused an ~2-fold increase in A:T → G:C mutations in *E. coli*, while the increase was significantly greater (~10-fold) in *E. coli* overexpressing Pol IV. The overexpression of Pol IV had its greater effect on A:T → G:C mutations than A:T → C:G and A:T → T:A mutations. The enhancement of A:T → G:C mutations depended on the Pol IV ability, since the expression of a mutant Pol IV lacking the polymerase activity did not increase the mutation frequency in *E. coli* CC106 exposed to H₂O₂. As *E. coli* CC106 can reverse Lac⁺ only through A:T → G:C transitions [20], these results indicate that A:T → G:C transitions are induced via an error-prone translesion DNA synthesis by Pol IV in *E. coli* cells.

4. Discussion

Recent progress in research about novel types of DNA polymerase in prokaryotes and eukaryotes has given us much information about the mechanism of bypass replication of damaged bases in DNA and mutation induction. DNA polymerases of the Y-family are involved in translesion DNA synthesis [1–3, 7–11]. Replication prevention is caused by base modifications induced by various DNA-damaging agents, such as ROS [1–3, 12, 15, 29, 30]. Purine and pyrimidine bases in DNA are easily oxidized by ROS, which leads to abnormal DNA behavior, including DNA replication prevention. In addition, damaged bases induce several types

of mutations, including base substitutions. Replication errors must occur at the sites of damaged bases to be fixed as mutations. Certain TLS DNA polymerases, such as Pol IV, catalyze the insertion of nucleotides opposite damaged bases and thereby may play a role in mutation induction [4–8]. Continuing of DNA replication beyond the lesion is required for maintenance of the genome integrity.

dTg blocks DNA synthesis by many prokaryotic and eukaryotic DNA polymerases one nucleotide before and opposite the lesion site [12, 15, 31]. On the other hand, some DNA polymerases inefficiently insert noncognate nucleotides opposite dTg [12, 22]. DNA polymerase η and κ are able to continue synthesis after having inserted dATP opposite the lesion [12, 21–23]. Pol ζ also contributes to the bypass of dTg as well as other lesions that block synthesis by replicative DNA polymerases [32]. In contrast, Pol IV did not have the ability to bypass dTg (Figure 2(b)).

There are four diastereomers of dTg [31, 33]. dTg exists in solution as either the 5R *cis-trans* pair or the 5S *cis-trans* pair, due to epimerization at the C₆ position. The bypass over dTg by Y-family DNA polymerases is stereospecific [21–23, 32]. Pol ζ bypasses the 5R epimers more efficiently [32], while Pol κ bypasses the 5S epimers more efficiently [22]. Pol Nu has been shown to be particularly adept at efficient and accurate translesion DNA synthesis past a 5S-thymine glycol [21].

8-oxodG is not a replicative block for replicative DNA polymerases, which incorporate dATP as frequently as dCTP opposite 8-oxodG in the template [12–16, 24]. As a result, G:C → T:A transversions occur at the site of 8-oxoG. We showed here that Pol IV inserted dATP opposite 8-oxoG in the template, but the insertion efficiency was very low (Figure 3(a)). These results indicate that Pol IV preferred to insert dCTP opposite 8-oxodG. Recently, Maga et al. [34] also reported that DNA polymerases λ and η can bypass 8-oxodG by insertion of dCTP opposite 8-oxodG in the template. Therefore, it is likely that these Pols are a principal player in mutation induction by template 8-oxodG.

Oxidation of the 5-methyl group of thymine produces the stable products 5-fodU and 5-hmdU in DNA [12, 25]. When 5-fodU is produced in a template DNA, base substitutions are induced at the site of the lesion [25, 26].

We previously showed that 5-fodU is removed from DNA in *E. coli* by three DNA glycosylases, MutM, endonuclease III (Nth), and endonuclease VIII [35]. The frequency of spontaneous mutations is significantly enhanced in *E. coli* *mutM nth nei* triple mutant compared with the wild-type strain [35]. The results in Figure 2(c) demonstrate, for the first time, that if 5-fodU remains in the template strand, Pol IV prefers to insert dATP opposite the lesion and hence induces no mutations.

5-hmU directs the insertion of only dATP during normal DNA replication [12, 15, 27]. The present results indicate that Pol IV preferred to insert dATP at the site of 5-hmdU (Figure 2(d)) and as a result does not make base substitutions. The results are in accord with the finding that a *Bacillus subtilis* bacteriophage (SPO1) has 5-hmdU in its DNA instead of thymine and 5-hmdU: dA base-pairs show the same behavior as T:A [36]. On the other hand, *E. coli* and mammalian cells have DNA glycosylases that remove 5-hmdU from DNA [12, 37, 38]. Why do cells possess repair enzymes for 5-hmdU that forms stable base pairs with adenine? There is another pathway of 5-hmdU generation: oxidation and deamination of 5-methylcytosine (5-mdC). 5-mC occurs naturally in DNA as a product of cytosine methylation [37]. Therefore, a normal base pair between 5-mdC and dG generates a mismatched base pair of 5-hmdU:dG, which would cause 5-mC (C):G to T:A transitions. Recently, we found that a 5-hmdU DNA glycosylase activity of MutM and Nth removes 5-hmdU from 5-hmdU: dG mispairs with 37~58 times greater efficiency, respectively, than that from 5-hmdU: dA base pairs [38].

2-oxodA (isoguanine) is a common lesion of adenine produced by ROS and ionizing radiation. It is a replicative block for several DNA polymerases [12]. However, 2-oxodA has a mutational potential comparable to that of 8-oxodG in bacteria and mammalian cells. Barone et al. [39] found that insertion opposite 2-oxodA is difficult for both KFlexo⁻ and the replicative Pol α . A template 2-oxodA might cause a transient replication block, thereby provoking recruitment of TLS polymerases. Archeal Dpo4 is efficient to insert nucleotides opposite 2-oxodA, while human Pol η inefficient [39]. Replication of a template 2-oxodA by these polymerases is mutagenic and causes base substitutions. On the other hand, Crespan et al. [40] showed that 2-oxodA can be efficiently and faithfully bypassed by a human DNA Pol λ in combination with proliferating cell nuclear antigen (PCNA) and replication protein A (RP-A). Thus, the efficiency and fidelity of TLS on the 2-oxodA template depend upon the DNA polymerase used.

