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Research Article

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

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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(α)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-^{32}P]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 μg/mL ampicillin and 30 μg/mL chloramphenicol. Overnight cultures (5 mL) were inoculated into 500 mL of prewarmed LB medium containing 50 μg/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 μg/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 sheered 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 × 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-^{32}P]ATP by T4 polynucleotide kinase and annealed with the appropriate template oligonucleotides.

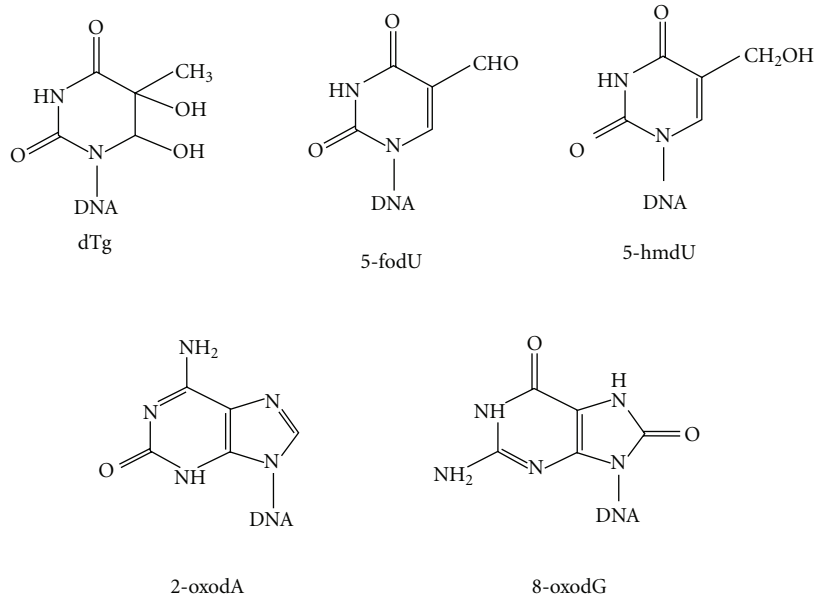


Figure 1: The structures of the studied oxidatively damaged bases, thymine glycol (dTg), 5-formyluracil (5-fodU), 5-hydroxymethyluracil (5-hmdU), 1,2-dihydro-2-oxoadenine (2-oxodA), and 7,8-dihydro-8-oxoguanine (8-oxodG).

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

Template 1	3′-CGTCGGGCCCCCTAAGTGATCAAG-5′
Template 2	3′-CGTCGGGCCCCCTA2GTGATCAAG-5′
Template 3	3′-CGTCGGGCCCCCTAGGTGATCAAG-5′
Template 4	3′-CGTCGGGCCCCCTA8GTGATCAAG-5′
Template 5	3′-ACGTCCAGCTCACATCTCCTAG -5′
Template 6	3′-ACGTCCAGCTCACATCFCCTAG -5′
Template 7	3′-ACGTCCAGCTCACATCHCCTAG -5′
Template 8	3′-CGACGGGCCCCCAATGAGAACAAG-5′
Template 9	3′-CGACGGGCCCCCAAXGAGAACAAG-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 μl) 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 μg/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 aspargine [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

