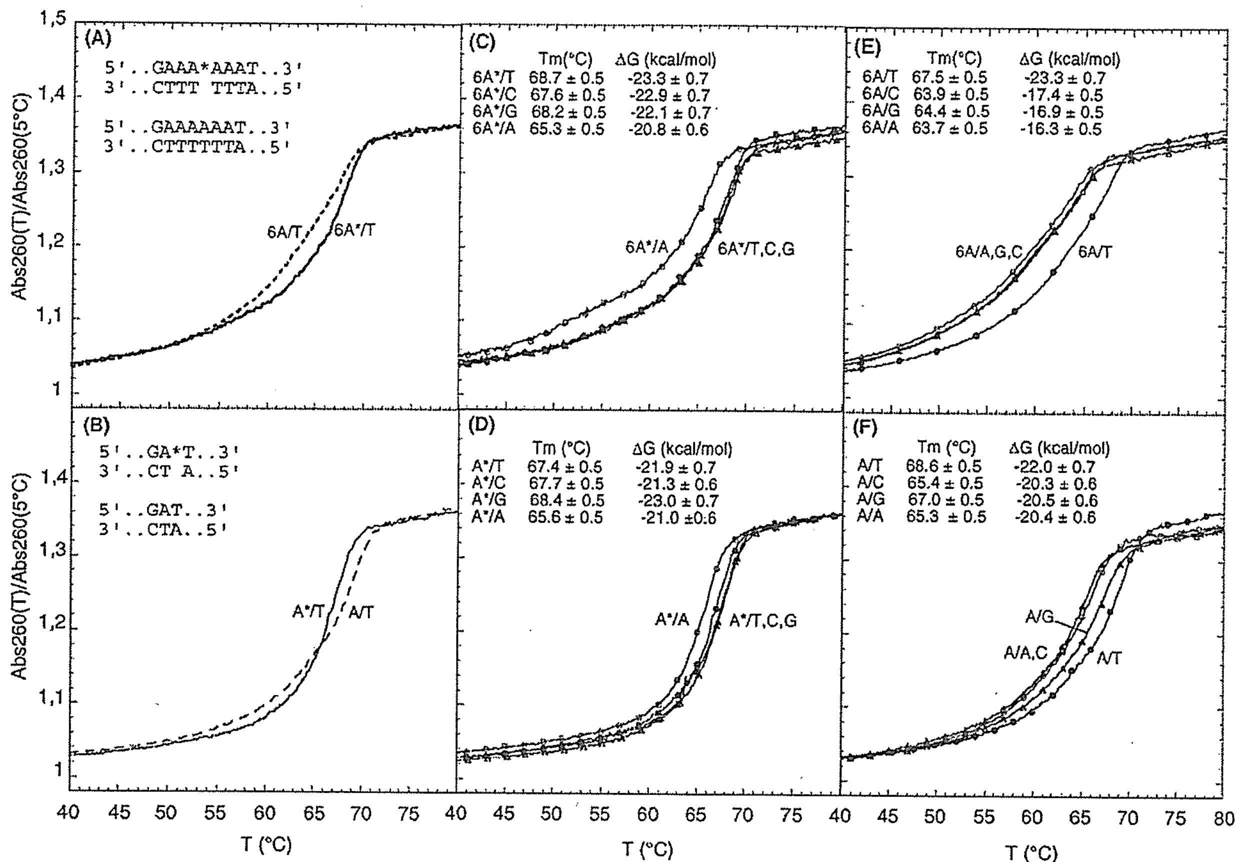

Fig. 5 – UV denaturation profiles of DNA duplexes with and without 2-OH-A. Effect of 2-OH-A:T pairing in the 6A repeat sequence (panel A) and in a random sequence (panel B); continuous line, 2-OH-A:T; dashed line, A:T. Thermal stability of DNA duplexes with A or 2-OH-A paired with T, G, C or A in the A-repeat sequence (panels E and C, respectively) and in the random sequence (panels F and D). Filled circle, A:T and 2-OH-A:T; empty circle, A:A and 2-OH-A:A; filled triangle, A:C and 2-OH-A:C; empty triangle, A:G and 2-OH-A:G.