We found that "incorrect" dCTP was effectively incorporated opposite 2-oxodA by Pol IV (Figure 3(b)), suggesting a stable base pair between 2-oxodA and dC. Thermodynamic analysis also showed that 2-oxodA forms a stable base pair with cytosine, guanine, and thymine, and to a lesser extent with adenine [18, 39]. The base-pairing scheme of 2-oxodA (isoguanine):dC has been postulated [41]. 2-oxodA:dC is a potent inducer of parallel-stranded DNA duplex structure. All imino protons associated with the 2-oxodA:dC basepairs are consistent with the formation of a stable duplex suggested by T_m measurements [18]. 2-oxodA might form stable

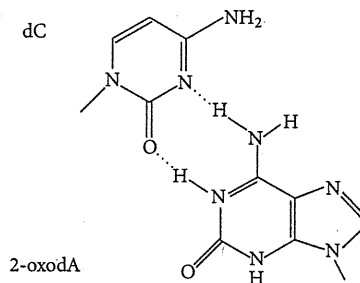


FIGURE 4: Possible wobble structure for base pairing of the 1,2-dihydro-2-oxoadenine (2-oxodA):dC.

reverse Watson-Crick basepair with the normal dC [41]. However, the base pairing would not be present in the active site of a DNA polymerase, as the sugar-triphosphate moiety could not fit properly in the active site. Even if it could fit, the sugar-triphosphate moiety would not be properly oriented for catalysis. Alternatively, a wobble structure with two hydrogen bonds is possible as shown in Figure 4. No equivalent structure is possible for 2-oxodA:dT.

2-oxodA is as mutagenic as 8-oxodG when a double-stranded shuttle vector DNA containing 2-oxodA is replicated in *E. coli* and mammalian cells. Bypass of 2-oxodA results in the formation of A:T \rightarrow G:C transitions and A:T \rightarrow T:A transversions during leading strand synthesis [12, 29, 30]. Moreover, we observed here that a template 2-oxodA directs the insertion of "incorrect" dCTP more efficiently than that of "correct" dTTP (Figure 3(b)) *in vitro*. Based on this together with the *in vivo* mutagenesis data (Table 3), we conclude that Pol IV has the ability to bypass 2-oxodA and induce A:T \rightarrow G:C transitions at the site of 2-oxodA. Pol IV overexpression has its greater effect on A:T \rightarrow G:C mutations than A:T \rightarrow C:G and A:T \rightarrow T:A mutations. The facts may reflect that Pol IV is accurately handling a lesion leading A:T \rightarrow C:G and A:T \rightarrow T:A mutations that some other DNA Pol tends to handle inaccurately. To prevent mutation induction, *E. coli* MutY and human MUTYH proteins have been shown to remove 2-oxodA from double-stranded DNA *in vitro* [42, 43].

The generation of 2-oxodA in double-stranded DNA by a Fenton-like reagent is less efficient than 8-oxodG [12, 30]. On the other hand, the yield of 2-oxodA is similar to that of 8-oxodG in the monomeric form [12, 30]. Thus, it is likely that most 2-oxodA that occurs in DNA arises through insertion of 2-oxo-dATP generated in the nucleotide pool. 2-oxo-dATP is inserted opposite G and T in the template by DNA polymerase III and DNA polymerase I *in vitro* [30, 44, 45]. On the other hand, calf thymus Pol α inserts 2-oxo-dATP opposite noncognant C in addition to cognant T, which would cause induction of G:C \rightarrow A:T transitions *in vivo*. Hydrogen peroxide treatment caused a ~2-fold increase in A:T \rightarrow G:C mutations in *E. coli*, while the increase was significantly greater (~10-fold) in *E. coli* overexpressing Pol IV (Table 3). These results indicate that Pol IV may involved in these ROS-enhanced A:T \rightarrow G:C mutations. It is also likely that the UmuDC gene products may play a critical role in the

mutagenesis by damaged nucleotides, such as 2-oxodA, that block DNA replication.

Pol IV is relatively efficient on 2-oxodA, 5-fodU and 5-hmdU, but inefficient on dTg and 8-oxodG. Of these five lesions, Pol IV is most efficient with 2-oxodA. In 2-oxodA the $-C=O$ is at C2 and in the minor groove. Pol IV has consistently proven to be relatively effective at handling bulky N2-dG adducts, which is the same positioning as the extra oxygen in 2-oxodA. This may be achieved by stabilization of the 2-oxodA conformation in the active site through specific interactions between Pol IV and 2-oxodA. The other lesions have bulk in the major groove. The 5-position of pyrimidines is relatively away from the DNA backbone, which might explain the ability of Pol IV to handle 5-fodU and 5-hmdU. While dTg has extra bulk at C5, it also has bulk at C4, and 8-oxodG has bulk at C8G. Both C4T and C8G are closer to the DNA backbone. Pol IV may have something in its structure that cause a steric impediment in the cases of the extra bulk at C4 in dTg and at C8 in 8-oxodG.

Certain TLS polymerases can perform proficient and moderately accurate bypass of particular types of DNA damage, while some other polymerases continue synthesis after having inserted "incorrect" bases opposite the lesion. The differences in the chemical structure of the lesions and the association between polymerases and the lesion and nucleotides inserted may affect how for that structural information in the altered bases contributes to nucleotide selection during insertion opposite these lesions by these polymerases.

