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Biochemical evidence of a physical interaction between *Sulfolobus solfataricus* B-family and Y-family DNA polymerases

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Abstract The hyper-thermophilic archaeon *Sulfolobus solfataricus* possesses two functional DNA polymerases, belonging to the B-family (Sso DNA pol B1) and to the Y-family (Sso DNA pol Y1). Sso DNA pol B1 recognizes the presence of uracil and hypoxanthine in the template strand and stalls synthesis 3–4 bases upstream of this lesion (“read-ahead” function). On the other hand, Sso DNA pol Y1 is able to synthesize across these and other lesions on the template strand. Herein we report evidence that Sso DNA pol B1 physically interacts with DNA pol Y1 by surface plasmon resonance measurements and immuno-precipitation experiments. The region of DNA pol B1 responsible for this interaction has been mapped in the central portion of the polypeptide chain (from the amino acid residue 482 to 617), which includes an extended protease hyper-sensitive linker between the N- and C-terminal modules (amino acid residues Asn482-Ala497) and the α -helices forming the “fingers” sub-domain (α -helices R, R' and S). These results have important implications for understanding the polymerase-switching mechanism on the damaged template strand during genome replication in *S. solfataricus*.

Keywords DNA replication · Genome stability · DNA polymerase · Translesion synthesis · Archaea · *Sulfolobus solfataricus*

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Abbreviations

Sso: *Sulfolobus solfataricus* ·
DNA pol: DNA polymerase ·
PAGE: Polyacrylamide gel electrophoresis ·
IPTG: Isopropyl- β -D-thiogalactopyranoside ·
PMSF: Phenylmethylsulfonyl fluoride ·
PVDF: Poly(vinylidene difluoride)

Introduction

Hyper-thermophilic organisms have adopted molecular mechanisms that allow them to maintain genome stability against massive DNA damage caused by exposure to high temperature (Nohmi 2006). Spontaneous deamination, depurination and oxidation of DNA take place at greatly accelerated rate at high temperatures (Lindahl and Nyberg 1974). The hydrolytic deamination of cytosine leads to the formation of uracil in DNA and G:U base pairs result in G:C to A:T transitions in a half of the progeny if not repaired before replication. In addition, deamination of adenine results in formation of adenine that can pair with cytosine, thereby inducing A:T to G:C transitions if not repaired (Lindahl 1993). However, the spontaneous mutation rate in the hyper-thermophilic archaeon *Sulfolobus acidocaldarius* is reported to be similar to that of *Escherichia coli* (Grogan et al. 2001).

Interestingly, B-family DNA polymerases from hyper-thermophilic archaea are able to sense the presence of uracil in the template strand and tightly bind to uracil containing oligonucleotides (Lasken et al. 1996; Greagg et al. 1999). DNA polymerization is stalled when uracil is encountered four bases ahead of the primer-template junction. This “read-ahead” function appears to be a peculiar feature of the archaeal B-family DNA pols because B-family DNA polymerases from other organisms (i.e., *E. coli* bacteriophage T4 or yeast and mammals) and thermophilic bacterial A-family enzymes (i.e., *Thermus aquaticus* DNA pol) are able to read through uracil

residues on the template strand without halting DNA synthesis. Furthermore, the B-family DNA pol from *S. solfataricus* (Sso DNA pol B1) was reported to recognize the presence of hypoxanthine in the template, in addition to uracil, and to stall synthesis 3–4 bases upstream of this lesion (Gruz et al. 2003). Stalling of Sso DNA pol B1 at the template uracil has been also visualized by atomic force microscopy (Asami et al. 2006). The “read-ahead” function of the archaeal hyper-thermophilic B-family DNA pols is likely to operate as an additional safeguard mechanism against increased level of deaminated bases in the template DNA strand during genome duplication at high temperature. Analysis of the X-ray crystal structure of some archaeal B-family DNA pols revealed the presence of a special binding pocket for the uracil in the extreme N-terminal portion and site-specific mutagenesis studies confirmed this structural prediction for the B-family DNA pol of *Pyrococcus furiosus* (Fogg et al. 2002).

Interestingly, crenarchaea from the genus *Sulfolobus* possess a DNA pol belonging to the Y-family, in addition to the B-family enzyme (Kulaeva et al. 1996; Gruz et al. 2001). A peculiar feature of Y-family DNA pols is their ability to bypass various lesions in template DNA in an error-prone or error-free manner (Wang 2001; Freidberg et al. 2002). Thus, this family of DNA polymerases plays a critical role in genome stability in bacterial and eukaryotic organisms. Y-family DNA pols from *Sulfolobales* are extremely interesting enzymes since the X-ray structure of two of them (*S. acidocaldarius* and *S. solfataricus*) has been solved providing important structural information about the DNA lesion bypass mechanism (Ling et al. 2001; Silvian et al. 2001; Zhou et al. 2001). The Y-family DNA polymerase from *S. solfataricus* (Sso DNA pol Y1) has been reported to bypass a variety of DNA lesions (Gruz et al. 2001) and to catalyze erroneous incorporation of oxidized dNTPs (such as 8-OH-dGTP and 2-OH-dATP) into nascent DNA (Shimizu et al. 2003).

Herein we report evidence that Sso DNA pol B1 physically interacts with DNA pol Y1 by surface plasmon resonance measurements and immuno-precipitation experiments. The region responsible for this interaction has been mapped in the central portion of DNA pol B1 polypeptide chain (from the amino acid residue 482 to 617). The results of this analysis have important implications for understanding genome stability mechanisms in the hyper-thermophilic crenarchaeon *S. solfataricus*.

Materials and methods

Proteins

His-tagged Sso DNA pol B1 and the DNA pol Y1 were purified from *E. coli* over-expressing strains as described by Lou et al. (2004) and by Shimizu et al. (2003), respectively.

The truncated forms of Sso DNA pol B1 were produced by PCRs using the pET-DNA pol B1 plasmid

DNA as the template. To produce the proteins Sso DNA pol B1-721, -617 and -481 the 5'-primer, named *Nt-Bam*, had the following sequence: 5'-GGGTTT GGATCCGAATGACTAAGCAACTTACCTTA-3', the *Bam*HI restriction site is underlined; the 3'-primers were respectively: B-721-*Hind* (5'-TTGGAAGCTTCTATGTTTCAGTCCAAATACACCGTA-3'); B-617-*Hind* (5'-TTGGAAGCTTCTATCTCTTCTTCACTAACATCCCTT-3'); B-481-*Hind* (5'-GGGTTTAAGCTTCTATTTCGCTAAGATTTCTTCCCTT-3'). These latter oligonucleotides contain a stop codon immediately upstream the *Hind*III restriction site. The PCR amplified DNA fragment were purified, digested with *Bam*HI and *Hind*III and cloned into the *E. coli* expression vector pTRC-*HisC* (Invitrogen). All the cloned DNA fragments were sequenced to rule out the presence of undesired mutations.

To produce the recombinant proteins *E. coli* competent cells of the strain BL21(DE3) Rosetta (Novagen) were transformed with each expression plasmid. Transformed cells were grown in 0.5 litre of LB medium supplemented with chloramphenicol (at 30 µg/ml) and ampicillin (at 100 µg/ml). When the culture reached an optical density of 0.7 unit at 600 nm expression of the recombinant protein was induced by adding IPTG at 0.2 mM into the medium. Then the bacterial culture was incubated at 37°C for additional 2 h. Cells were harvested by centrifugation at 8,000 rpm for 10 min at 10°C using a GS-3 Sorvall rotor. Cell pellets were stored at -20°C until use.

Pellets of *E. coli* recombinant cells expressing the truncated protein Sso DNA pol B1-481 or -721 were resuspended in 10 ml of Buffer A (20 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 1 mM DTT, 50 mM NaCl) containing protease inhibitors (PMSF at 50 µg/ml, benzamide at 0.2 µg/ml, aprotinin at 1 µg/ml). Cells were lysed by two consecutive passages through a French press apparatus (Aminco Co., Silver Spring, MD) at 1,500 p.s.i.. The cell extract was centrifuged at 30,000 rpm for 30 min at 10°C in a Beckman 70.0 Ti rotor. The supernatant was incubated at 70°C per 15 min and transferred into ice per 10 min. The heat-treated samples were centrifuged at 30,000 rpm for 30 min at 10°C in a Beckman 70.0 Ti rotor. The supernatants, filtered through a 0.22 µm filter, were loaded onto a Ni²⁺-NTA chelate agarose super-flow column (Qiagen) equilibrated in Buffer A. Bound proteins were eluted by a stepwise gradient of imidazole (10–500 mM) in Buffer A containing 20% glycerol. Collected fraction (1 ml) were analysed by SDS-PAGE and those containing the recombinant protein were pooled and dialysed against Buffer B (20 mM Tris-HCl, pH 8.0, 1 mM DTT, 200 mM NaCl) overnight at 10°C. The sample was concentrated and stored at -20°C.

Pellets of *E. coli* recombinant cells expressing the truncated protein Sso DNA pol B1-617 were treated as described before. The cell extract was incubated for 10 min at 65°C and then transferred to ice for 10 min. The sample was centrifuged at 30,000 rpm for 30 min

using a Beckman 70.0 Ti rotor. The supernatant was filtered through a 0.22 μm filter and subjected to anionic-exchange chromatography on a Mono Q HR 10/10 column (Amersham/Pharmacia Biosciences) equilibrated in Buffer A. The column was developed with a linear gradient of NaCl (0–1 M). Collected fractions (1 ml) were analysed by SDS-PAGE and those containing the recombinant protein were pooled. The sample was dialysed against Buffer C (10 mM Tris-HCl, pH 8.5, 2.5 mM MgCl_2) overnight at 10°C and loaded onto a Heparin Sepharose column equilibrated in Buffer C. Fractions containing the recombinant protein were pooled and dialysed against Buffer B overnight at 10°C, concentrated and stored at -20°C.