the B family replicative Pol α. This distinguishes 2-OH-A from 8-oxoG, which is easily bypassed by several replicative polymerases via the mutagenic incorporation of an A opposite the lesion [1]. Thus, unlike 8-oxo-G, a template 2-OH-A might cause a permanent or transient replication block thereby provoking recruitment of a TLS polymerase. Among the enzymes we tested, Dpo4 was the least sensitive to 2-OH-A, while human Pol η was inefficient in bypassing the lesion. This raises the possibility that of the human Y family polymerases the Dpo4 homolog, polymerase κ, rather than Pol η, might be better equipped to bypass this oxidized purine. The different efficiencies of 2-OH-A bypass by Dpo4 and Pol η resemble their behaviour with AP sites and may reflect differences in their respective "little finger" subdomains [30,31], probably the critical features that allow lesion bypass [21].

Each polymerase showed a strong preference for insertion of T opposite 2-OH-A although our incorporation data suggested a range of alternative permissible base pairings that were affected by the sequence context of the oxidized base. These findings are consistent with the known ability of DNA 2-OH-A to adopt multiple tautomeric forms that are influenced by temperature, solvent polarity and neighbouring bases [32–34]. In particular, a shift from the prevailing keto tautomer (N1-H) towards the enol form (O2H) is likely to affect the probability that the lesion is accommodated as 2-OH-A:T or with other partners through classical W–C bonding or wobble base pairs. They are also consistent with our Tm measurements, which indicated that 2-OH-A forms stable base pairs not only with T, but also with C or G. Thus, the different abilities of DNA polymerases to accommodate otherwise unfavourable 2-OH-A tautomers within their active sites might influence replication fidelity [35].

A previous investigation of mutational spectra in MMR-defective cells indicated that AT>GC and AT>TA base substitutions (and to a minor extent AT>CG) might derive from miscoding by an oxidized purine [15]. These were the most frequent mutations observed following replication of 2-OH-A by Dpo4 and Pol η under conditions of limited polymerase engagement. The same analysis also revealed that frameshifts, with deletion of the 2-OH-A adduct, were significantly increased following replication by Dpo4. This

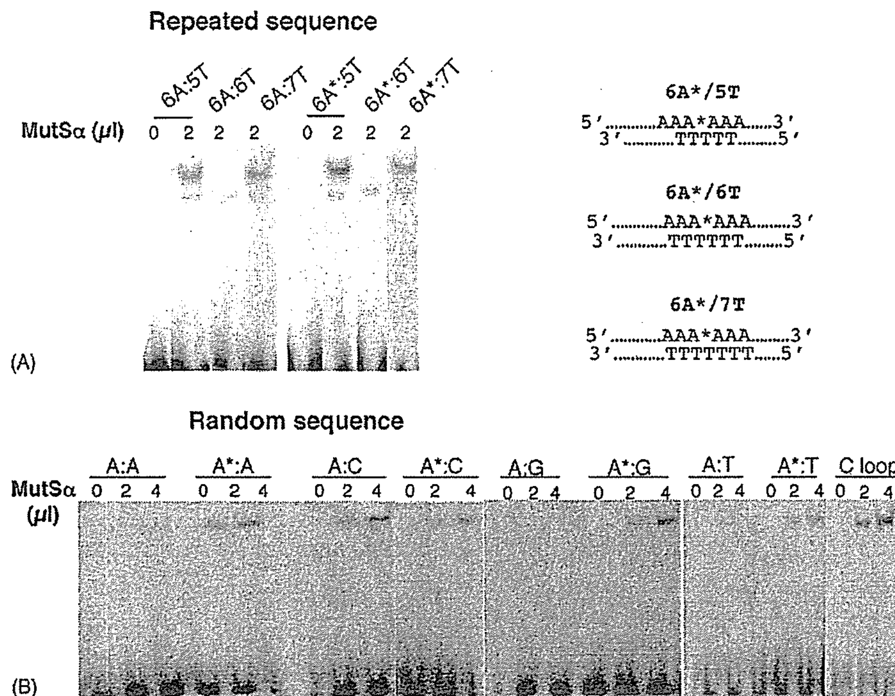

Fig. 6 – MutSα binding to 2-OH-A. End-labelled oligonucleotide duplexes of the repeated sequences shown in panel A were incubated with MutSα. In panel B is shown MutSα binding to 2-OH-A in random sequences. Products were analysed by non-denaturing PAGE as described in Section 2.