5. Conclusion

In this paper, we examined whether Pol IV can bypass oxidatively damaged bases and is involved in mutation induction at the damaged sites. Recombinant Pol IV was incubated *in vitro* with chemically synthesized oligonucleotides containing dTg, 5-fodU, 5-hmdU, 8-oxodG, and 2-oxodA. Pol IV preferred to insert dA opposite 5-fodU and 5-hmdU, while it did not insert any nucleotides opposite dTg. Pol IV inserted dA and dC opposite template 8-oxodG, while the Pol IV's ability was low. Pol IV inserted dCTP more efficiently than dTTP opposite 2-oxodA in DNA, suggesting that 2-oxodA in the template DNA has mutagenic potential leading to A:T → G:C transitions. Hydrogen peroxide treatment caused a ~2-fold increase in A:T → G:C mutations in *E. coli*, while the increase was significantly greater (~10-fold) in *E. coli* overexpressing Pol IV. These results indicate that Pol IV may play an important role in mutagenesis by 2-oxodA in *E. coli*.

Acknowledgments

The authors thank Dr. Elizabeth Nakajima for critically reading the paper. The authors wish to express their gratitude to the reviewers for helpful and excellent comments and discussion. This paper was financially supported in part by Grants-in-Aid for Science Research nos. 19510056, 21510056 from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to Q-M. Zhang-Akiyama) and Global

Center of Excellence Program "Formation of a Strategic Base for Biodiversity and Evolutionary Research (A06): from Genome to Ecosystem". The authors are also grateful to Takeda Science Foundation (Osaka) and the Central Research Institute of Electric Power Industry (Tokyo) for supporting Q-M. Zhang-Akiyama.

References

- [1] H. Ohmori, E. C. Friedberg, R. P. P. Fuchs et al., "The Y-family of DNA polymerases," *Molecular Cell*, vol. 8, no. 1, pp. 7–8, 2001.
- [2] D. F. Jarosz, P. J. Beuning, S. E. Cohen, and G. C. Walker, "Y-family DNA polymerases in *Escherichia coli*," *Trends in Microbiology*, vol. 15, no. 2, pp. 70–77, 2007.
- [3] A. R. Lehmann, A. Niimi, T. Ogi et al., "Translesion synthesis: Y-family polymerases and the polymerase switch," *DNA Repair*, vol. 6, no. 7, pp. 891–899, 2007.
- [4] J. Wagner, P. Gruz, S.-R. Kim et al., "The dinB gene encodes a novel *E. coli* DNA polymerase, DNA pol IV, involved in mutagenesis," *Molecular Cell*, vol. 4, no. 2, pp. 281–286, 1999.
- [5] M. Tang, P. Pham, X. Shen et al., "Roles of *E. coli* DNA polymerases IV and V in lesion-targeted and untargeted SOS mutagenesis," *Nature*, vol. 404, no. 6781, pp. 1014–1018, 2000.
- [6] R. S. Galhardo, R. Do, M. Yamada et al., "DinB upregulation is the sole role of the SOS response in stress-induced mutagenesis in *Escherichia coli*," *Genetics*, vol. 182, no. 1, pp. 55–68, 2009.
- [7] R. Napolitano, R. Janel-Bintz, J. Wagner, and R. P. P. Fuchs, "All three SOS-inducible DNA polymerases (Pol II, Pol IV and Pol V) are involved in induced mutagenesis," *EMBO Journal*, vol. 19, no. 22, pp. 6259–6265, 2000.
- [8] S.-R. Kim, K. Matsui, M. Yamada, P. Gruz, and T. Nohmi, "Roles of chromosomal and episomal dinB genes encoding DNA pol IV in targeted and untargeted mutagenesis in *Escherichia coli*," *Molecular Genetics and Genomics*, vol. 266, no. 2, pp. 207–215, 2001.
- [9] D. F. Jarosz, V. G. Godoy, J. C. Delaney, J. M. Essigmann, and G. C. Walker, "A single amino acid governs enhanced activity of DinB DNA polymerases on damaged templates," *Nature*, vol. 439, no. 7073, pp. 225–228, 2006.
- [10] B. Yuan, H. Cao, Y. Jiang, H. Hong, and Y. Wang, "Efficient and accurate bypass of N2-(1-carboxyethyl)-2'-deoxyguanosine by DinB DNA polymerase in vitro and in vivo," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 25, pp. 8679–8684, 2008.
- [11] A. Kumari, I. G. Minko, M. B. Harbut, S. E. Finkel, M. F. Goodman, and R. S. Lloyd, "Replication bypass of interstrand cross-link intermediates by *Escherichia coli* DNA polymerase IV," *Journal of Biological Chemistry*, vol. 283, no. 41, pp. 27433–27437, 2008.
- [12] S. Bjelland and E. Seeberg, "Mutagenicity, toxicity and repair of DNA base damage induced by oxidation," *Mutation Research*, vol. 531, no. 1-2, pp. 37–80, 2003.
- [13] J. Cadet, T. Delatour, T. Douki et al., "Hydroxyl radicals and DNA base damage," *Mutation Research*, vol. 424, no. 1-2, pp. 9–21, 1999.
- [14] B. N. Ames, M. K. Shigenaga, and T. M. Hagen, "Oxidants, antioxidants, and the degenerative diseases of aging," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 17, pp. 7915–7922, 1993.