Surface plasmon resonance measurements

Real-time interactions of Sso DNA pol B1 and DNA pol Y1 were monitored using the surface plasmon resonance biosensor system Biacore 2000 (Biacore). Sso DNA pol B1 was diluted to a concentration of 20 $\mu\text{g/ml}$ in buffer 10 mM sodium acetate pH 3.6 and coupled to the carboxy-methylated dextran modified gold surface of a CM5 sensor chip, according to the manufacturer's instruction manual. Sso DNA pol Y1 was diluted at 40 $\mu\text{g/ml}$ in buffer 10 mM sodium acetate pH 5.0 and immobilised using the same procedure. Under these conditions, surfaces containing densities of about 2,100 resonance units of Sso DNA pol B1 and 7,000 resonance units of Sso DNA pol Y1 were generated. To collect sensorgrams the indicated proteins at various concentrations were passed over the sensor surface at a flow rate of 10 $\mu\text{l/min}$. Recorded sensorgrams were normalised to a baseline of zero resonance unit and analysed using the BIA Evaluation software.

Immuno-precipitation experiments

Protein A Sepharose CL-4B resin (250 μg) was re-suspended in Binding Buffer (50 mM Tris-HCl pH 7.0, 40 mM NaCl, 20 mM MgCl_2 , 2.5 mM 2-mercaptoethanol) and conjugated with anti-Sso DNA pol Y1 antibodies. Mixtures (final volume: 40 μl) were prepared which contained in Binding Buffer: 9 μg of Sso DNA pol B1 and 7 μg of Sso DNA pol Y1, 9 μg of Sso DNA pol B1 alone (negative control experiment), 7 μg of Sso DNA pol Y1 alone (positive control experiment). To each mixture 80 μl of Protein A Sepharose resin conjugated with anti-Sso DNA pol Y1 antibodies were added. The samples were incubated for 1 h at room temperature with gentle shaking. The resin of each mixture was washed with 5 ml of Washing Buffer (50 mM Tris-HCl pH 7.0, 300 mM NaCl, 1 mM MgCl_2 , 1% Triton-X-100) and then re-suspended in 60 μl of SDS-PAGE Sample Buffer 1 \times (62 mM Tris-HCl pH 6.8, 1% glycerol, 0.5% SDS, 0.5% 2-mercapto-ethanol, 0.01% blue bromophenol). Samples were run on a 10% polyacrylamide denaturing

gel. After the electrophoretic run the gel was transferred to a PVDF membrane, which was cut into two halves: the upper part was analysed using anti-His antibodies conjugated with horseradish peroxidase (Qiagen) and the ECL + system (Amersham/Pharmacia Biosciences). The lower half of the membrane was analysed using anti-Sso DNA polY1 antibodies and the anti-rabbit IgG antibodies conjugated with alkaline phosphatase as the primary and secondary antibody, respectively.

Results

Direct physical interaction between Sso DNA pol B1 and DNA pol Y1

The physical interaction between Sso DNA pol B1 and DNA pol Y1 was monitored using the surface plasmon resonance Biacore 2000 system. In an initial set of experiments Sso DNA pol B1 solutions of increasing concentrations were passed over a DNA pol Y1-immobilised CM5 sensor chip. Fig. 1 shows an example of overlaid sensorgrams obtained with four different concentrations of DNA polB1 (from 0.15 to 1.2 μM). The amplitude of the curves is proportional to the concentration of the analyte, suggesting a direct physical association of the two proteins. The dissociation rate of DNA pol B1 was very low and the equilibrium dissociation constant (K_D) was in the order of 1×10^{-8} M. Evidence for a physical interaction between the two *Sulfolobus* DNA pols was obtained in similar experiments where DNA pol Y1 was used as the analyte and DNA pol B1 as the ligand (data not shown).

The Sso DNA pol B1/DNA pol Y1 interaction was also tested by immuno-precipitation experiments using the purified recombinant proteins. Protein A Sepharose beads conjugated with anti-DNA pol Y1 antibodies were added to mixtures of the two polymerases. As shown in Fig. 2, DNA pol B1 was pulled down with

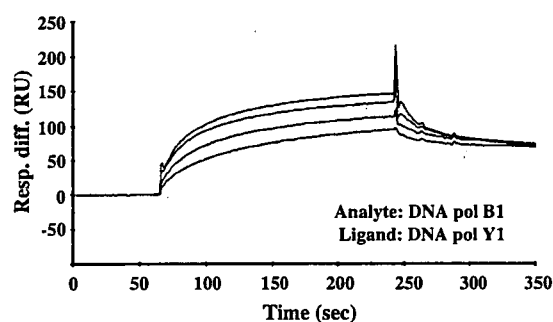


Fig. 1 Physical interaction between Sso DNA pol B1 and DNA pol Y1 detected by surface plasmon resonance measurements. An overlaid plot of sensorgrams was obtained by fluxing DNA pol B1 at various concentrations over a DNA pol Y1-immobilised sensor chip (lower to upper curve DNA pol B1 was at 0.15, 0.3, 0.6 and 1.2 μM , respectively), as described in Materials and methods

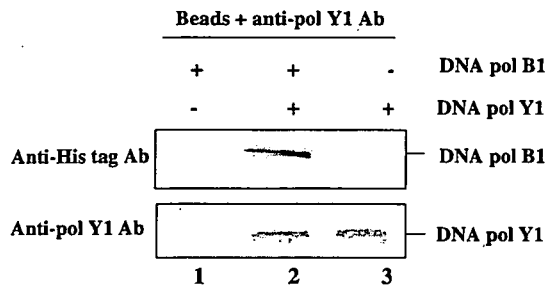


Fig. 2 Physical interaction between Sso DNA pol B1 and DNA pol Y1 detected by immuno-precipitation experiments. Immuno-precipitation experiments were carried out using Protein A Sepharose beads conjugated with anti-DNA pol Y1 antibodies. The following protein samples were analysed: *lane 1*, Sso DNA pol B1 alone (9 μ g, negative control experiment); *lane 2*, a mixture of Sso DNA pol B1 (9 μ g) and Sso DNA pol Y1 (7 μ g); *lane 3*, Sso DNA pol Y1 alone (7 μ g, positive control experiment). After the electrophoretic run the gel was transferred to a PVDF membrane, which was cut into two halves: the upper part was analysed using anti-His antibodies using the ECL⁺ system to detect the His-tagged Sso DNA pol B1. The lower half of the membrane was analysed using anti-Sso DNA pol Y1 antibodies and the anti-rabbit IgG antibodies conjugated with alkaline phosphatase as the primary and secondary antibody, respectively

DNA pol Y1 in these experiments confirming that a physical association takes place between the two proteins, as also indicated by the surface plasmon resonance analyses.

Production of Sso DNA pol B1 truncated forms

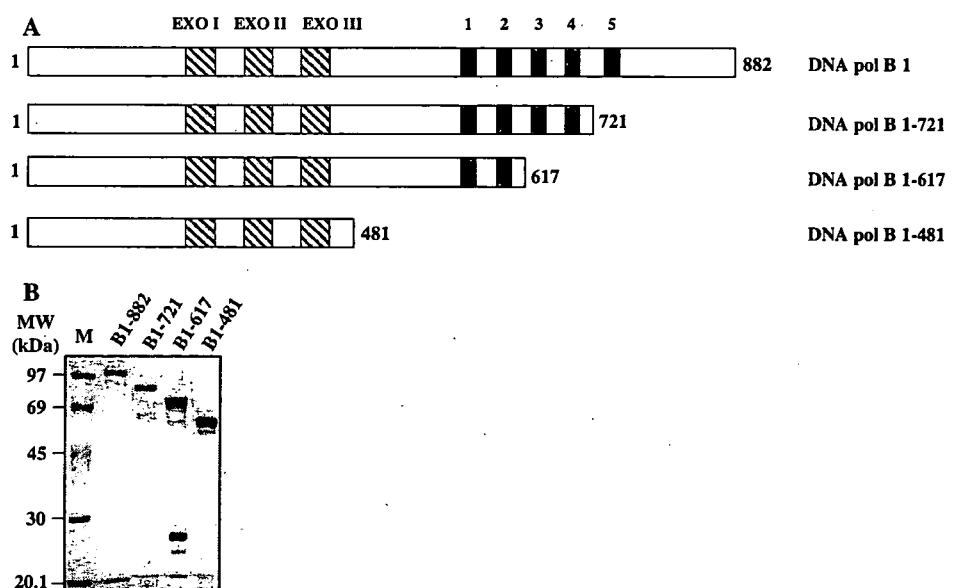
In previous studies we demonstrated that the Sso DNA pol B1 possesses a modular organization with regions that are hyper-sensitive to the proteolytic digestion (Pisani and Rossi 1994; Pisani et al. 1996). The resolution

of the Sso DNA pol B1 crystallographic structure by X-ray diffraction analysis confirmed the domain organization of the protein (Savino et al. 2004). Based on this knowledge, we designed truncated forms of DNA pol B1 with the aim of identifying the protein region responsible for the physical interaction with DNA pol Y1 (see Fig. 3). The DNA pol B1-721 deleted protein lacks the whole "thumb" sub-domain (residues 721–882) which is responsible for the interaction with the DNA template strand. The truncated form DNA pol B1-617 lacks almost the entire polymerase module, excluding a small portion of the "palm" sub-domain (the beta-sheets 19–21 and the alpha-helix Q) and the "fingers" sub-domain. The truncated protein DNA pol B1-481 consisted of the protein N-terminal half including the uracil-binding pocket and the proof-reading exonuclease domain. These three C-terminally deleted forms of DNA pol B1 were produced in *E. coli* as His-tagged proteins using the pTRC-*His* vector system and found to be soluble. Their purification was readily attained by heat-treatment of the cell extracts and chromatographic procedures (see Fig. 3).