polymerase has a very low frameshift fidelity, which is consistent with its ability to accommodate unpaired nucleotides in the active site [30]. It is also known to generate deletions at noniterated nucleotides [36]. Together with the apparent difficulties experienced by replicative polymerases at a template 2-OH-A, these findings strengthen the likelihood that Y polymerase-mediated TLS occurs at this lesion.

Little or no information is available on the repair of 2-OH-A formed by in situ oxidation of DNA adenine. We show here that the major human MMR recognition complex, MutSα, recognizes 2-OH-A-containing oligonucleotides. Recognition by MutSα was context dependent. MutSα efficiently recognized oligonucleotide duplexes containing 2-OH-A in structures mimicking insertion/deletion loop (IDL) within a 6A repeat. In this regard, the behaviour of the oxidized adenine was indistinguishable from its normal homolog. Within the same type of IDL structure, the behaviour of 2-OH-A differs from DNA 8-oxoG, which appears to assume a conformation that renders it invisible to MutSα [37]. The ability of MutSα to recognize 2-OH-A within an IDL context may have relevance in vivo as it is consistent with the apparent contribution of the oxidized base to microsatellite instability at the A_{26} BAT26 sequence in MMR-defective cells [15].

Although duplexes containing 8-oxoG:C base pairs are not recognized [38,39], MutSα bound each of the four 2-OH-A-containing base pairs to a similar extent. Alterations in base geometry and local flexibility are likely to be major determinants of mismatch recognition [28,40]. Recognition of 2-OH-A:T pairs might again reflect the unique ability of 2-OH-A to adopt multiple tautomeric forms, some of which are associated with wobble base pairs and local distortion [33]. A 2-OH-A:T pair in a fully paired A6 repeat sequence was not recognized by MutSα, however. A_n repeats of this kind are known to assume unusual conformations in which the A:T base pairs show large propeller twist angles and form bifurcated hydrogen bonds involving the N6 amino group of A and the O4 atoms of two adjacent Ts in the opposite strand. We speculate that this particular structural arrangement might confer a greater resistance to deformation and thereby prevent DNA from adopting the structural changes needed to trigger MutSα recognition.

Notwithstanding the precise mechanism by which MutSα recognizes 2-OH-A-containing base pairs, our findings clearly indicate how MMR might help control the steady-state levels of DNA 2-OH-A. The major source of DNA 2-OH-A is acknowledged to be the oxidized dNTP pool [5]. MMR would reverse this incorporation and prevent the accumulation of 2-OH-A in DNA. This role is consistent with the higher levels of DNA 2-OH-A observed in $msh2^{-/-}$ ES cells in comparison to wild type ES cells [41].

In summary, we have shown that replication fork block is the likely outcome of a replicative DNA polymerase encountering a template 2-OH-A. Our findings indicate that a specialized bypass polymerase might overcome the block at the expense of replication fidelity. In addition, MutSα recognition of 2-OH-A-containing substrates indicates that MMR might contribute to mutation avoidance by acting on 2-OH-A-containing base pairs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dnarep.2006.11.002.