- [15] S. S. Wallace, "Biological consequences of free radical-damaged DNA bases," *Free Radical Biology and Medicine*, vol. 33, no. 1, pp. 1–14, 2002.
- [16] S. S. David, V. L. O'Shea, and S. Kundu, "Base-excision repair of oxidative DNA damage," *Nature*, vol. 447, no. 7147, pp. 941–950, 2007.
- [17] G. L. Dianov, T. Thybo, I. I. Dianova, L. J. Lipinski, and V. A. Bohr, "Single nucleotide patch base excision repair is the major pathway for removal of thymine glycol from DNA in human cell extracts," *Journal of Biological Chemistry*, vol. 275, no. 16, pp. 11809–11813, 2000.
- [18] H. Sugiyama, S. Ikeda, and I. Saito, "Remarkably stable parallel-stranded oligonucleotides containing 5-methylisocytosine and isoquinine," *Journal of the American Chemical Society*, vol. 118, no. 41, pp. 9994–9995, 1996.
- [19] H. Sugiyama, S. Matsuda, K. Kino, Q.-M. Zhang, S. Yonei, and I. Saito, "New synthetic method of 5-formyluracil-containing oligonucleotides and their melting behavior," *Tetrahedron Letters*, vol. 37, no. 50, pp. 9067–9070, 1996.
- [20] C. G. Cupples and J. H. Miller, "A set of *lacZ* mutations in *Escherichia coli* that allow rapid detection of each of the six base substitutions," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 86, no. 14, pp. 5345–5349, 1989.
- [21] K.-I. Takata, T. Shimizu, S. Iwai, and R. D. Wood, "Human DNA polymerase η (POLN) is a low fidelity enzyme capable of error-free bypass of 5S-thymine glycol," *Journal of Biological Chemistry*, vol. 281, no. 33, pp. 23445–23455, 2006.
- [22] P. L. Fischhaber, V. L. Gerlach, W. J. Feaver, Z. Hatahet, S. S. Wallace, and E. C. Friedberg, "Human DNA polymerase κ bypasses and extends beyond thymine glycols during translesion synthesis in vitro, preferentially incorporating correct nucleotides," *Journal of Biological Chemistry*, vol. 277, no. 40, pp. 37604–37611, 2002.
- [23] R. Kusumoto, C. Masutani, S. Iwai, and F. Hanaoka, "Translesion synthesis by human DNA polymerase η across thymine glycol lesions," *Biochemistry*, vol. 41, no. 19, pp. 6090–6099, 2002.
- [24] G. W. Hsu, M. Ober, T. Carell, and L. S. Beese, "Error-prone replication of oxidatively damaged DNA by a high-fidelity DNA polymerase," *Nature*, vol. 431, no. 7005, pp. 217–221, 2004.
- [25] S. Bjelland, H. Ånensen, I. Knævelsrud, and E. Seeberg, "Cellular effects of 5-formyluracil in DNA," *Mutation Research*, vol. 486, no. 2, pp. 147–154, 2001.
- [26] I. Miyabe, Q.-M. Zhang, H. Sugiyama, K. Kino, and S. Yonei, "Mutagenic effects of 5-formyluracil on a plasmid vector during replication in *Escherichia coli*," *International Journal of Radiation Biology*, vol. 77, no. 1, pp. 53–58, 2001.
- [27] Q.-M. Zhang, H. Sugiyama, I. Miyabe et al., "Replication in vitro and cleavage by restriction endonuclease of 5-formyluracil- and 5-hydroxymethyluracil-containing oligonucleotides," *International Journal of Radiation Biology*, vol. 75, no. 1, pp. 59–65, 1999.
- [28] Z. Nackerdien, K. S. Kasprzak, G. Rao, B. Halliwell, and M. Dizdaroglu, "Nickel(II)- and cobalt(II)-dependent damage by hydrogen peroxide to the DNA bases in isolated human chromatin," *Cancer Research*, vol. 51, no. 21, pp. 5837–5842, 1991.
- [29] H. Kamiya and H. Kasai, "Mutations induced by 2-hydroxyadenine on a shuttle vector during leading and lagging strand syntheses in mammalian cells," *Biochemistry*, vol. 36, no. 37, pp. 11125–11130, 1997.
- [30] H. Kamiya, "Mutagenicities of 8-hydroxyguanine and 2-hydroxyadenine produced by reactive oxygen species," *Biological and Pharmaceutical Bulletin*, vol. 27, no. 4, pp. 475–479, 2004.
- [31] H. Miller, A. S. Fernandes, E. Zaika et al., "Stereoselective excision of thymine glycol from oxidatively damaged DNA," *Nucleic Acids Research*, vol. 32, no. 1, pp. 338–345, 2004.
- [32] R. E. Johnson, S.-L. Yu, S. Prakash, and L. Prakash, "Yeast DNA polymerase zeta (ζ) is essential for error-free replication past thymine glycol," *Genes and Development*, vol. 17, no. 1, pp. 77–87, 2003.
- [33] K. L. Brown, T. Adams, V. P. Jasti, A. K. Basu, and M. P. Stone, "Interconversion of the cis-5R,6S- and trans-5R,6R-thymine glycol lesions in duplex DNA," *Journal of the American Chemical Society*, vol. 130, no. 35, pp. 11701–11710, 2008.
- [34] G. Maga, G. Villani, E. Crespan et al., "8-oxo-guanine bypass by human DNA polymerases in the presence of auxiliary proteins," *Nature*, vol. 447, no. 7144, pp. 606–608, 2007.
- [35] Q.-M. Zhang, I. Miyabe, Y. Matsumoto, K. Kino, H. Sugiyama, and S. Yonei, "Identification of repair enzymes for 5-formyluracil in DNA: *nth*, *nei*, and *mutM* proteins of *Escherichia coli*," *Journal of Biological Chemistry*, vol. 275, no. 45, pp. 35471–35477, 2000.
- [36] R. G. Kallen, M. Simon, and J. Marmur, "The occurrence of a new pyrimidine base replacing thymine in a bacteriophage DNA: 5-hydroxymethyl uracil," *Journal of Molecular Biology*, vol. 5, pp. 248–250, 1962.
- [37] V. Rusmintratrip and L. C. Sowers, "An unexpectedly high excision capacity for mispaired 5-hydroxymethyluracil in human cell extracts," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 26, pp. 14183–14187, 2000.
- [38] M. Hori, S. Yonei, H. Sugiyama, K. Kino, K. Yamamoto, and Q.-M. Zhang, "Identification of high excision capacity for 5-hydroxymethyluracil mispaired with guanine in DNA of *Escherichia coli* MutM, Nei and Nth DNA glycosylases," *Nucleic Acids Research*, vol. 31, no. 4, pp. 1191–1196, 2003.
- [39] F. Barone, S. D. McCulloch, P. Macpherson et al., "Replication of 2-hydroxyadenine-containing DNA and recognition by human MutSa," *DNA Repair*, vol. 6, no. 3, pp. 355–366, 2007.
- [40] E. Crespan, U. Hübscher, and G. Maga, "Error-free bypass of 2-hydroxyadenine by human DNA polymerase λ with proliferating cell nuclear antigen and replication protein A in different sequence contexts," *Nucleic Acids Research*, vol. 35, no. 15, pp. 5173–5181, 2007.
- [41] X.-L. Yang, H. Sugiyama, S. Ikeda, I. Saito, and A. H.-J. Wang, "Structural studies of a stable parallel-stranded DNA duplex incorporating isoguanine: cytosine and isocytosine: guanine basepairs by nuclear magnetic resonance spectroscopy," *Biophysical Journal*, vol. 75, no. 3, pp. 1163–1171, 1998.
- [42] Y. Ushijima, Y. Tominaga, T. Miura, D. Tsuchimoto, K. Sakumi, and Y. Nakabeppu, "A functional analysis of the DNA glycosylase activity of mouse MUTYH protein excising 2-hydroxyadenine opposite guanine in DNA," *Nucleic Acids Research*, vol. 33, no. 2, pp. 672–682, 2005.
- [43] K. Hashiguchi, Q. M. Zhang, H. Sugiyama, S. Ikeda, and S. Yonei, "Characterization of 2-hydroxyadenine DNA glycosylase activity of *Escherichia coli* MutY protein," *International Journal of Radiation Biology*, vol. 78, no. 7, pp. 585–592, 2002.