Interaction of the Sso DNA pol B1 truncated forms with the DNA pol Y1

The physical interaction of the Sso DNA pol B1 deleted forms with DNA pol Y1 was probed by surface plasmon resonance measurements. In these experiments solutions of each purified protein of various concentrations were passed over a DNA pol Y1-immobilised CM5 sensor chip. The protein DNA pol B1-481 was unable to interact with DNA pol Y1 suggesting that the C-terminal polymerase domain (residues 482–882) contains the region critical for the interaction with DNA pol Y1.

Fig. 3 Diagrammatic representation of Sso DNA pol B1 deleted forms used in this study. **a** The position of the B Family DNA pols sequence similarity motifs is indicated (Blanco et al. 1991). **b** Electrophoretic analysis of purified DNA pol B1 and its deleted forms (5 μ g) on a 10% SDS-polyacrylamide gel and Coomassie Blue staining. The molecular weight of size markers run in the *lane M* are reported on the left part of the panel



Surface plasmon resonance measurements were also carried out using either Sso DNA pol B1-617 or -721 truncated proteins as the analytes. Both these truncated forms were found to physically associate with the DNA pol Y1 with a binding affinity comparable with the full-sized DNA pol B1. As shown in Fig. 4, similar sensorgrams were obtained by fluxing each DNA pol B1 C-terminal deleted form over a DNA pol Y1-immobilised sensor chip. These results suggest that the protein region from residue 482 to 617 is critical for the DNA pol B1/DNA pol Y1 interaction.

Discussion

The hyper-thermophilic crenarchaeon *S. solfataricus* has two functional DNA polymerases, DNA pol B1 and DNA pol Y1. We previously reported that these DNA pols are expressed at about the same level in *S. solfataricus* cells by quantitative Western blot analysis suggesting that both enzymes play an important role in genome maintenance (Gruz et al. 2003). Sso DNA pol B1 is able to recognize the deaminated bases uracil and hypoxanthine on the template DNA strand and to stop synthesis 3–4 bases upstream of these lesions. In addition, Sso DNA pol B1 also stops nucleotide incorporation 1 base before 8-oxo-guanine. In contrast, Sso DNA pol Y1 belongs to the family of translesion synthesis DNA pols and readily bypasses all these damaged bases in the template strand. Our analysis has revealed that Sso DNA pol B1 and DNA pol Y1 directly interact in vitro, as assessed by surface plasmon resonance measurements and immuno-precipitation experiments. The results of the protein-protein interaction experiments carried out with the DNA pol B1 truncated forms suggest that the region responsible for this physical interaction spans amino acid residues 482–617. This portion of the Sso DNA pol B1 polypeptide chain includes the extended protease hyper-sensitive linker between the N- and C-terminal modules of the

enzyme (amino acid residues Asn482-Ala497) and the α -helices forming the “fingers” sub-domain (α -helices R, R' and S; Savino et al. 2004). The extended linker was demonstrated to be highly flexible and exposed to the solvent in a limited proteolysis study (Pisani et al. 1996) and this notion has been subsequently confirmed by the X-ray crystallographic analysis of Sso DNA pol B1 (Savino et al. 2004). In fact, the 3D structure of this region is not traced in the protein structural model because of its poorly or completely undefined electron density due to high conformational mobility. It is tempting to speculate that interaction with DNA pol Y1 could stabilize this linker region and protect it against the protease attack in vivo.

Mouse and human REV1 subunit of DNA pol ζ were reported to physically interact with the trans lesion synthesis DNA pols ι , η and κ and this interaction was proposed to play a pivotal role in the multi-enzyme, multi-step process of lesion bypass in mammal cells (Guo et al. 2003; Ohashi et al. 2004). In *S. solfataricus* cells, stalling of DNA pol B1 at the template uracil or hypoxanthine may generate single strand gap downstream of the lesion site. Gap filling is likely to be carried out by homologous recombination or translesion synthesis by DNA pol Y1. This latter event requires a switch from DNA pol B1 to DNA pol Y1 at the damaged base. Based on the results of our biochemical analysis, we propose that the physical interaction between Sso DNA pol B1 and DNA pol Y1 could play a role in the polymerase switching mechanism at the lesion site during chromosomal replication. However, we were unable to detect any effect on the uracil-bypass efficiency by Sso DNA pol Y1 in the presence of the full-sized DNA pol B1 or its C-terminal truncated forms. It is quite likely that other factors participate in the translesion synthesis process in *S. solfataricus* cells. After lesion bypass by DNA pol Y1, additional DNA repair enzymes might be recruited at the stalled replication fork, such as DNA glycosylases. These enzymes have been identified in various hyper-thermophilic archaea (Sartori and Jiricny 2003; Chung et al. 2003). It has been shown that the uracil DNA glycosylases physically interacts with the PCNA-like sliding clamp in *Pyrobaculum aerophilum* (Yang et al. 2002) and in *S. solfataricus* (Dionne and Bell 2005). This interaction could be responsible for the recruitment of these and other DNA repair enzymes at the replication fork in vivo. However, our attempts failed to detect a direct physical interaction between Sso DNA pol Y1 and any of the three *S. solfataricus* PCNA-like homologs by surface plasmon resonance experiments suggesting that this interaction is not stable enough to be detected by this technique. Analysis of the physical and/or functional interaction between the *S. solfataricus* DNA pols and the enzymes responsible for repairing deaminated bases in DNA removal will provide insights into genome maintenance mechanisms in this hyper-thermophilic crenarchaeon.

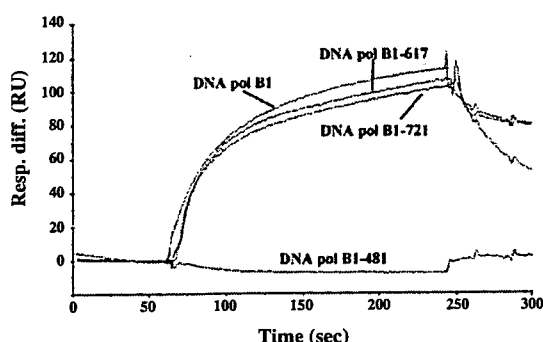


Fig. 4 Physical interaction of the Sso DNA pol B1 truncated forms with DNA pol Y1 detected by surface plasmon resonance measurements. Plot of sensorgrams obtained by fluxing each indicated protein at 0.3 μ M over a DNA pol Y1-immobilised sensor chip, as described in Materials and methods

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Efficient and Erroneous Incorporation of Oxidized DNA Precursors by Human DNA Polymerase η

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Efficient and Erroneous Incorporation of Oxidized DNA Precursors by Human DNA Polymerase η [†]

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ABSTRACT: Altered oxidative metabolism is a property of many tumor cells. Oxidation of DNA precursors, i.e., dNTP pool, as well as DNA is a major source of mutagenesis and carcinogenesis. Here, we report the remarkable nature of human DNA polymerase η that incorporates oxidized dNTPs into a nascent DNA strand in an efficient and erroneous manner. The polymerase almost exclusively incorporated 8-hydroxy-dGTP (8-OH-dGTP) opposite template adenine (A) at 60% efficiency of normal dTTP incorporation, and incorporated 2-hydroxy-dATP (2-OH-dATP) opposite template thymine (T), guanine (G), or cytosine (C) at substantial rates. The synthetic primers having 8-hydroxy-G paired with template A or 2-hydroxy-A paired with template T, G, or C at the termini were efficiently extended. In contrast, human DNA polymerase ι incorporated 8-OH-dGTP opposite template A with much lower efficiency and did not incorporate 2-OH-dATP opposite any of the template bases. It did not extend the primers having the oxidized bases at the termini either. We propose that human DNA polymerase η may participate in oxidative mutagenesis through the efficient and erroneous incorporation of oxidized dNTPs during DNA synthesis.

Reactive oxygen species (ROS¹), such as superoxide, hydrogen peroxide, hydroxyl radicals, and singlet oxygen, are produced through normal cellular metabolism, and formation of such radicals is further enhanced by irradiation or chemical exposure (1, 2). ROS generates a variety of altered purines and pyrimidines in DNA (3, 4), and oxidation of DNA plays important roles in mutagenesis, carcinogenesis, and aging (5, 6). Several lines of evidence indicate, however, that oxidation of DNA precursors in the nucleotide pool, i.e., dNTPs, is another important cause of genome instability (7–9). Indeed, the frequency of A:T-to-C:G transversion muta-

tions increases more than 1000-fold over the wild-type level in *Escherichia coli mutT* mutants, which are deficient in the ability to hydrolyze oxidized dGTP, i.e., 7,8-dihydro-8-oxo-dGTP (8-hydroxy-dGTP, 8-OH-dGTP, Figure 1A) (10, 11). 8-OH-dGTP leads to A:T-to-C:G mutations when it is incorporated opposite adenine (A) in the template DNA because the incorporated 8-OH-G in DNA can pair with incoming dCMP in the next round of DNA replication (8, 12). The high spontaneous A:T-to-C:G mutations in the *mutT* strain are almost completely suppressed when the *mutT* cells are cultured in anaerobic conditions, indicating the essential role of oxygen in the mutagenesis (13). Another oxidized nucleotide, i.e., 1,2-dihydro-2-oxo-dATP (2-hydroxy-dATP, 2-OH-dATP, Figure 1B), can induce G:C-to-T:A transversions when it is incorporated opposite guanine (G) in the template (14, 15). The sanitizing enzyme, i.e., Orf135, in *E. coli* degrades 2-OH-dATP, and G:C-to-T:A mutations occur in the *orf135*-deficient strain more frequently than in the wild-type strain (16, 17).

