Involvement of Y-Family DNA Polymerases in Mutagenesis Caused by Oxidized Nucleotides in *Escherichia coli*

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Escherichia coli DNA polymerase IV incorporated 2-hydroxy-dATP opposite template guanine or thymine and 8-hydroxy-dGTP exclusively opposite adenine in vitro. Mutator phenotypes in *sod/fur* strains were substantially diminished by deletion of *dinB* and/or *umuDC*. DNA polymerases IV and V may be involved in mutagenesis caused by incorporation of the oxidized deoxynucleoside triphosphates.

Excess oxidation is a major threat to the genomic integrity of most living organisms. Reactive oxygen species oxidize deoxynucleoside triphosphates (dNTPs), as well as DNA, and some of the oxidized dNTPs have been shown to be mutagenic when they are incorporated in DNA. 8-Oxo-7,8-dihydro-2′-deoxyguaniosine 5′-triphosphate (8-OH-dGTP) leads to A · T-to-C · G transversions when it is incorporated opposite adenine (A) in the template (5, 14). To counteract the mutagenic 8-OH-dGTP, *Escherichia coli* has a sanitizing enzyme, MutT, that hydrolyzes 8-OH-dGTP (20). When the *mutT* gene is inactivated, the frequency of mutation of A · T to C · G increases more than a thousandfold compared with the wild-type frequency (35). In the case of 2-oxo-1,2-dihydro-2′-deoxyadenosine 5′-triphosphate (2-OH-dATP), G · C-to-T · A transversions occur when it is incorporated opposite guanine (G) in the template (14, 16). Another sanitizing enzyme, Orf135, degrades 2-OH-dATP in *E. coli*, and G · C-to-T · A mutations occur in an *orf135*-deficient strain more frequently than in the wild-type strain (15, 17).

The members of the Y family of DNA polymerases (DNA Pols) are involved in error-free and error-prone translesion synthesis (TLS) of damaged template DNA in various species, including humans (13, 26). Recently, involvement of Y-family DNA polymerases in the incorporation of damaged dNTPs was suggested by in vitro experiments performed with purified DNA Pols (28). The archaeal Y-family DNA Pols from *Sulfolobus* sp. and the human DNA Pols exclusively incorporate 8-OH-dGTP opposite A in the template DNA and incorporate 2-OH-dATP opposite G and thymine (T). Thus, it would be interesting to examine the in vivo roles of Y-family DNA Pols in the incorporation of mutagenic dNTPs into DNA. *Escherichia coli* strain QC1736 seems to be an appropriate background to investigate the roles of Y-family DNA Pols (DNA Pol IV and Pol V encoded by *dinB* and *umuDC*, respectively) in the mutagenesis

caused by oxidized nucleotides. Iron metabolism is deregulated in this strain due to the lack of the Fur protein, a negative regulator of iron uptake (29). This strain also lacks both superoxide dismutases (SodA and SodB), which cata-
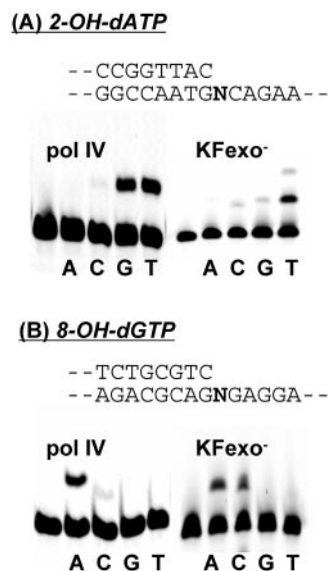

FIG. 1. Incorporation of oxidized nucleotides by DNA polymerases. The incorporation of 2-OH-dATP (A) and 8-OH-dGTP (B) into DNA by DNA Pol IV and KF exo⁻ of *E. coli* was assayed as described previously (28). Cy3-conjugated primer, annealed to the template at a 1:1 ratio (0.1 μM), was incubated with DNA Pol IV (0.1 μM) or KF exo⁻ (0.02 U), and then 50 μM 2-OH-dATP (A) or 50 μM 8-OH-dGTP (B) was added. No other dNTPs were added to the reaction mixtures. All the reactions were carried out at room temperature for 30 min. The reaction products were analyzed on 15% denatured polyacrylamide gels, and the bands were visualized using a Molecular Imager FX Pro system (Bio-Rad, Richmond, CA). The oligonucleotide sequences of the primer and template were 5′-Cy3-CGCGCGAAGAC CGGTTAC-3′ and 5′-GAAGGGATCCTTAAGACNGTAACCGGTCTT CGCGCG-3′, respectively, for 2-OH-dATP and 5′-Cy3-CGGAGCTCGGT CGGCGTCTGCGTC and 5′-AGCCGCAGGAGNGACGCAGACGCC GACCGAGCTCCG-3′, respectively, for 8-OH-dGTP (N = A, C, G, or T). Parts of the sequences of the primer and template are shown. The unlabeled lanes on the left indicate the positions of Cy3-labeled primers without extension.