- [44] H. Kamiya, H. Maki, and H. Kasai, "Two DNA polymerases of *Escherichia coli* display distinct misinsertion specificities for 2-Hydroxy-dATP during DNA synthesis," *Biochemistry*, vol. 39, no. 31, pp. 9508–9513, 2000.
- [45] K. Satou, H. Harashima, and H. Kamiya, "Mutagenic effects of 2-hydroxy-dATP on replication in a HeLa extract: induction of substitution and deletion mutations," *Nucleic Acids Research*, vol. 31, no. 10, pp. 2570–2575, 2003.

Mutagenic potency of *Helicobacter pylori* in the gastric mucosa of mice is determined by sex and duration of infection

Alexander Sheh^a, Chung Wei Lee^{a,b}, Kenichi Masumura^c, Barry H. Rickman^b, Takehiko Nohmi^c, Gerald N. Wogan^{a,1}, James G. Fox^{a,b,2}, and David B. Schauer^{a,b,2}

^aDepartment of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139; ^bDivision of Comparative Medicine, Massachusetts Institute of Technology, Cambridge, MA 02139; and ^cDivision of Genetics and Mutagenesis, National Institute of Health Sciences, Tokyo 158-8501, Japan

Contributed by Gerald N. Wogan, July 19, 2010 (sent for review April 27, 2010)

Helicobacter pylori is a human carcinogen, but the mechanisms evoked in carcinogenesis during this chronic inflammatory disease remain incompletely characterized. We determined whether chronic *H. pylori* infection induced mutations in the gastric mucosa of male and female *gpt* delta C57BL/6 mice infected for 6 or 12 mo. Point mutations were increased in females infected for 12 mo. The mutation frequency in this group was 1.6-fold higher than in uninfected mice of both sexes ($P < 0.05$). A:T-to-G:C transitions and G:C-to-T:A transversions were 3.8 and 2.0 times, respectively, more frequent in this group than in controls. Both mutations are consistent with DNA damage induced by oxidative stress. No increase in the frequency of deletions was observed. Females had more severe gastric lesions than males at 6 mo postinfection (MPI; $P < 0.05$), but this difference was absent at 12 MPI. In all mice, infection significantly increased expression of *IFN γ* , *IL-17*, *TNF α* , and *iNOS* at 6 and 12 mo, as well as *H. pylori*-specific IgG1 levels at 12 MPI ($P < 0.05$) and IgG2c levels at 6 and 12 MPI ($P < 0.01$ and $P < 0.001$). At 12 MPI, IgG2c levels in infected females were higher than at 6 MPI ($P < 0.05$) and also than those in infected males at 12 MPI ($P < 0.05$). Intensity of responses was mediated by sex and duration of infection. Lower *H. pylori* colonization indicated a more robust host response in females than in males. Earlier onset of severe gastric lesions and proinflammatory, Th1-biased responses in female C57BL/6 mice may have promoted mutagenesis by exposing the stomach to prolonged oxidative stress.

gpt delta mouse | *Helicobacter* | inflammation | mutagenesis | sexual dimorphism

Acting through multiple, complex mechanisms that are incompletely understood, chronic inflammation is a significant risk factor for several major human malignancies, including stomach cancer. Chronic inflammation induced by *Helicobacter pylori* infection increases lifetime risk of developing gastritis, duodenal and gastric ulcers, mucosa-associated lymphoid tissue lymphoma, mucosal atrophy, and gastric carcinoma (1, 2). Indeed, *H. pylori* has been classified by International Agency for Research on Cancer as a group I human carcinogen on the basis of its impact on gastric cancer incidence, the second most frequent cause of cancer-related death worldwide (3). Among postulated mechanisms through which infection may contribute to increased cancer risk are overproduction of reactive oxygen and nitrogen species (RONS) by inflammatory cells, and the consequent induction of mutations critical for tumor initiation in cells of inflamed tissues (4). The inflammatory response to infection results in increased production of RONS, including superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), nitric oxide (NO), peroxynitrite ($ONO_2^{\cdot-}$), and nitrous anhydride (N_2O_3), in vitro (5, 6) and in vivo (7–9). *H. pylori* can also directly activate RONS-producing enzymes, such as inducible NO synthase (iNOS) and spermine oxidase, in gastric epithelial cells, causing DNA damage and apoptosis (5, 6). Chronic inflammatory states increase levels of DNA adducts, such as etheno adducts, 8-oxoG, and other mutagenic precursors, in vitro and in vivo (10–12), but

tend not to alter the frequency of deletions (13). RONS also can damage DNA indirectly by creating adduct-forming electrophiles via lipid peroxidation (14, 15). RONS have been shown to induce mutations in *H. pylori* by inducing a hypermutation state in the bacteria (16).