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ORIGINAL PAPER

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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*H1 restriction site is underlined; the 3′-primers were respectively: B-721-*Hin*d (5′-TTGGAAGCTTCTATG TTTCAGTCCAAATACACCGTA-3′); B-617-*Hin*d (5′-TTGGAAGCTTCTATCTCTTCTTCACTAACATCC CCTT-3′); B-481-*Hin*d (5′-GGGTTTAAGCTTCTATT TCGCTAAGATTTCTTCCTT-3′). These latter oligonucleotides contain a stop codon immediately upstream the *Hin*dIII restriction site. The PCR amplified DNA fragment were purified, digested with *Bam*HI and *Hin*dIII and cloned into the *E. coli* expression vector pTRC-*His*C (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, benzamidine 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 Ni^{2+}-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 μg/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 μg/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 μl/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× (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

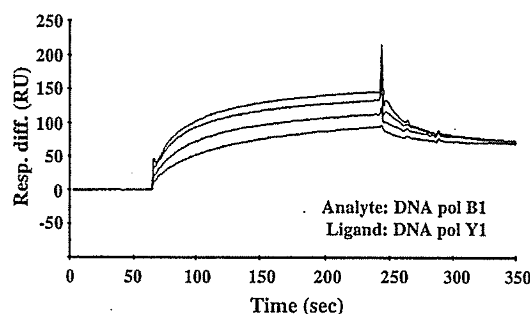

Fig. 1 Physical interaction between Sso DNA pol B1 and DNA pol Y1 detected by surface plasmon resonance measurements. An overlaid plot of sensorgrams was obtained by fluxing DNA pol B1 at various concentrations over a DNA pol Y1-immobilised sensor chip (*lower to upper curve* DNA pol B1 was at 0.15,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

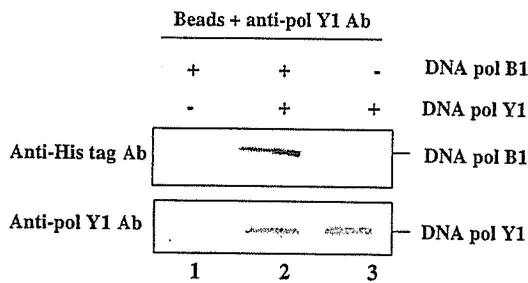

Fig. 2 Physical interaction between Sso DNA pol B1 and DNA pol Y1 detected by immuno-precipitation experiments. Immuno-precipitation experiments were carried out using Protein A Sepharose beads conjugated with anti-DNA pol Y1 antibodies. The following protein samples were analysed: *lane 1*, Sso DNA pol B1 alone (9 μg, negative control experiment); *lane 2*, a mixture of Sso DNA pol B1 (9 μg) and Sso DNA pol Y1 (7 μg); *lane 3*, Sso DNA pol Y1 alone (7 μg, 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 polY1 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.

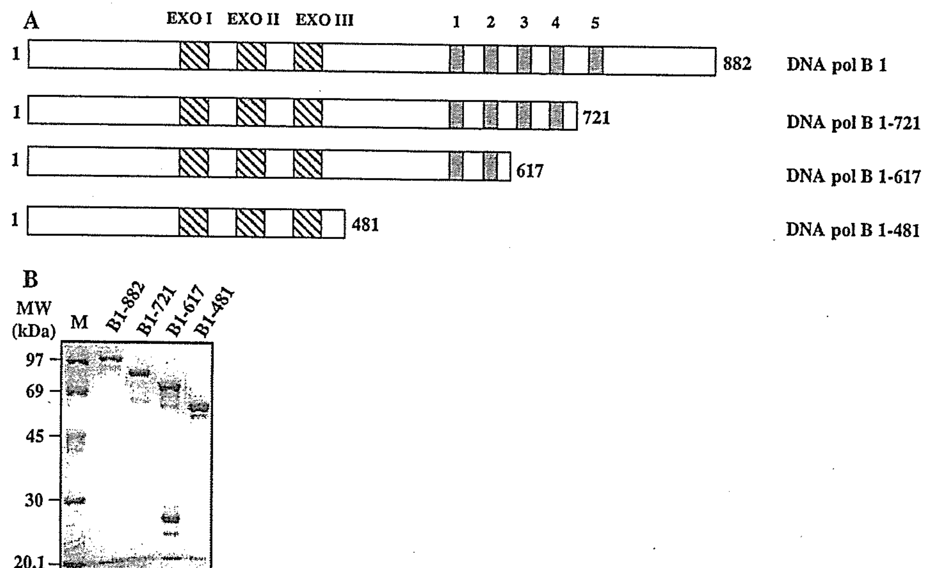

Fig. 3 Diagrammatic representation of Sso DNA pol B1 deleted forms used in this study. **a** The position of the B Family DNA pols sequence similarity motifs is indicated (Blanco et al. 1991). **b** Electrophoretic analysis of purified DNA pol B1 and its deleted forms (5 μg) on a 10% SDS-polyacrylamide 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.