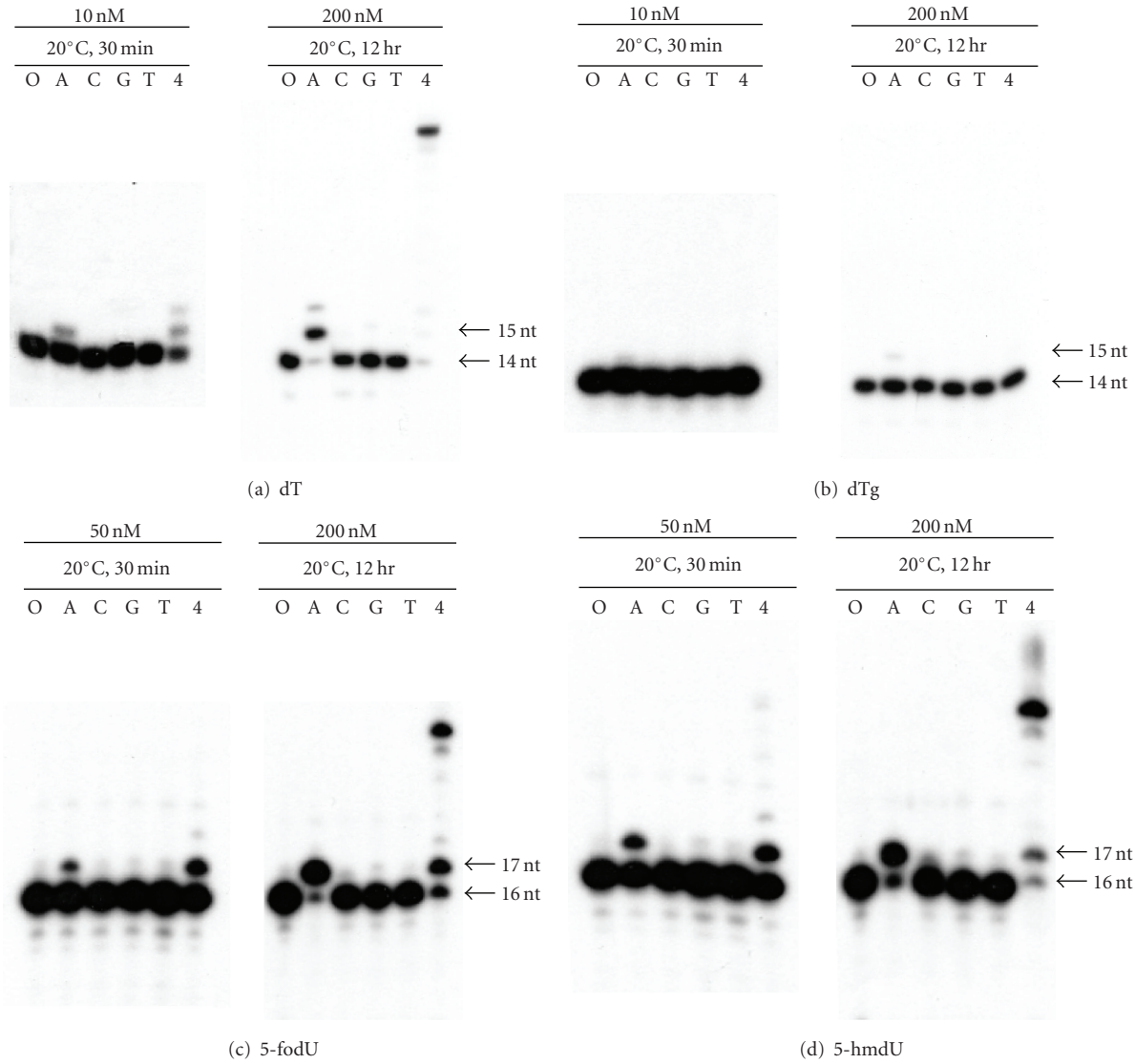


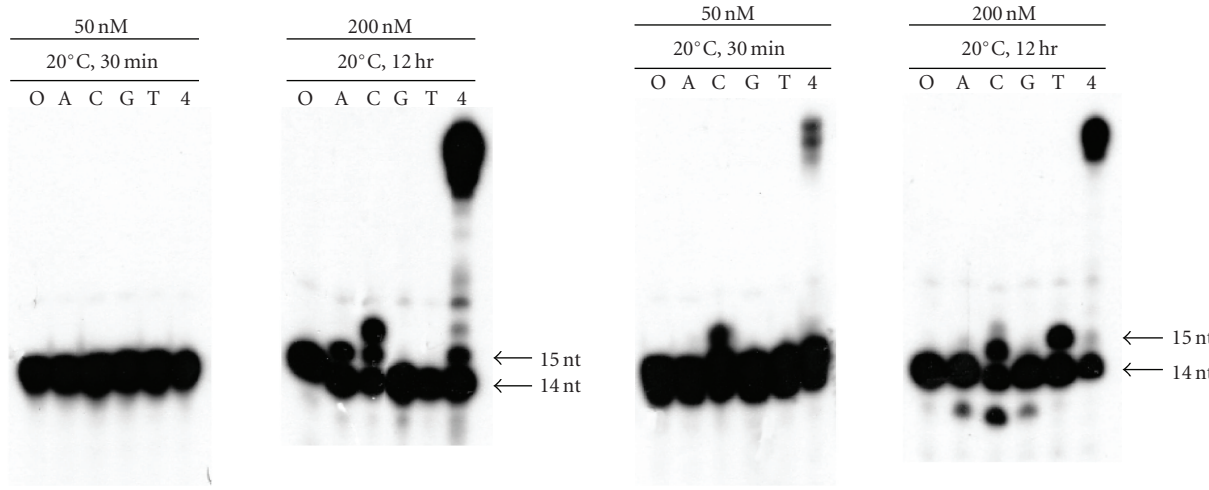
Figure 2: Primer extension assay for Pol IV to bypass dTg, 5-fodU, and 5-hmdU in the template oligonucleotides. Primers were labeled at the 5′-terminal by polynucleotide kinase and annealed with appropriate template oligonucleotide. Primer/templates (50 fmol) in a 10-μl reaction mixture was incubated 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 occasionary 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 → 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.



(a) 8-oxodG (b) 2-oxodA

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-μl 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′ → 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	Km (fmol/ mL /min)	V_{max} (min^{-1})	k_{cat}	k_{cat}/Km
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-μl 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 μM), 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 *kcat*/Km 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 → G:C transitions.

3.5. Overexpression of Pol IV Causes A:T → 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_2O_2[a]	10 mM H_2O_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 H_2O_2 for 1 hr. The mutant Pol IV (DinB003) was generated by site-specific mutagenesis replacing aspartic acid in position 103 with aspargine [4]. The mutation frequency was expressed as number of mutants/10^8 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 aspargine [4].

2-oxodA has been shown to occur in DNA by treatment of cells with hydrogen peroxide (H_2O_2) [12, 28]. Hence, the *E. coli* cells were exposed to H_2O_2 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 H_2O_2. 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 C_6 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 KFexo$^-$ 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

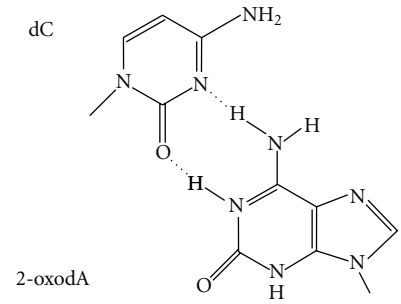


Figure 4: Possible wobble structure for base pairing of the 1,2-dihydro-2-oxoadenine (2-oxodA):dC.

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 → G:C transitions and A:T → 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 → G:C transitions at the site of 2-oxodA. Pol IV overexpression has its greater effect on A:T → G:C mutations than A:T → C:G and A:T → T:A mutations. The facts may reflect that Pol IV is accurately handling a lesion leading A:T → C:G and A:T → 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 → A:T transitions *in vivo*. 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 (Table 3). These results indicate that Pol IV may involved in these ROS-enhanced A:T → 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*.

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