A widely used experimental model is the *H. pylori* SS1-infected C57BL/6 mouse, which is susceptible to chronic infection and develops robust gastritis and premalignant lesions similar to those occurring in humans (17, 18). To date, limited investigation has focused on genetic damage associated with infection in these animals, but available data are still incomplete. Enhanced DNA fragmentation was observed in gastric cells of infected mice (19), in which dsDNA breaks were also detected by TUNEL assay (20, 21). Mutagenicity in reporter genes recovered from gastric DNA of male and female Big Blue transgenic mice 6 mo after infection with *H. pylori* or *Helicobacter felis* has also been reported (22). In *H. pylori*-infected male mice, point mutation frequency was increased at 6 mo post infection (MPI), but decreased to control levels by 12 MPI, suggesting that the animals may have adapted to infection (22). Female mice infected with *H. felis* also had an increased frequency of point mutations at 7 MPI, compared with uninfected controls (23). Mutagenesis resulting from infection has also been associated with p53 status. Mutations were found in the *lacI* reporter genes of a small number of *H. felis*-infected female TSG-p53/Big Blue mice harboring either one ($p53^{+/-}$) or two ($p53^{+/+}$) WT p53 alleles (23). A 2-fold increase in mutations was found in DNA from the gastric mucosa of infected $p53^{+/-}$ mice, and also in uninfected $p53^{+/-}$ mice; the mutation frequency in infected $p53^{+/-}$ mice was further increased by approximately threefold. The intensity of inflammation was estimated to be significantly higher in infected $p53^{+/-}$ mice than in infected $p53^{+/+}$ animals, and gastric epithelial proliferation was similarly increased with infection in both latter treatment groups. By contrast, in another study, infection of Big Blue transgenic mice (sex not specified) with the SS1 strain of *H. pylori* for 3.5 mo resulted in no significant increase in gastric mutations over uninfected controls (13).

We used the *gpt* delta mouse to measure the accumulation of gastric mutations associated with *H. pylori* SS1 infection in male and female animals at 6 and 12 MPI. This experimental system comprises λ -EG10-based transgenic C57BL/6 mice harboring tandem arrays of 80 copies of the bacterial *gpt* gene at a single site on chromosome 17. The model was specifically designed to facilitate the in vivo detection of point mutations by 6-thioguanine

Author contributions: A.S., C.W.L., J.G.F., and D.B.S. designed research; A.S., C.W.L., and B.H.R. performed research; K.M. and T.N. contributed new reagents/analytic tools; A.S., B.H.R., G.N.W., J.G.F., and D.B.S. analyzed data; and A.S., G.N.W., and J.G.F. wrote the paper. The authors declare no conflict of interest.

¹To whom correspondence should be addressed. E-mail: wogan@mit.edu.

²J.G.F. and D.B.S. contributed equally to this work.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1009017107/-DCSupplemental.

(6-TG) selection, and deletions up to 10 kb in length by selection based on sensitivity to P2 interference (Spi⁻) (24). When phage DNA rescued from mouse tissues is introduced into appropriate *Escherichia coli* strains, both point mutations and large deletions can be efficiently detected (24). We also characterized histopathologic changes, expression of inflammatory cytokines, and expression of iNOS in gastric mucosa 6 and 12 mo after initial infection. In addition, we compared responses of male and female mice to assess the influence of sex. We found that *H. pylori* infection induced significant increases in the frequency of point mutations in the gastric mucosa of female, but not male, *gpt* delta mice. The accumulation of point mutations was therefore sex-dependent and was mediated by the duration of infection and the severity of disease.

Results and Discussion

Pathology, Cytokine and iNOS Expression, and Serologic Responses to *H. pylori* Infection and *H. pylori* Levels. Inflammatory histopathologic changes resulting from *H. pylori* infection were evaluated (Fig. S1) and gastric histological activity index (GHAI) scores calculated, with the following results. Scores were significantly increased in infected animals of both sexes at 6 and 12 MPI (female, $P < 0.001$ and $P < 0.01$, respectively; and male, $P < 0.05$ and $P < 0.001$, respectively; Fig. 1). Regarding specific types of lesions represented in the GHAI, infected females experienced higher levels of hyperplasia ($P < 0.05$), epithelial defects ($P < 0.001$), and dysplasia ($P < 0.05$) compared with infected males at 6 MPI, and thus had correspondingly higher ($P < 0.05$) GHAI scores at this time point. At 6 MPI, infected animals of both sexes displayed mild to moderate inflammation, comprised chiefly of submucosal and mucosal infiltrates of mononuclear and granulocytic cells. In addition, in infected males, mucous metaplasia, intestinal metaplasia, oxyntic gland atrophy, and hyalinosis were all significantly elevated compared with controls (all $P < 0.01$; intestinal metaplasia, $P < 0.001$); no significant increases occurred in foveolar hyperplasia, epithelial defects, or dysplasia. In infected females, inflammation ($P < 0.05$), epithelial defects ($P < 0.001$), mucous metaplasia ($P < 0.01$), hyalinosis ($P < 0.001$), as well as premalignant lesions of oxyntic gland atrophy ($P < 0.001$), intestinal metaplasia ($P < 0.05$), and dysplasia ($P < 0.05$) were significantly elevated compared with uninfected controls. At 12 MPI, there were no differences in gastric lesion severity between infected males and females; both groups had similar mucosal changes. Infected male mice at 12 MPI displayed significantly

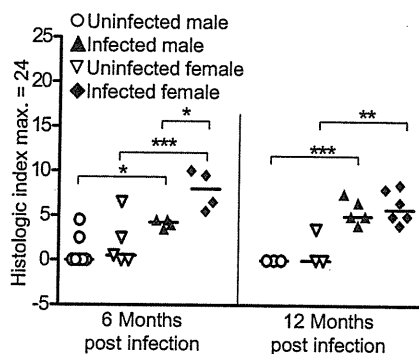


Fig. 1. *H. pylori* infection elicits more gastric pathologic processes in female mice at 6 mo. *H. pylori* infection increased the GHAI in both male and female C57BL/6 mice at 6 and 12 mo. At 6 mo of infection, infected females had significantly more pathologic processes than infected males. Uninfected males (○; 6 MPI, $n = 7$; 12 MPI, $n = 3$), uninfected females (▽; 6 MPI, $n = 5$; 12 MPI, $n = 3$), infected males (▼; 6 MPI, $n = 4$; 12 MPI, $n = 5$) and infected females (◆; 6 MPI, $n = 4$; 12 MPI, $n = 6$). Bar represents the mean. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

increased epithelial defects but decreased intestinal metaplasia than infected males at 6 MPI ($P < 0.01$ and $P < 0.001$).