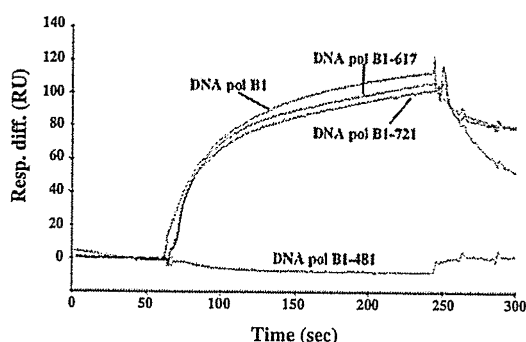

Fig. 4 Physical interaction of the Sso DNA pol B1 truncated forms with DNA pol Y1 detected by surface plasmon resonance measurements. Plot of sensorgrams obtained by fluxing each indicated protein at 0.3 μM over a DNA pol Y1-immobilised sensor chip, as described in Materials and methods

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.

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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-

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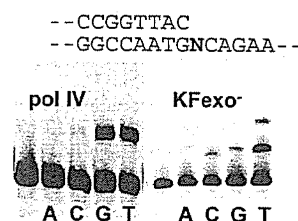
(A) 2-OH-dATP



(B) 8-OH-dGTP

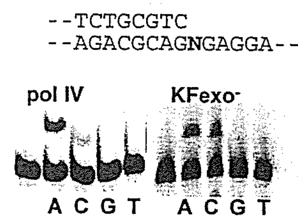


FIG. 1. Incorporation of oxidized nucleotides by DNA polymerases. The incorporation of 2-OH-dATP (A) and 8-OH-dGTP (B) into DNA by DNA Pol IV and KF exo⁻ of *E. coli* was assayed as described previously (28). Cy3-conjugated primer, annealed to the template at a 1:1 ratio (0.1 μM), was incubated with 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.

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*)$_{XIII}$] carrying F′ *lacIZ- proB*$^+$; *lacZ* has a mutation (GAG to TAG) at codon 461		7
CC104	Derivative of strain P90C [*araA*(*lac proB*)$_{XIII}$] 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 frequency 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 · C → T · 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 · T → C · 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 · C to T · 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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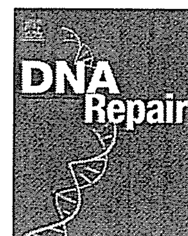

Specificity of replicative and SOS-inducible DNA polymerases in frameshift mutagenesis: Mutability of *Salmonella typhimurium* strains overexpressing SOS-inducible DNA polymerases to 30 chemical mutagens