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TABLE 1. Strains used in this study[a]

Strain	Characteristics	P1 transduction or conjugation	Reference or source
CC101	Derivative of strain P90C [*araA*(*lac proB*)$_{\mathrm{xIII}}$] carrying F′ *lacIZ- proB*$^+$; *lacZ* has a mutation (GAG to TAG) at codon 461		7
CC104	Derivative of strain P90C [*araA*(*lac proB*)$_{\mathrm{xIII}}$] carrying F′ *lacIZ- proB*$^+$; *lacZ* has a mutation (GAG to GCG) at codon 461		7
AR30	Δ*dinB61*::*ble sulA211*		4
DE2302	*thr-1 ara-14 leuB6* Δ(*gpt proA*)*62 lacY1 tsx-33 supE44 galK2 hisG4 rpsL31 xyl-5 mtl-1 arg3 thi-1 uvrA6* Δ(*umuDC*)*595*::*cat fadR615*::Tn*10 purB58*		34
EC8	*thr-1 ara-14 leuB6* Δ(*gpt-proA*)*62 lacY1 tsx-33 supE44 galK2 hisG4 rpsL31 xyl-5 mtl-1 argE3 thi-1 uvrA6* Δ(*umuDC*)*596*::*ermGT fadR*$^+$ *purB*$^+$		11
KY1056sFtet101	AB1157 derivative; harboring F′ derived from CC101, which has Tn*10* in it for selection of F′		K. Yamamoto
KY1056sFtet104	AB1157 derivative; harboring F′ derived from CC104, which has Tn*10* in it for selection of F′		K. Yamamoto
YG6125A	AB1157 derivative; harboring F′ derived from CC101, which has Tn*10* in it for selection of F′ and Δ*dinB*::*kan*		This study
YG6125B	AB1157 derivative; harboring F′ derived from CC104, which has Tn*10* in it for the selection of F′ and Δ*dinB*:*kan*		This study
QC1736	Δ(*argF-lac*)*U169 rpsL* Δ*sodA3 sodB*::MudPR *fur*::*kan*; Cm^r Km^r		29
YG6177	Like QC1736 but Δ*dinB61*::*ble*; Cm^r Km^r Zc^r	AR30 (P1) → QC1736	This study
YG6180	Like QC1736 but Δ*umuDC*(*596*)::*ermGT*; Cm^r Km^r	DE2302/EC8 (P1) → QC1736	This study
YG6124	Like QC1736 Δ*dinB61*::*ble*; Δ*umuDC*(*596*)::*ermGT*; Cm^r Km^r Zc^r	DE2302/EC8 (P1) → YG6177	This study
YG6175[b]	Like QC1736 but harboring F′ from CC101; Cm^r Km^r Tc^r	KY1056sFtet101 → QC1736	This study
YG6176[b]	Like QC1736 but harboring F′ from CC104; Cm^r Km^r Tc^r	KY1056sFtet104 → QC1736	This study
YG6178[b]	Like QC1736 but Δ*dinB61*::*ble* and harboring F′ from CC101; Cm^r Km^r Tc^r Zc^r	YG6125A → YG6177	This study
YG6179[b]	Like QC1736 but Δ*dinB61*::*ble* and harboring F′ from CC104; Cm^r Km^r Tc^r Zc^r	YG6125B → YG6177	This study
YG6181[b]	Like QC1736 but Δ*umuDC*(*596*)::*ermGT* and harboring F′ derived from CC101; Cm^r Km^r Tc^r	KY1056sFtet101 → YG6180	This study
YG6182[b]	Like QC1736 but Δ*umuDC*(*596*)::*ermGT* and harboring F′ from CC104; Cm^r Km^r Tc^r	KY1056sFtet104 → YG6180	This study
YG6126[b]	Like QC1736 but Δ*dinB61*::*ble* and Δ*umuDC*(*596*)::*ermGT* and harboring F′ from CC101; Cm^r Km^r Tc^r Zc^r	YG6125A → YG6124	This study
YG6127[b]	Like QC1736 but Δ*dinB61*::*ble* and Δ*umuDC*(*596*)::*ermGT* and harboring F′ from CC104; Cm^r Km^r Tc^r Zc^r	YG6125B → YG6124	This study