Oxidized dNTPs also cause genome instability in mammalian cells. Spontaneous tumorigenesis in mice deficient in *Mth1*, a mammalian counterpart of *mutT*, is much enhanced in lung, liver, and stomach, and the MTH1 protein hydrolyzes both 8-OH-dGTP and 2-OH-dATP (18, 19). Recent studies with mismatch repair defective cells suggest that the majority of mutations in human cells that are deficient in mismatch repair functions do not arise from spontaneous replication errors, but from the incorporation of oxidized dNTPs (20, 21). Thus, it is of great interest in

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¹ Abbreviations: ROS, reactive oxygen species; 8-OH-dGTP, 7,8-dihydro-8-oxo-dGTP; A, adenine; 2-OH-dATP, 1,2-dihydro-2-oxo-dATP; G, guanine; Pol ι , DNA polymerases; C, cytosine; TLS, translesion DNA synthesis; T, thymine; Pol η , DNA polymerase η ; Pol ι , DNA polymerase ι ; HPLC, high performance liquid chromatography.

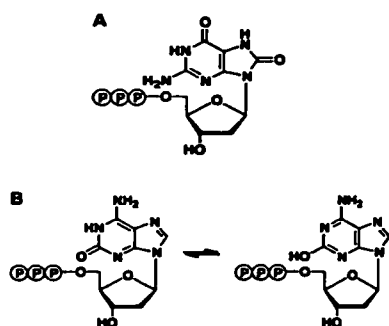


FIGURE 1: Structures of 8-OH-dGTP (A) and 2-OH-dATP (B). P represents a phosphate group.

the mechanisms as to how these oxidized dNTPs induce genome instability and oxidative carcinogenesis.

When the oxidized dNTPs escape from the sanitation by the enzymes, they will be incorporated into nascent DNA by DNA polymerases (Pols). However, oxidized dNTPs are in general poor substrates for Pols (22). For example, the efficiency of incorporation of 8-OH-dGTP by Pol δ is more than 10^4 -fold lower than that of incorporation of normal dGTP, and the enzyme prefers to incorporate 8-OH-dGTP opposite template cytosine (C) (23). 8-OH-dGTP is poorly incorporated into DNA by T7 Pol exo^- , HIV reverse transcriptase, *E. coli* Pol II, and Klenow exo^- as well (24). An exception may be human Pol β , which incorporates 8-OH-dGTP into DNA with an efficiency of 10–20% of normal dGTP incorporation and favors its incorporation opposite template A (25). 2-OH-dATP is also a poor substrate for mammalian Pols. The efficiencies of incorporation of 2-OH-dATP opposite template T and C by Pol α are more than 100-fold and 1000-fold, respectively, lower than those of incorporation of normal dATP and dGTP (26).

The Y-family Pols are recently recognized Pols that comprise proteins from different species, including members of Archaea, Bacteria, and Eukarya (27). The most distinct feature of this family of enzymes is their ability to bypass various lesions, such as ultraviolet light photoproducts, in template DNA (28–30). Some bypass reactions, i.e., translesion DNA synthesis (TLS), catalyzed by these enzymes are error prone while others are error free (31). Thus, this family of Pols seems to be involved in mutagenesis and DNA-damage tolerance (32). Interestingly, some of the Y-family Pols are shown to incorporate oxidized dNTPs into DNA in an erroneous manner. Archaeal Y-family Pols from *Sulfolobus* sp. and a bacterial Y-family Pol, i.e., DNA Pol IV (DinB) of *E. coli*, almost exclusively incorporate 8-OH-dGTP opposite template A, and 2-OH-dATP opposite template G and thymine (T) (33, 34). Further genetic analysis with *E. coli* *sod/fur* mutants suggests that both Y-family Pols of *E. coli*, i.e., Pol IV and Pol V (UmuD'C), are involved in oxidative mutagenesis caused by oxidized dNTPs (34). The enzymes may participate in sequential biochemical steps, such as incorporation of oxidized dNTPs into DNA and extension of primers having oxidized bases at the termini. Pol IV is also shown to be involved in induction of G:C-to-T:A mutations when 2-OH-dATP is directly introduced into *E. coli* cells by CaCl₂ treatment (35). Collectively, these results led us to postulate that certain human Y-family Pols might be involved in oxidative mutagenesis through incorrect and efficient incorporation of oxidized dNTPs into nascent DNA. In fact, human Pol η incorporates oxidized dNTPs into

DNA in an erroneous manner like the archaeal and bacterial Y-family Pols (33).

In this study, we quantitatively compared two human Y-family Pols, i.e., Pol η and Pol ι , in the specificity and efficiency of incorporation of 8-OH-dGTP and 2-OH-dATP into a nascent DNA strand. Surprisingly, Pol η incorporated 8-OH-dGTP opposite template A at almost the same efficiency as incorporation of normal dTTP, and incorporated 2-OH-dATP opposite template T, G, and C at substantial rates. In contrast, Pol ι poorly incorporated 8-OH-dGTP opposite template A and did not incorporate 2-OH-dATP opposite any of the bases in template DNA. Since Pol η is constitutively associated with replication factories (36, 37) and involved in multiple DNA transactions (38–40), we propose that this enzyme may be involved in mutagenesis through the efficient and erroneous incorporation of oxidized dNTPs into DNA strand.

EXPERIMENTAL PROCEDURES

Materials. Human Pol η and Pol ι were purified as described (41, 42). 8-OH-dGTP and 2-OH-dATP were prepared as described (26, 43), and no discernible impurities were detected by high performance liquid chromatography (HPLC). dNTPs (ultrapure grade) were purchased from GE Healthcare Bio-Sciences KK (Tokyo).

DNA Substrates. Oligonucleotides containing 8-OH-G and 2-OH-A were prepared by the phosphoramidite method on controlled pore glass supports (1 μ mol) by using a Beckman OLIGO1000 DNA synthesizer. After automated synthesis, the oligomers were detached from the support, deprotected, and purified by HPLC. The oligomers were identified by complete digestion of the oligomers with alkaline phosphatase and P1 nuclease to 2'-deoxymononucleosides. Other oligonucleotides were purchased from BEX Corp. (Tokyo). A Cy3-labeled 18-mer primer (5'-CGCGCGAAGACCG-GTTAC-3') or Cy3-labeled 19-mer primers (5'-CGCGC-GAAGACCGGTTACN-3' where N represents either 8-OH-G or 2-OH-A) were annealed to 36-mer templates (5'-GAAGGGATCCTTAAGACXGTAACCGGTCTTCGCGG-3' where X represents A, C, G, or T) at a molar ratio of 1:1 to generate substrates. The 5'-Cy3-primer/templates generated by annealing the 18-mer primer to the 36-mer template were named substrate 1, and those generated by annealing the 19-mer primer having 8-OH-G or 2-OH-A to the 36-mer templates were named substrate 2 or 3, respectively. In control reactions, Cy3-labeled 19-mer primers (5'-CGCGC-GAAGACCGGTTACN-3' where N represents either G or A) were annealed to the 36-mer templates. The 5'-Cy3-primer/templates generated by annealing the 19-mer primer having G or A to the 36-mer templates were named substrate 4 or 5, respectively.

Steady-State Kinetic Analyses. The standard reaction mixtures (10 μ L) contained 40 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 10 mM dithiothreitol, 250 μ g/mL bovine serum albumin, 60 mM KCl, 2.5% glycerol, 1 nM Pol η or Pol ι , 100 nM primer/template DNA (substrate 1, 2, or 3), and a single oxidized dNTP (8-OH-dGTP or 2-OH-dATP) or normal dNTP (dATP, dTTP, dGTP, or dCTP). The activity of Pol ι was also measured in the same reaction mixtures but without KCl (44). In that case, the concentration of MgCl₂ was reduced to 1 mM instead of 5 mM. Six different

concentrations of oxidized dNTP or normal dNTP were used to determine the kinetic parameters, i.e., k_{cat} and K_m (see below). The mixtures were incubated at 37 °C, and the reactions were terminated by adding 10 μ L of stop solution (98% formamide, 10 mM EDTA, 10 mg/mL Blue Dextran). After heat denaturation for 10 min at 100 °C, the samples were separated by electrophoresis with 15% denaturing polyacrylamide gel containing 8 M urea and visualized by the Molecular Imager FX Pro System (Bio-Rad Laboratories, CA).

When the kinetic parameters for incorporation of oxidized or normal dNTPs into DNA were determined, the reaction mixtures contained substrate 1. The reaction time and the concentrations of oxidized dNTP and normal dNTP varied depending on the rates of reactions and the K_m values. For example, the incubation time was 1 min and the concentrations of 8-OH-dGTP varied from 0.05 to 10 μ M for the analysis of incorporation of 8-OH-dGTP opposite template A by Pol η . Identical reaction conditions were used for the analyses of incorporation of a single normal dNTP opposite a template canonical base by Pol η . On the other hand, the incubation time was 10 min and the range of concentrations of 8-OH-dGTP was 5 to 100 μ M to determine the parameters for incorporation of 8-OH-dGTP opposite template A by Pol ι . For the kinetic analyses of extension of primers having oxidized bases at the 3'-termini by Pol η , the incubation time was 5 min and the concentrations of dGTP varied from 0.05 to 10 μ M with the template/primer DNAs of substrate 2 or 3.

Data Analyses. Gel band intensities were measured using the Molecular Imager FX Pro System and Quantity One software (Bio-Rad Laboratories, CA), and nucleotide incorporation parameters were determined (45). Less than 20% of the primers were extended in these steady-state kinetic analyses, ensuring single-hit kinetics (46). For each DNA substrate, the rate of incorporation was plotted as a function of dNTP concentration, and the V_{max} and K_m values were determined by nonlinear regression fitting using the Sigma-Plot software (Systat Software Inc., CA). k_{cat} was calculated by dividing V_{max} by the enzyme concentration. The fidelity of incorporation, i.e., F_{inc} , was calculated using the equation $F_{inc} = (k_{cat}/K_m)_{incorrect}/(k_{cat}/K_m)_{correct}$. All values are means \pm standard errors from three experiments.