H. pylori infection significantly increased transcription profiles at 6 and 12 MPI as follows (details in Fig. S2): *IFN*γ (males, $P < 0.001$; females, $P < 0.01$ at 6 and 12 MPI); *TNF*-α (males, $P < 0.01$ at 6 and 12 MPI; females, $P < 0.01$ and $P < 0.05$); *IL*-17 (males, $P < 0.05$ and $P < 0.001$; females, $P < 0.05$ and $P < 0.01$). *iNOS* expression was increased in male and female mice at 6 and 12 MPI (males, $P < 0.05$ and $P < 0.01$; females, $P < 0.01$ and $P < 0.001$) compared with controls at 12 mo. *IL*-10 expression was not significantly affected by infection in animals of either sex ($P > 0.05$).

H. pylori infection also resulted in a Th1-predominant IgG2c response in infected mice as previously reported (25, 26) (Fig. 2). *H. pylori*-specific IgG2c levels were higher in infected than in uninfected animals at 6 and 12 MPI ($P < 0.01$ and $P < 0.001$, respectively). At 12 MPI, infected females had significantly higher

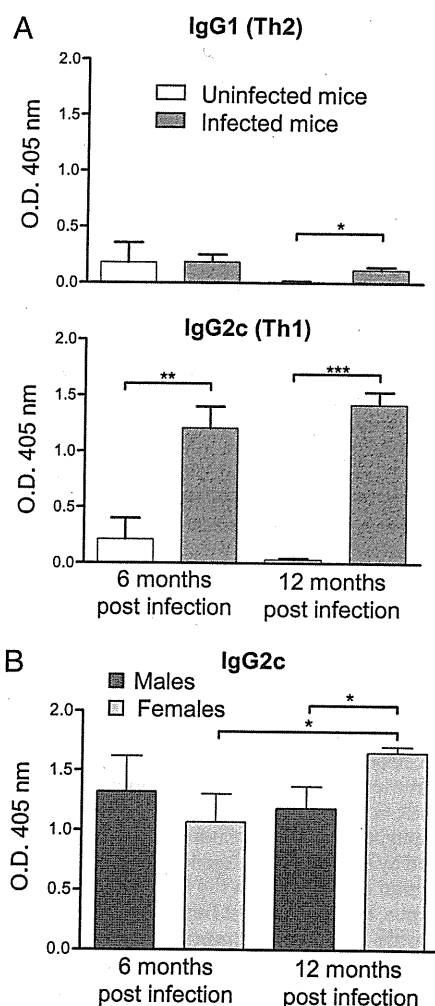


Fig. 2. The effect of *H. pylori* infection on *H. pylori*-specific IgG1 and IgG2c. Serum levels of IgG were measured by ELISA in uninfected and *H. pylori*-infected mice. (A) *H. pylori*-infected mice (gray bars; 6 MPI, $n = 9$; 12 MPI, $n = 12$) developed a greater IgG1 response after 12 mo of infection compared with uninfected mice (white bars; 6 MPI, $n = 6$; 12 MPI, $n = 6$; $P < 0.05$). *H. pylori*-infected mice developed a greater IgG2c response after 6 and 12 mo of infection ($P < 0.01$ and $P < 0.001$, respectively). (B) At 12 mo, infected female mice (light gray bars; 6 MPI, $n = 4$; 12 MPI, $n = 6$) had substantially increased IgG2c compared with infected males (dark gray bars; 6 MPI, $n = 5$; 12 MPI, $n = 6$) at 12 mo and infected females at 6 mo ($P < 0.05$, both). Data are mean (SE) of mice in different treatment groups. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

IgG2c levels than infected males ($P < 0.05$). Duration of infection also increased IgG2c levels in infected females, with higher levels noted at 12 versus 6 MPI ($P < 0.05$). *H. pylori*-specific IgG1 (Th2) levels were higher in infected than in uninfected animals at 12 MPI ($P < 0.05$). Females had higher Th1/Th2 ratios than males: at 6 MPI, 8.58 vs. 4.01; and at 12 MPI, 12.9 vs. 7.51.

We and others have demonstrated that *H. pylori* levels are inversely correlated with the degree of pathology and the immune response as measured by antibody and cytokine production (25, 27). At 6 MPI, *H. pylori* was detectable by quantitative PCR in all infected males, but not in one female. At 12 MPI, there was a salient difference in levels of *H. pylori* colonization between males and females; *H. pylori* was undetectable in four infected females, but undetectable in only one male (Fig. 3).

Frequency and Nature of Mutations. We determined the frequency of *gpt* point mutations in gastric DNA isolated from infected and uninfected mice by selection of mutants based on 6-TG resistance. Recognizing the possible confounding effect of clonal expansion of sibling mutants (i.e., jackpot mutations), we sequenced the *gpt* genes from all 566 recovered mutants. Any mutation found to duplicate another at the same site within an individual sample was excluded from subsequent frequency calculations. After this adjustment, at 12 MPI the *gpt* mutation frequency in infected females ($7.5 \pm 2.0 \times 10^{-6}$; $P < 0.05$) was significantly (1.6-fold) higher than that in all control animals ($4.7 \pm 1.1 \times 10^{-6}$), whereas the frequency in infected males ($6.3 \pm 5.4 \times 10^{-6}$; $P = 0.49$) was not. At 6 MPI, *gpt* mutation frequency in infected animals of either sex (females, $5.6 \pm 2.0 \times 10^{-6}$; $P = 0.72$; males, $7.2 \pm 2.2 \times 10^{-6}$; $P = 0.57$) was not significantly different from that of controls ($6.2 \pm 3.0 \times 10^{-6}$; Fig. 4).