[a] The deletion strains for *dinB* encoding DNA Pol IV were constructed by P1 transduction as indicated. The *umuDC* deletion encoding DNA Pol V was introduced into QC1736 and YG6177 by two-step P1 transduction (11). (P1) indicates that P1vir phage lysate was prepared in the strain. F′ with a mutation for specific detection of changes from G · C to T · A or from A · T to C · G was separately introduced by conjugation as indicated. The arrows indicate the directions of transfer for P1 transduction and conjugation. Chloramphenicol, kanamycin, tetracycline, and zeocin were used at concentrations of 10 μg/ml, 25 μg/ml, 10 μg/ml, and 50 μg/ml, respectively. Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance; Zc^r, zeocin resistance.
[b] Strain used for the LacZ reversion assay.

lyze the breakdown of the superoxide anion. Thus, both iron overload and superoxide stress occur in strain QC1736, which leads to high rates of spontaneous mutation from A · T to C · G and from G · C to T · A (24). The hot spots and sequence contexts of A · T-to-C · G mutations are almost identical to those in a *mutT* strain (25). In contrast, the hotspots of G · C-to-T · A mutations are very different from those in *mutM mutY* strains, in which 8-OH-G in DNA acts as a major mutagenic lesion. Expression of the cDNA of the human counterpart of *E. coli* MutT, MTH1, which hydrolyzes both 8-OH-dGTP and 2-OH-dATP (12), suppresses the mutator phenotype of the strain. Thus, it has been concluded that the targets contributing to the oxidative mutagenesis in the *sod/fur* mutant are oxidized dNTPs, such as 8-OH-dGTP and 2-OH-dATP, rather than DNA (25).

We first examined the specificity with which the purified native form of DNA Pol IV (31) incorporates 2-OH-dATP and 8-OH-dGTP in vitro. DNA Pol IV predominantly incorporated 2-OH-dATP opposite template G and T, and the fre-

quency of incorporation opposite G was almost equal the frequency of incorporation opposite T (Fig. 1 A). In contrast, Klenow fragment exo$^-$ (KF exo$^-$) (New England Biolabs, Massachusetts) predominantly incorporated 2-OH-dATP opposite T. DNA Pol IV almost exclusively incorporated 8-OH-dGTP opposite A, and KF exo$^-$ incorporated 8-OH-dGTP opposite A and cytosine (C) (Fig. 1 B). These in vitro results suggest possible involvement of Y-family DNA polymerases in oxidative mutagenesis through misincorporation of the oxidized dNTPs during DNA synthesis in *E. coli*.

To examine the in vivo roles of Y-family DNA Pols, mutation frequencies were compared for *sod/fur* strains with and without Pol IV and Pol V (Table 1). Both A · T-to-C · G and G · C-to-T · A transversion frequencies were reduced by 80 to 90% by deletion of either *dinB* or *umuDC* or both in the *sod/fur* strains (Table 2). Interestingly, the double mutants (Δ*dinB* Δ*umuDC*) exhibited levels of mutation frequency similar to those of single mutants (Δ*dinB* or Δ*umuDC*). These results suggest that the base substitutions by erroneous incorporation

TABLE 2. Mutation frequencies for the *sodAB fur* strains with and without DNA Pol IV and DNA Pol V[a]

Expt	Mutation frequency (10^{-6})							
	Pol IV⁺/Pol V⁺ (YG6176)	Pol IV⁻/Pol V⁺ (YG6179)	Pol IV⁺/Pol V⁻ (YG6182)	Pol IV⁻/Pol V⁻ (YG6127)	Pol IV⁺/Pol V⁺ (YG6175)	Pol IV⁻/Pol V⁺ (YG6178)	Pol IV⁺/Pol V⁻ (YG6181)	Pol IV⁻/Pol V⁻ (YG6126)
$G \cdot C \rightarrow T \cdot A$								
Expt I	28.2 ± 3.5	3.66 ± 0.32	2.72 ± 0.20	3.57 ± 0.42				
Expt II	35.6 ± 2.5	2.61 ± 0.12	2.82 ± 0.15	3.23 ± 0.30				
$A \cdot T \rightarrow C \cdot G$								
Expt I					12.8 ± 0.61	2.49 ± 0.18	3.24 ± 0.25	2.43 ± 0.15
Expt II					10.0 ± 0.60	2.08 ± 0.16	3.7 ± 0.21	1.86 ± 0.19
Expt III					9.1 ± 0.80	2.38 ± 0.47		