RESULTS

Efficient and Erroneous Incorporation of 8-OH-dGTP and 2-OH-dATP into DNA by Pol η . Human Pol η almost exclusively incorporates 8-OH-dGTP opposite template A and incorporates 2-OH-dATP opposite template T, G, and C (33). To compare two human Y-family Pols, we at first examined the specificity of human Pol ι incorporating 8-OH-dGTP and 2-OH-dATP into DNA. To this end, we used substrate 1 having four different bases in the N position in the template and incubated it with Pol ι in the presence of either 8-OH-dGTP or 2-OH-dATP. For the incorporation of 8-OH-dGTP, the primer annealed to the template strand having A in the N position was elongated, and no other primers annealed to templates having C, G, or T in the N position were extended (Figure 2A). These results indicate that Pol ι almost exclusively incorporates 8-OH-dGTP opposite template A as in the case of Pol η . For the incorporation of 2-OH-dATP, no

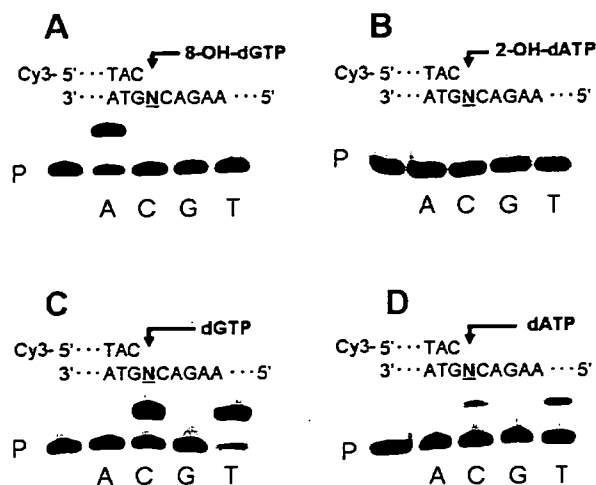


FIGURE 2: Incorporation of 8-OH-dGTP (A), 2-OH-dATP (B), dGTP (C), and dATP (D) into DNA by Pol η . For analysis of incorporation of oxidized dNTPs (A and B), the reaction mixtures described in Experimental Procedures contained 1 nM Pol η , 50 μ M either 8-OH-dGTP or 2-OH-dATP, and 100 nM substrate 1. No normal dNTPs were included. For analysis of incorporation of normal dNTPs (C and D), the reaction mixtures contained 5 nM Pol ι , 50 μ M either dGTP or dATP, and 100 nM substrate 1. In the reaction, KCl was omitted from the reaction mixtures and the concentration of MgCl $_2$ was 1 mM. The mixtures were incubated for 15 min at 37 °C, and the samples were processed as described in Experimental Procedures. The N in the template strand represents A, C, G, or T.

Table 1: Kinetic Parameters for Incorporation of 8-OH-dGTP and 2-OH-dATP by Pol η and Pol ι

Pol	template base/dNTP	K_m (μ M)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{mM}^{-1} \text{min}^{-1}$)	F_{inc}
Pol η	A/dTTP	3.8 ± 0.52	15.4 ± 0.82	4.1	1
	A/8-OH-dGTP	4.2 ± 0.35	10.1 ± 0.35	2.4	0.59
	C/dGTP	1.2 ± 0.22	1.9 ± 0.1	1.6	1
	C/8-OH-dGTP	22.5 ± 8.3	0.3 ± 0.05	0.013	0.008
	C/dGTP	1.2 ± 0.22	1.9 ± 0.1	1.6	1
	C/2-OH-dATP	1.7 ± 0.55	0.1 ± 0.007	0.06	0.038
	G/dCTP	1.6 ± 0.12	9.5 ± 0.22	5.9	1
	G/2-OH-dATP	3.6 ± 0.6	0.35 ± 0.02	0.1	0.017
Pol ι	T/dATP	5.2 ± 0.47	15 ± 0.61	2.9	1
	T/2-OH-dATP	3.0 ± 0.52	0.55 ± 0.03	0.18	0.062
	A/dTTP	14.4 ± 3.0	5.1 ± 0.67	0.35	1
	A/8-OH-dGTP	132 ± 45.4	1.4 ± 0.31	0.011	0.031
	A/dTTP ^a	0.86 ± 0.14^a	2.24 ± 0.08^a	2.6^a	1^a
	A/8-OH-dGTP ^a	48.5 ± 7.37^a	2.17 ± 0.15^a	0.045^a	0.017^a

^a The activities were measured without KCl in the reaction mixtures.

primers were elongated regardless of the bases in the template strand (Figure 2B). The activity of Pol ι is enhanced by omission of KCl from the reaction mixtures (44). We conducted, therefore, the experiments in the absence of KCl. Although the activity was enhanced several times (Table 1), the specificity was unchanged. 8-OH-dGTP was almost exclusively incorporated opposite template A, and no 2-OH-dATP was incorporated into DNA regardless of the template bases (data not shown). In control reactions, Pol ι incorporated normal dGTP opposite template C and T and incorporated normal dATP opposite template T and slightly opposite template C (Figure 2C,D). Thus, we conclude that, although the incorporation of 8-OH-dGTP opposite template A is a common feature to Y-family Pols, the specificity incorporating 2-OH-dATP into DNA is markedly distinct even within two human homologues.

To compare the efficiency of two Pols for the incorporation of oxidized dNTPs quantitatively, we determined the steady-state kinetic parameters, i.e., K_m and k_{cat} (Table 1). The most remarkable feature of the kinetic data is the high efficiency of Pol η incorporating 8-OH-dGTP opposite template A. The K_m and k_{cat} values ($4.2 \mu\text{M}$ and 10.1 min^{-1} , respectively) were similar to those of incorporation of normal dTTP opposite template A ($3.8 \mu\text{M}$ and 15.4 min^{-1} , respectively). Thus, the F_{inc} , i.e., $(k_{cat}/K_m)_{8\text{-OH-dGTP}}/(k_{cat}/K_m)_{dTTP}$, was about 0.6 ($=2.4/4.1$), which suggests that Pol η incorporates 8-OH-dGTP opposite template A with the efficiency of about 60% of that of incorporation of normal dTTP. In addition, the incorporation of 8-OH-dGTP by Pol η was strongly biased opposite template A compared to template C. The k_{cat}/K_m value of incorporation of 8-OH-dGTP opposite template A was more than 180 times greater than that of incorporation of 8-OH-dGTP opposite template C (2.4 versus 0.013). In contrast, Pol ι incorporates 8-OH-dGTP opposite template A with much lower efficiency. The k_{cat}/K_m value of incorporation of 8-OH-dGTP opposite template A was more than 200 and 30 times, respectively, less than that of incorporation of 8-OH-dGTP opposite template A by Pol η (0.011 versus 2.4) and normal dTTP by Pol ι (0.011 versus 0.35). The k_{cat}/K_m value of incorporation of normal dTTP and 8-OH-dGTP opposite template A by Pol ι was enhanced several times by omission of KCl from the reaction mixtures (0.35 versus 2.6 and 0.011 versus 0.045). Nevertheless, the k_{cat}/K_m value of incorporation of 8-OH-dGTP opposite template A was more than 50 times less than that of incorporation of 8-OH-dGTP opposite template A by Pol η (0.045 versus 2.4) and normal dTTP by Pol ι (0.045 versus 2.6).

We then determined the kinetic parameters of incorporation of 2-OH-dATP opposite template C, G, or T by Pol η (Table 1). We did not determine the parameters for Pol ι because the enzyme did not incorporate 2-OH-dATP into DNA (Figure 2B). The K_m values for the incorporation of 2-OH-dATP by Pol η were indistinguishable from those for the incorporation of normal dNTPs opposite template C, G, or T. However, the k_{cat} values for the incorporation of 2-OH-dATP were 20 to 30 times less than those of incorporation of normal dNTPs. Nevertheless, the F_{inc} values, i.e., $(k_{cat}/K_m)_{2\text{-OH-dATP}}/(k_{cat}/K_m)_{dNTP}$, opposite template C, G, and T were 0.038, 0.017, and 0.062, respectively, suggesting that Pol η incorporates 2-OH-dATP opposite multiple template bases (except for A) with efficiency of 2 to 6% of that of incorporation of normal dNTPs. The order of k_{cat}/K_m values of incorporation of 2-OH-dATP opposite template bases was T (0.18) > G (0.10) > C (0.06).