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ABSTRACT

DNA replication is frequently hindered because of the presence of DNA lesions induced by endogenous and exogenous genotoxic agents. To circumvent the replication block, cells are endowed with multiple specialized DNA polymerases that can bypass a variety of DNA damage. To better understand the specificity of specialized DNA polymerases to bypass lesions, we have constructed a set of derivatives of *Salmonella typhimurium* TA1538 harboring plasmids carrying the *polB*, *dinB* or *mucAB* genes encoding *Escherichia coli* DNA polymerase II, DNA polymerase IV or DNA polymerase RI, respectively, and examined the mutability to 30 chemicals. The parent strain TA1538 possesses CGCGCGCG hotspot sequence for −2 frameshift. Interestingly, the chemicals could be classified into four groups based on the mutagenicity to the derivatives: group I whose mutagenicity was highest in strain YG5161 harboring plasmid carrying *dinB*; group II whose mutagenicity was almost equally high in strain YG5161 and strain TA98 harboring plasmid carrying *mucAB*; group III whose mutagenicity was highest in strain TA98; group IV whose mutagenicity was not affected by the introduction of any of the plasmids. Introduction of plasmid carrying *polB* did not enhance the mutagenicity except for benz[a]anthracene. We also introduced a plasmid carrying *polA* encoding *E. coli* DNA polymerase I to strain TA1538. Strikingly, the introduction of the plasmid reduced the mutagenicity of chemicals belonging to groups I, II and III, but not the chemicals of group IV, to the levels observed in the derivative whose SOS-inducible DNA polymerases were all deleted. These results suggest that (i) DNA polymerase IV and DNA polymerase RI possess distinct but partly overlapping specificity to bypass lesions leading to −2 frameshift, (ii) the replicative DNA polymerase, i.e., DNA polymerase III, participates in the mutagenesis and (iii) the enhanced expression of *E. coli polA* may suppress the access of Y-family DNA polymerases to the replication complex.

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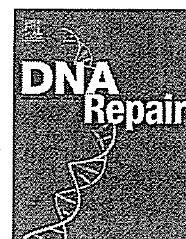

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1. Introduction

Cellular DNA is continuously exposed to a variety of endogenous and exogenous genotoxic agents. Although DNA repair mechanisms are in operational to remove DNA lesions, DNA polymerases have to often encounter the lesions that are escaped from the repair mechanisms. DNA damages, such as ultraviolet light (UV) photoproducts or carcinogen adducts, strongly block the progress of DNA replication, and thus daughter-strand gaps are generated downstream of the lesions [1]. In *Escherichia coli*, the single-stranded regions are recognized by RecA protein, which mediates recombination to fill in the gaps with a homologous DNA sequence that is derived from the replicated sister chromatids [2]. In addition, the binding of RecA to the single-stranded regions activates RecA protein itself to mediate the cleavage of LexA repressor, which triggers the expression of more than 30 genes in the chromosome. This damage-inducible gene expression is referred to as an SOS response [3]. Interestingly, some of DNA polymerases in *E. coli* (see below) are regulated as part of the SOS response, and the SOS-inducible DNA polymerases appear to be involved in translesion DNA synthesis (TLS), which directly bypasses the lesions to fill in the gaps [4–6]. Some TLS reactions they catalyze are error-prone, i.e., incorporating incorrect bases in the nascent strand, while others are error free [7]. Thus, filling in the gaps by TLS appears to contribute to mutagenesis as well as to DNA damage tolerance, while filling-in reactions by homologous recombination are supposed to be non-mutagenic.

In *E. coli*, there are five DNA polymerases, i.e., DNA polymerases I–V [8]. DNA polymerase I encoded by *polA* is the first DNA polymerase to be described and is involved in lagging strand DNA synthesis, i.e., processing of Okazaki fragment, DNA repair and initiation of ColE1 plasmids such as pBR322 [9,10]. DNA polymerase II encoded by *polB* is a damage (SOS)-inducible DNA polymerase [11,12] and is involved in the process to restart the synthesis of damaged DNA [13,14]. This polymerase is reported to bypass 2-acetylaminofluorene adducts, which results in −2 frameshift [15,16]. Unlike DNA polymerase I, which belongs to A-family DNA polymerase, DNA polymerase II is a member of B family, in which mammalian replicative DNA polymerases such as DNA polymerase delta are included [17]. DNA polymerase III holoenzyme, which is composed of multiple subunits, is responsible for the chromosome replication of *E. coli* and the catalytic subunit is encoded by *dnaE* (or *polC*) [18,19]. This enzyme is classified into C family, in which prokaryotic replicative DNA polymerases are categorized [8]. DNA polymerase IV and DNA polymerase V belong to Y family, whose members are mostly involved in TLS, and the expression of *dinB* and *umuDC* encoding DNA polymerase IV and DNA polymerase V, respectively, is regulated as part of the SOS response [20–24]. DNA polymerase IV is shown to be involved in −1 frameshift mutagenesis induced by 4-nitroquinoline N-oxide and benzo[a]pyrene [25,26], and DNA polymerase V is known to play important roles in mutagenesis induced by UV and a variety of genotoxic compounds [27,28]. However, current knowledge about the roles of replicative, i.e., DNA polymerase I and DNA polymerase III, and SOS-inducible specialized DNA polymerases, i.e., DNA polymerase II, DNA polymerase IV and DNA polymerase V, in mutagenesis is still limited because synthetic oligonucleotides bearing specific DNA lesions are required for in vitro and in vivo analyses to address the question.