[a] The mutagenicity assay was carried out as described previously (32). Briefly, a single colony was inoculated into 2 ml of M9-glucose minimal medium, and then the overnight culture was diluted 1,000-fold. Eight to twelve diluted cultures were prepared, and they were cultivated overnight. One milliliter of each culture was harvested and washed twice with phosphate buffer (pH 7.4), and then the cell pellet was suspended in phosphate buffer. All of the suspension was spread on one plate for mutation, and a portion of the diluted suspension was used for determining survival. Twenty amino acids were added in the assays (both liquid medium and plates) for growth of *sodAB fur* strains because the production of amino acids is inhibited by oxygen radicals (3). The values are means ± standard errors.

of 2-OH-dATP or 8-OH-dGTP require both DNA Pol IV and DNA Pol V functions.

The dNTP pool and DNA are continuously exposed to a variety of exogenous and endogenous damaging agents, including reactive oxygen species, and the incorporation of oxidized dNTPs into DNA is a major source of spontaneous mutagenesis and carcinogenesis (1). Here we obtained biochemical and genetic evidence that DNA Pol IV and Pol V may be involved in oxidative mutagenesis through misincorporation of altered nucleotides (i.e., 2-OH-dATP and 8-OH-dGTP) during DNA synthesis. This is consistent with the report by Satou et al. (27) that DNA Pol IV promotes mutation of $G \cdot C$ to $T \cdot A$ in *E. coli* when 2-OH-dATP is directly introduced into cells by $CaCl_2$ treatment. It has also been suggested that SOS-inducible polymerases, including Pol IV and Pol V, are involved in mutagenesis caused by increases in the normal levels of dNTPs (33). It has been reported that more than one DNA polymerase is involved in mutagenesis when the Y-family DNA polymerases are involved in TLS. For benzo[a]pyrene-induced mutagenesis, both Pol IV and Pol V are required for a −1 frameshift TLS (23). DNA lesions induced by other chemicals, including 3-methylcholanthrene or dimethylbenzo[a]anthracene, also require both DNA Pol IV and Pol V for a −2 frameshift in a CG repetitive sequence in *Salmonella enterica* serovar Typhimurium (18, 21). Thus, we speculate that DNA Pol IV and Pol V are involved in sequential biochemical steps, such as incorporation and extension of oxidized dNTPs during chromosome replication. One of these polymerases might incorporate oxidized dNTPs into DNA in an erroneous manner, and the other might extend the mutagenic primer termini containing the oxidized deoxynucleoside monophosphate, thereby inducing base substitutions. It is obvious, however, that more experiments are needed to elucidate the precise mechanisms.

DNA Pol IV is controlled by σ^s, and the level of expression of Pol IV in the stationary phase decreases significantly when the *rpoS* gene encoding σ^s is defective (10, 19). Thus, Pol IV appears to be regulated not only by the SOS response but also by the σ^s-dependent stress response. In stationary-phase cells, the amount of cellular mismatch repair proteins decreases at least 10-fold (8). Hence, the error-prone nature of Pol IV is expected to be more significant. In fact, DNA Pol IV is responsible for some of the adaptive mutations in stationary-

phase cells (9, 22), Interestingly, adaptive mutagenesis is approximately fourfold more frequent in a *sodA sodB* strain than in the parental strain, and this mutagenesis is suppressed under anaerobic conditions (2). Therefore, DNA Pol IV might be involved in stationary-phase mutagenesis by either incorporation of oxidized dNTPs or extension of primers having oxidized deoxynucleoside monophosphates or both, although it is possible that DNA Pol IV induces mutations by error-prone bypass across oxidized bases in template DNA.

The oxidized nucleotide pools also cause a problem in mammalian cells. Spontaneous tumorigenesis in lungs, livers, and stomachs is enhanced in mice that are deficient in *Mth1* (30). In addition, a recent study suggested that the majority of mutations in human cells that are deficient in mismatch repair do not arise from spontaneous replication errors but from the incorporation of oxidized dNTPs (6). Thus, it might be interesting to examine the roles of mammalian Y-family DNA Pols in genome instability caused by oxidation of the nucleotide pool.

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