Efficient Extension of Primers Having 8-OH-G or 2-OH-A at the 3'-Termini by Pol η . To investigate whether Pol η and Pol ι can extend the primers upon incorporation of oxidized dNTPs during DNA synthesis, we examined whether these Pols extend primers having either 8-OH-G or 2-OH-A at the 3'-termini. We annealed the primers to the template strands where the terminal oxidized base was paired with template A, C, G, or T and incubated the primer/templates, i.e., substrate 2 or 3, with Pol η or Pol ι in the presence of four normal dNTPs. Pol η extended the primer having 8-OH-G when the terminal oxidized base was paired with template A or C (Figure 3A). Virtually no extension was observed, however, when the terminal 8-OH-G was paired with template G or T. Pol η could also extend primers having

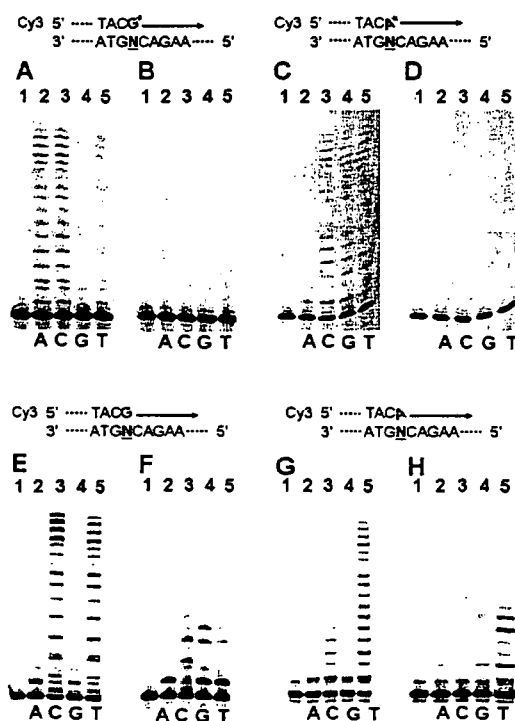


FIGURE 3: Extension of primers having 8-OH-G or 2-OH-A at the termini by Pol η and Pol ι . Primers having 8-OH-G were extended by Pol η (A) or Pol ι (B), and those having 2-OH-A were extended by Pol η (C) or Pol ι (D). As control reactions, primers having G were extended by Pol η (E) or Pol ι (F) and those having A were extended by Pol η (G) or Pol ι (H). The reaction mixtures described in Experimental Procedures contained 1 nM Pol η (A and C) or Pol ι (B and D), 200 μM four normal dNTPs (dATP, dCTP, dGTP, and dTTP), and 100 nM substrate 2 (A and B) or substrate 3 (C and D). The reaction mixtures were incubated for 5 min at 37 °C. In control reactions, the reaction mixtures contained 10 nM Pol η (E and G) or Pol ι (F and H), 200 μM four normal dNTPs, and 100 nM substrate 4 (E and F) or substrate 5 (G and H). In control reactions with Pol ι (F and H), KCl was omitted from the reaction mixtures and the concentration of MgCl_2 was 1 mM. The reaction mixtures were incubated for 5 min (E and G) or 15 min (F and H) at 37 °C. The samples were processed as described in Experimental Procedures. Lane 1 (the leftmost lane) represents the position of the primers alone. The N in the template strands represents A (lane 2), C (lane 3), G (lane 4), and T (lane 5).

2-OH-A when the terminal oxidized base was paired with template C, G, or T (Figure 3C). In contrast, Pol ι was unable to extend any primers containing either 8-OH-G or 2-OH-A at the termini regardless of the pairing template bases (Figure 3B,D). In control reactions, Pol η extended the primers having G at the termini when the terminal base was paired with template C or T (Figure 3E) and extended the primers having A at the termini when the terminal base was paired with template T (Figure 3G). Weak extension was observed when the terminal A was paired with template C. Pol ι was a poor extender even without oxidized bases at the termini of primers. It weakly extended primers when the terminal G or A was paired with canonical template bases (Figure 3F,H). These results suggest that Pol η extends the primers upon incorporation of 8-OH-dGTP opposite template A or C, and extends the primers upon incorporation of 2-OH-dATP opposite template C, G, or T. Two Y-family Pols are distinct for their ability to extend the primers having oxidized bases at the termini.

We then determined the steady-state kinetic parameters for the extension, i.e., incorporation of next dGTP opposite

Table 2: Kinetic Parameters for Incorporation of dGTP with Primers Having Terminal 8-OH-G or 2-OH-A Pairing with Multiple Template Bases by Pol η ^a

Template base/dNTP	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\mu\text{M}^{-1}\text{min}^{-1}$)
dGTP — G* — A C	0.98 ± 0.11	2.4 ± 0.08	2.4
dGTP — G* — C C	2.5 ± 0.38	2.6 ± 0.14	1.0
dGTP — A* — C C	7.3 ± 0.89	9.9 ± 0.61	1.4
dGTP — A* — G C	6.9 ± 1.3	7.3 ± 0.7	1.1
dGTP — A* — T C	2.8 ± 0.25	8.9 ± 0.28	3.2

^a G* = 8-OH-G; A* = 2-OH-A.

template C, by Pol η from the primers having either 8-OH-G or 2-OH-A at the 3'-termini (Table 2). We did not determine the parameters for Pol ι because it did not extend the primers having 8-OH-G or 2-OH-A (Figure 3B,D). The terminal 8-OH-G in the primer was paired with template A or C while the terminal 2-OH-A was paired with template C, G, or T. The primer/templates, i.e., substrates 2 or 3, were incubated with Pol η in the presence of dGTP without other dNTPs. For the extension from the primer having 8-OH-G at the termini, Pol η extended the primer more efficiently when the terminal oxidized base was paired with template A than template C. The K_m value for the extension from the primer having 8-OH-G paired with template A was 2.5 times less than that for the extension from the primer having the oxidized base paired with template C (0.98 versus 2.5 μM) although the k_{cat} values were very similar between the two substrates (2.4 versus 2.6 min^{-1}). Thus, the ratio, i.e., $(k_{cat}/K_m)_{8\text{-OH-G:A}}/(k_{cat}/K_m)_{8\text{-OH-G:C}}$, was calculated to be 2.4 ($=2.4/1.0$), suggesting that Pol η more than two times more efficiently extends the primer having 8-OH-G paired with template A compared to that having 8-OH-G paired with template C. For the extension from the primer having 2-OH-A at the 3'-termini, Pol η extended the primer most efficiently when the oxidized base was paired with template T followed by template C and template G. The K_m value for the extension from the primer having 2-OH-A paired with template T (2.8 μM) was 2 to 3 times less than those for the extension from the primers having 2-OH-A paired with template G or template C (6.9 or 7.3 μM), and the k_{cat} values were not very different among the three substrates (8.9, 7.3, or 9.9 min^{-1}). Thus, the ratio of k_{cat}/K_m values, i.e., $(k_{cat}/K_m)_{2\text{-OH-A:T}}:(k_{cat}/K_m)_{2\text{-OH-A:G}}:(k_{cat}/K_m)_{2\text{-OH-A:C}}$, was 1:0.34:0.44, which suggests that Pol η extends the primers having terminal 2-OH-A paired with template G or C at rates of 30 to 40% of that of extension from the primer having 2-OH-A paired with template T.

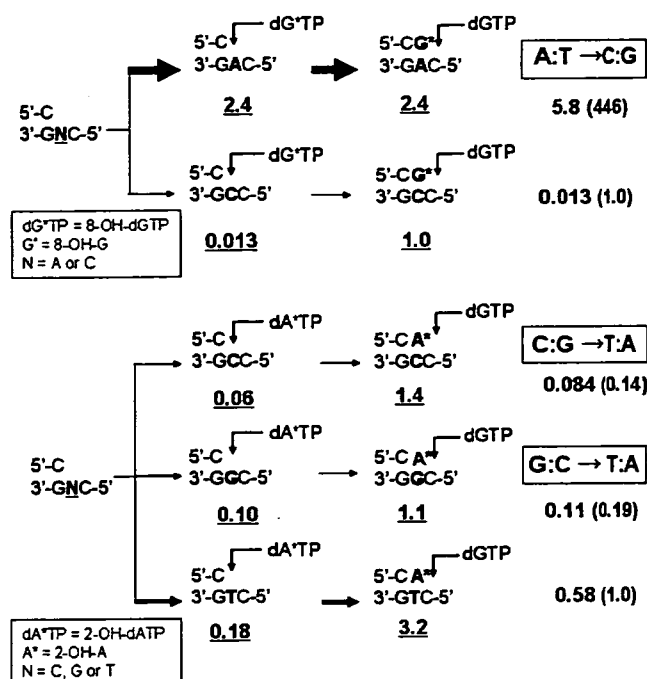


FIGURE 4: Total catalytic efficiency of incorporation of 8-OH-dGTP and 2-OH-dATP into DNA and the following primer extension by Pol η . The total efficiency of incorporation and extension was calculated as the product of the individual k_{cat}/K_m values, e.g., $2.4 \times 2.4 = 5.8$ for the incorporation of 8-OH-dGTP opposite template A and the extension by incorporation of normal dGTP opposite template C. The individual k_{cat}/K_m value for each step is underlined. Relative efficiency is presented in parentheses. The relative values were calculated by setting the efficiency of incorporation of 8-OH-dGTP opposite template C and the following extension or incorporation of 2-OH-dATP opposite template T and the following extension as 1.0.

DISCUSSION

Oxidation is a major cause of spontaneous DNA damage that may contribute to mutagenesis, carcinogenesis, and aging (5, 6). Oxidative damage in DNA is caused not only by direct oxidation of bases in DNA but also by incorporation of oxidized dNTPs in the nucleotide pool into DNA during chromosome replication (7, 47). In this study, we have revealed the remarkable nature of Pol η that incorporates 8-OH-dGTP opposite template A almost as efficiently as the incorporation of normal dTTP (Table 1). In addition, Pol η extends the primer having 8-OH-G paired with template A more than two times more efficiently than the same primer having 8-OH-G paired with template C (Table 2). Calculation of the total efficiency by multiplying the individual k_{cat}/K_m values suggests that Pol η prefers template A more than 400 times ($446 = 5.8/0.013$) to template C for the incorporation and extension (Figure 4). The efficient and erroneous incorporation of 8-OH-dGTP and the following extension may lead to A:T-to-C:G transversions because the incorporated 8-OH-G opposite template A may pair with dCMP in the next round of DNA replication (8, 43, 48). Besides 8-OH-dGTP, Pol η incorporates 2-OH-dATP opposite template T, G, and C with efficiency of 2 to 6% of that of incorporation of normal dNTPs (Table 1). The F_{inc} values of incorporation of 2-OH-dATP opposite template T and C are about 20 and 50 times higher than the corresponding values by calf thymus Pol α (26). Moreover, Pol η can extend the primers having terminal 2-OH-A paired with template T, G, and C

(Figure 3, Table 2). The relative preference of Pol η to template T:G:C for incorporation and extension is calculated as 1:0.19:0.14 (Figure 4). Incorporation of 2-OH-dATP opposite template G or C and the following extension may lead to G:C-to-T:A and C:G-to-T:A mutations since the incorporated 2-OH-A can pair with dTMP during the next round of DNA replication (14, 49). Thus, we suggest that Pol η has a potential to enhance mutagenesis through the efficient and erroneous incorporation of 8-OH-dGTP and 2-OH-dATP and the following extension during DNA replication (see below). It is possible, however, that other Pols may be involved in extension steps once oxidized dNTPs are inserted into nascent DNA by Pol η .