After sequencing and accounting for clonal expansion, 236 from infected and 156 mutants from uninfected mice were used to identify effects of *H. pylori* infection on types of mutations causing loss of *gpt* function, with the results summarized in Table 1 and Table S1. G:C-to-A:T transitions and G:C-to-T:A transversions were the most prevalent types of mutations in both infected and uninfected animals, representing 33% to 50% and 17% to 31%, respectively, of total mutations in various treatment groups; mutant frequencies varied from 2.1 to 3.0×10^{-6} in these transitions and 0.9 – 1.8×10^{-6} in the transversions. The 1.6-fold higher total mutations observed in infected females compared with age-matched controls at 12 MPI was attributable mainly to two types of mutations: 3.8 times more A:T-to-G:C transitions ($P < 0.05$) and 2.0 times more G:C-to-T:A transversions ($P <$

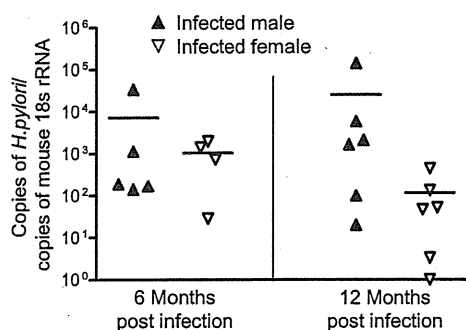


Fig. 3. *H. pylori* levels in the stomach were lower in infected females at 12 MPI. Values represent the number of *H. pylori* organisms per copies of mouse 18s rRNA. At 6 MPI, one infected female mouse was under the threshold of detection (15 copies of *H. pylori*). At 12 MPI, four infected females and one infected male were undetectable. Infected males (▲; 6 MPI, $n = 5$; 12 MPI, $n = 6$) and infected females (▼; 6 MPI, $n = 4$; 12 MPI, $n = 6$). Bar represents the mean.

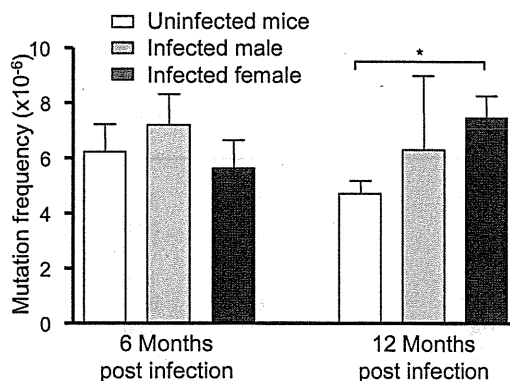


Fig. 4. Twelve-month infection with *H. pylori* increases the frequency of point mutations in female mice. The mutation frequency of point mutations was determined by the *gpt* assay in uninfected mice (white bars; 6 MPI, $n = 13$; 12 MPI, $n = 15$), *H. pylori*-infected males (light gray bars; 6 MPI, $n = 4$; 12 MPI, $n = 5$) and *H. pylori*-infected females (dark gray bars; 6 MPI, $n = 9$; 12 MPI, $n = 11$). Control mice of both sexes were grouped for this analysis. Data are mean (SEM) of mutation frequency of mice in different treatment groups. * $P < 0.05$.

0.05). These two mutations are consistent with the expected mutational spectrum induced by RONS (28) and have been found elevated in another animal model of chronic inflammation (13). An increase in G:C-to-T:A transversions was also reported in a previous study documenting *H. pylori*-induced mutations (22).

A:T-to-G:C transitions can be formed by deamination of adenine to hypoxanthine or creation of ethenoadenine. Deamination is mediated by N_2O_3 , the autoxidation product of $NO\cdot$, which directly nitrosates primary amines on DNA bases (28, 29). Hypoxanthine resembles guanine and mispairs with cytosine, resulting in the observed mutation. Alternatively, A:T-to-G:C transitions can also be created indirectly by lipid peroxidation by RONS, forming etheno adducts in DNA such as highly mutagenic ethenoadenine (30, 31). G:C-to-T:A transversions have also been associated with increased *iNOS* expression levels, pointing to the involvement of RONS (32). Cells cocultivated with activated macrophages predominantly develop G:C to T:A transversions caused by exposure to $NO\cdot$, $O_2^{\cdot-}$, and H_2O_2 (33). G:C-to-T:A transversions are also caused by adducts produced by oxidative stress or lipid peroxidation, such as 8-oxodG, edC, and M1G (34, 35). The presence of 8-oxodG, a major product of oxidative damage to DNA, results in mispairing of guanine with adenine during replication, thus inducing transversions (36). Although 8-oxodG is believed to be the main cause of this mutation, etheno adducts formed by lipid peroxidation may also play a significant role in mutagenesis observed *in vivo* (37).

Fig. S3 shows the mutation spectrum detected in the *gpt* gene, of which the following features are noteworthy. In both uninfected and infected mice, hotspots occurred at nucleotides C64, G110, G115, and G418; C64, G110, and G115 are CpG sites and are known hotspots in this assay (38, 39). Hotspots occurring in infected mice were located at A8, G116, and G143; A8 and G116 were found predominantly in infected females at both 6 and 12 mo, whereas G143, located at a CpG site, was a hotspot in infected animals of both sexes. Uninfected mice had hotspots at G406, G416, and A419, one of which (G416) occurred mainly in males. Mutations in infected males were concentrated mainly in hotspots common to both uninfected and infected animals, whereas in infected females there was an increase in mutations throughout the *gpt* gene at non-G:C sites. This result suggests that the chemistry of DNA damage in mice with a stronger host response to infection may have differed from that in mice with mild or minimal gastritis.