Although Pol η was initially identified as an error-free bypass Pol across a *cis-syn* thymine–thymine dimer in DNA (28, 29), it appears to play important roles in several DNA transactions besides TLS (50). Pol η interacts with RAD51 and has an activity to extend D loop, which is an intermediate of homologous recombination (38, 39). It also participates in mutagenesis at A:T base pairs in immunoglobulin genes during somatic hypermutation (40). In addition, Pol η is identified in replication factories in cells not deliberately exposed to DNA damaging agents (36, 37), suggesting that it might constitutively gain access to the genomic DNA and contribute to mutagenesis and/or damage avoidance even without external DNA damage. Interestingly, *E. coli* Y-family Pols, i.e., Pol IV (DinB) and variants of Pol V (UmuD' C), are shown to be involved in the chromosome replication when the cells are treated with hydroxyurea, which does not induce DNA damage but depletes the nucleotide pool (51). In this case, the Y-family Pols appear to rescue the stalled replication by efficient incorporation of dNTPs in the depleted nucleotide pool at the expense of increased mutations. By analogy, we envisage that Pol η might be localized in replication factories even without external DNA damage and contribute to rescue the stalled replication by incorporation of oxidized dNTPs when the nucleotide pool is heavily oxidized and/or the ratio of oxidized versus normal dNTPs is substantially enhanced. The replicative Pols might have difficulty to extend the primers without Pol η in the situation since the oxidized dNTPs are generally difficult substrates for replicative Pols. However, the incorporation by Pol η is erroneous and thus may induce mutations as described above. In fact, the mutagenicity of 8-OH-dGTP directly incorporated into human cells is reduced significantly when the expression of Pol η is suppressed by RNAi technique (Kamiya et al., unpublished results). It is demonstrated that both 8-OH-dGTP and 2-OH-dATP are significant contributors to mutations in mismatch-repair-defective cells and incorporation of oxidized DNA precursors is a significant influence on microsatellite instability in repair-defective human tumor cells (21). We suggest, therefore, that Pol η may be involved in mutagenesis by the incorporation of oxidized dNTPs in various DNA transactions when the nucleotide pool is oxidized and imbalanced.

Unlike Pol η , Pol ι has a limited ability to incorporate oxidized dNTPs and extend the primers having the oxidized bases at the termini. It could not incorporate 2-OH-dATP into DNA and extend the primers having 8-OH-G or 2-OH-A regardless of the template bases under the experimental conditions (Figure 2B, Figure 3B,D). The only activity we could detect in this study was the incorporation of 8-OH-

dGTP opposite template A (Figure 2A, Table 1). Pol ι is known to form Hoogsteen base pairing in the active site where the template A is rotated to the *syn* conformation while the incoming dTTP is in the *anti* conformation (52). Thus, the template A may be driven to the *syn* conformation when 8-OH-dGTP is incorporated. The Hoogsteen base pairing, i.e., template A (*syn*):8-OH-dGTP (*syn* or *anti*), may be the basis for the limited catalysis of incorporation of 8-OH-dGTP opposite template A. Nevertheless, Pol ι could not extend the primers having 8-OH-G at the termini. This may resemble the situation where Pol ι fails to further elongate the primers upon incorporation of C or T opposite 1,*N*²-propano-2'-deoxyguanosine, which forms a *syn* conformation in the template DNA strand (53). Although it is possible that other Pols such as Pol κ may help the extension step upon incorporation of 8-OH-G opposite template A by Pol ι , it seems unlikely that Pol ι is heavily involved in oxidative mutagenesis through the incorporation of oxidized dNTPs into nascent DNA.

In this study, we have revealed the remarkable nature of Pol η that incorporates 8-OH-dGTP and 2-OH-dATP into DNA in an efficient and erroneous manner. We assume that the incoming 8-OH-dGTP adopts the *syn* conformation in the active site of Pol η , which allows the formation of Hoogsteen base pairing with the *anti* form of template A. The active site large enough to accommodate bulky DNA adducts may permit the *syn* conformation of incoming 8-OH-dGTP, which is favored when it pairs with A in a DNA duplex (54). The Hoogsteen base pairing, i.e., 8-OH-dGTP (*syn*) and template A (*anti*), which might be opposite to the conformation of pairing bases in the active site of Pol ι , could be the basis for the efficient and erroneous incorporation of 8-OH-dGTP by Pol η . Interestingly, yeast and human Pol η can bypass 8-OH-G in DNA in an efficient but error-free manner by insertion of dCMP opposite the oxidized base in the template strand (55). In these cases, the large active site of Pol η is thought to allow the *anti* conformation of 8-OH-G in the template strand, which introduces barriers for other Pols (56). Since oxidative damage in the nucleotide pool as well as DNA is a major source of spontaneous mutagenesis and carcinogenesis, the present study warrants the validity of further research on the roles of Pol η in the oxidative mutagenesis in human cells.

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Miscoding Properties of 2'-Deoxyinosine, a Nitric Oxide-Derived DNA Adduct, during Translesion Synthesis Catalyzed by Human DNA Polymerases

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Chronic inflammation involving constant generation of nitric oxide ([•]NO) by macrophages has been recognized as a factor related to carcinogenesis. At the site of inflammation, nitrosatively deaminated DNA adducts such as 2'-deoxyinosine (dI) and 2'-deoxyxanthosine are primarily formed by [•]NO and may be associated with the development of cancer. In this study, we explored the miscoding properties of the dI lesion generated by Y-family DNA polymerases (pols) using a new fluorescent method for analyzing translesion synthesis. An oligodeoxynucleotide containing a single dI lesion was used as a template in primer extension reaction catalyzed by human DNA pols to explore the miscoding potential of the dI adduct. Primer extension reaction catalyzed by pol α was slightly retarded prior to the dI adduct site; most of the primers were extended past the lesion. Pol η and pol $\kappa\Delta C$ (a truncated form of pol κ) readily bypassed the dI lesion. The fully extended products were analyzed by using two-phased PAGE to quantify the miscoding frequency and specificity occurring at the lesion site. All pols, that is, pol α , pol η , and pol $\kappa\Delta C$, promoted preferential incorporation of 2'-deoxycytidine monophosphate (dCMP), the wrong base, opposite the dI lesion. Surprisingly, no incorporation of 2'-deoxythymidine monophosphate, the correct base, was observed opposite the lesion. Steady-state kinetic studies with pol α , pol η , and pol $\kappa\Delta C$ indicated that dCMP was preferentially incorporated opposite the dI lesion. These pols bypassed the lesion by incorporating dCMP opposite the lesion and extended past the lesion. These relative bypass frequencies past the dC:dI pair were at least 3 orders of magnitude higher than those for the dT:dI pair. Thus, the dI adduct is a highly miscoding lesion capable of generating A \rightarrow G transition. This [•]NO-induced adduct may play an important role in initiating inflammation-driven carcinogenesis.

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Keywords: inflammation; nitric oxide; DNA adduct; translesion synthesis; nonradioactive analysis

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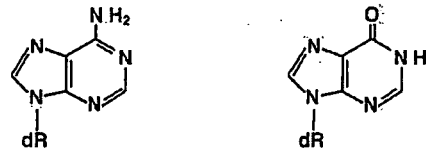
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Abbreviations used: [•]NO, nitric oxide; dI, 2'-deoxyinosine; dX, 2'-deoxyxanthosine; dA, 2'-deoxyadenosine; 8-Oxo-dG, 8-oxo-2'-deoxyguanosine; dNTP, 2'-deoxynucleoside triphosphate; Alexa546, Alexa Fluor 546 dye; pol α , human DNA polymerase α ; pol η , human DNA polymerase η ; pol κ , human DNA polymerase κ ; pol $\kappa\Delta C$, a truncated form of pol κ ; F_{ins} , frequency of insertion; F_{ext} , frequency of extension; 8-NO₂-dG, 8-nitro-2'-deoxyguanosine; dCMP, 2'-deoxycytidine monophosphate; dTMP, 2'-deoxythymidine monophosphate; dTTP, 2'-deoxythymidine triphosphate; dCTP, 2'-deoxycytidine triphosphate; dGTP, 2'-deoxyguanosine triphosphate; Cy3, Cyanin 3; endo V, endonuclease V.

Introduction

Chronic inflammation involving constant generation of nitric oxide (NO) by macrophages has been recognized as a factor related to carcinogenesis.¹⁻³ NO can attack neighboring epithelial and stromal cells by damaging the DNA, altering their genome stability. There are two possible major pathways for the NO reaction (Fig. 1). One pathway involves the combination of NO and superoxide with the formation of highly toxic peroxynitrite (ONOO^-). Spontaneous hydrolysis of peroxynitrite under physiological conditions generates secondary radical species (NO_2 , OH , and CO_3^-) that induce oxidation and nitration of diverse DNA adducts^{4,5} such as 8-oxo-2'-deoxyguanosine (8-OxodG) and 8-nitro-2'-deoxyguanosine (8- NO_2 -dG).^{6,7} A second involves nitrosative deamination of DNA base by NO via formation of several nitrosating agents, which predominantly exist as nitrous anhydride (N_2O_3) at physiological pH.⁸ The DNA base products by nitrosative deamination mainly involve conversion of adenine to hypoxanthine [2'-deoxyinosine (dI)] (Fig. 2), guanine to xanthine [2'-deoxyxanthosine (dX)], and cytosine to uracil.⁹⁻¹²

The spectrum of nitrosative DNA adducts in N_2O_3 -treated plasmid DNA was composed of approximately 2% dG-dG cross-links, 4-6% abasic sites, and 25-35% each of dI, dX, and 2'-deoxyuracil.^{12,13} Moreover, dI and dX, as well as lipid peroxidated adducts, were increased in the cellular DNA of tissues from the NO -overproducing SJL mouse model of inflammation.¹⁴ The increased production of NO was associated with an increased mutation frequency.¹⁵ When human TK6 cells were exposed to NO , the increase in mutation rates observed at hypoxanthineguanine



2'-deoxyadenosine (dA)

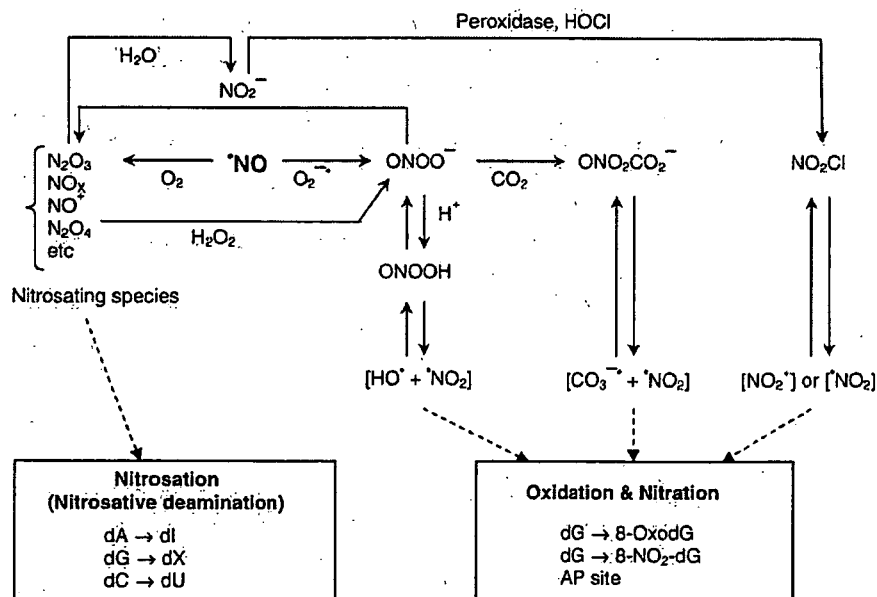
2'-deoxyinosine (dI)

Fig. 2. Structures of dA and dI adducts.

phosphoribosyltransferase and thymidine kinase gene loci correlated with the 40-fold increase of dI and dX more than did that of the controls in the cellular DNA.¹⁰ Thus, the dI adduct is one of the major NO -derived DNA lesions and may contribute to the burden of carcinogenesis in inflammation tissue.

Site-specifically dI-modified oligodeoxynucleotides have been used as DNA templates for investigating the miscoding events using only mouse pol α and rat pol β .¹⁶ The previous report showed that rat pol β inserted only 2'-deoxycytidine monophosphate (dCMP) opposite the dI lesion and that mouse pol α tended to incorporate dCMP and 2'-deoxythymidine monophosphate (dTMP) opposite the lesion. However, the miscoding events were investigated with the detection of mismatched base pairs by loss of a restriction enzyme recognition site. No quantitative analysis, therefore, has been performed for determination of miscoding events generated by dI.

Human DNA pol η ¹⁷ and pol κ ^{18,19} that are associated with translesion synthesis past a variety of DNA lesions^{20,21} were examined. We have here explored the miscoding properties of the dI lesion that occurred during DNA replication catalyzed by

Fig. 1. Possible pathways for the formation of NO -induced DNA adducts.

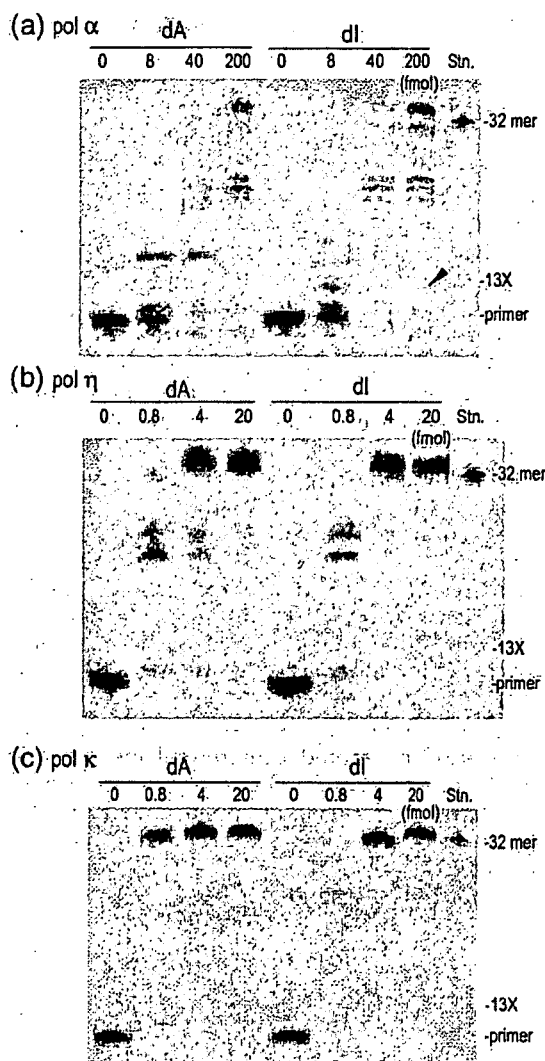


Fig. 4. Primer extension reactions catalyzed by pol α , pol η , or pol $\kappa\Delta C$ on dI-modified DNA template. Using unmodified or dI-modified 38-mer templates primed with an Alexa546-labeled 10-mer, we performed primer extension reactions at 25 °C for 30 min in a buffer containing four dNTPs (100 μ M each) and varying amounts of (a) pol α (0, 8, 40, and 200 fmol), (b) pol η , or (c) pol $\kappa\Delta C$ (0, 0.8, 4, and 20 fmol), as described in Materials and Methods. The whole amount of the reaction mixture was subjected to 20% denaturing PAGE (30 \times 40 \times 0.05 cm). An Alexa546-labeled 32-mer (5'AGAGGAAAGTAGCGAAGGAATTCATCAGCATG) was used as a marker of fully extended product. 13X represents the adducted position.

Kinetic studies on dI-modified templates

Steady-state kinetic studies were performed using pol α , pol η , and pol $\kappa\Delta C$ to determine the frequency of dNTP incorporation (F_{ins}) opposite the dI lesion and chain extension (F_{ext}) from the primer terminus using the same sequence context that was used for the two-phased PAGE assay (Table 1). With pol α , the F_{ins} value for deoxythymidine triphosphate (dTTP) (1.21×10^{-2}), the correct base, opposite the dI was 59

times lower than that for 2'-deoxycytidine triphosphate (dCTP) (0.718). The relative bypass frequency ($F_{ins} \times F_{ext}$) past the dC:dI pair was approximately 2100 times higher than that for the dT:dI pair. F_{ins} and F_{ext} values for dA:dI and dG:dI were not detectable. When pol η was used, the F_{ins} value for dCTP (0.551), the wrong base, opposite the dI was 27 times higher than that for dTTP (2.07×10^{-2}) and was 17 and 75 times higher than that for 2'-deoxyadenosine triphosphate and 2'-deoxyguanosine triphosphate (dGTP), respectively. The F_{ext} value for the dC:dI pair was also higher than that for other 2'-deoxynucleoside monophosphates paired with dI. As a result, the $F_{ins} \times F_{ext}$ value past dC:dI was at least 3 orders of magnitude higher than that past other pairs. Similarly, with pol κ , $F_{ins} \times F_{ext}$ past dC:dI was much higher than that for other base pairs. F_{ins} and F_{ext} values for dTTP were 119 and 55 times lower than that for dCTP, respectively. Thus, all pols, that is, pol α , pol η , and pol $\kappa\Delta C$, exclusively promote misincorporation of dCMP opposite the dI lesion during translesion synthesis, since $F_{ins} \times F_{ext}$ values of other dNTPs were considerably lower than that for dCTP, the wrong base.

Discussion

Primer extension reactions catalyzed by DNA pols are a powerful method to explore translesion synthesis past DNA adducts and their accompanying kinetic parameters of nucleotide insertion and extension. A 32 P-labeled oligodeoxynucleotide at the 5'-terminus is widely employed in such analyses; however, the handling of hazardous radioisotopes is intricate for use and waste disposal. Indeed, use of radioisotopes is restricted in many countries including Japan. As an alternative to 32 P, we used fluorescent dyes, Alexa546 and Cyanin 3 (Cy3), to label the 5'-terminus of the oligodeoxynucleotide used as primers and standard markers. The detection limits of Alexa546- and Cy3-labeled primers were approximately 120 and 240 times lower than that of the 32 P-labeled primer, respectively. However, when Alexa546-labeled primers (500 fmol) were annealed with DNA template (750 fmol) and the assays of primer extension and kinetic studies were carried out in this work, the resultant data were quantitative and reproducible (Figs. 6 and 7). Alexa546 exhibits a more sensitive and photostable fluorescence than Cy3. Moreover, even under repeated thawing and melting, an Alexa546-labeled oligomer stored at -20 °C was not degraded for at least 6 months. Using this method, we determined the miscoding frequency and specificity of 8-OxodG, which is known to generate predominantly 2'-deoxyadenosine monophosphate misincorporation at the lesion site. The results obtained from Alexa546 labeling were consistent with that from 32 P labeling (data not shown). Thus, Alexa546 was applicable to two-phased PAGE. Therefore, we have used this fluorescent method to explore translesion synthesis past dI adducts and its miscoding specificity and frequency using two-phased PAGE.