Salmonella typhimurium is a Gram negative bacterium, whose genome sequence is 70–90% homologous to *E. coli* [29]. Some of *S. typhimurium* strains have been widely used to detect a variety of environmental mutagens and carcinogens as tester strains of Ames test [30]. One of such strain TA1538 possesses CGCGCGCG sequence in the *hisD* gene, which is a mutational hot spot for −2 (–CG) frameshift [31,32]. The strain bears a deep-rough *rfa* mutation, which increases the permeability to hydrophobic compounds such as polycyclic aromatic hydrocarbons (PAH) [33]. In addition, the strain is deficient in the capacity to excise bulky DNA adducts by the *uvrB* mutation, so that the DNA adducts are more likely to be bypassed rather than removed by repair enzymes [33].

In a previous study, we have systematically disrupted the genes of *S. typhimurium* TA1538 encoding SOS-inducible DNA polymerases, i.e., $polB_{ST}$, $dinB_{ST}$, $umuDC_{ST}$ and *samAB*; and concluded that different sets of DNA polymerases are engaged in lesion bypass in the CGCGCGCG sequence depending upon the environmental threats by chemicals [34]. We also proposed that not only SOS-inducible DNA polymerases but also the main replicative DNA polymerase, i.e., DNA polymerase III, plays important roles in −2 frameshift [34].

In this study, we generated a set of isogenic derivatives of *S. typhimurium* TA1538 by introducing plasmids carrying *polB*, *dinB* or *mucAB* encoding *E. coli* DNA polymerase II, DNA polymerase IV or DNA polymerase RI, respectively, and examined the mutability to 30 chemicals. We introduced the plasmid carrying *mucAB*, i.e., pKM101, instead of a plasmid carrying *E. coli umuDC*, because DNA polymerase RI is a homologue of *E. coli* DNA polymerase V [35], and the derivative of TA1538 harboring plasmid pKM101, i.e., strain TA98, has been widely used as a standard tester strain of Ames test [30]. We also introduced a plasmid carrying the *polA* gene of *E. coli* to strain TA1538 and examined the mutability to investigate the possible involvement of DNA polymerase I in TLS leading to frameshift. Intriguingly, the introduction of the *polA* plasmid completely suppressed the mutations depending on the activities of $dinB_{ST}$ and $umuDC_{ST}$ of *S. typhimurium*. Collectively, the present results suggest that (1) DNA polymerase IV and DNA polymerase RI has distinct but partly overlapping specificity to bypass lesions leading to −2 frameshift, (2) the replicative DNA polymerase, i.e., DNA polymerase III, substantially contributes to −2 frameshift and (3) the enhanced expression of *E. coli* polymerase I inhibits the access of Y-family DNA polymerases to the replication complex where TLS occurs. In addition, our results raise an interesting possibility that strain YG5161 harboring plasmid pYG768 carrying *dinB* could be a superior tester strain to strain TA98 to detect the mutagenicity of environmental PAHs.

2. Materials and methods

2.1. Strains and plasmids

The strains and plasmids used in this study are listed in Table 1. Strain YG5160 and strain YG5161 were constructed

Table 1 – *S. typhimurium* strains and plasmids

Strain or plasmid	Description	Source
Strains		
TA1535	*hisG46, gal,* Δ *(chl, uvrB, bio), rfa*	Maron and Ames [30]
TA1537	*hisC3076, gal,* Δ *(chl, uvrB, bio), rfa*	Maron and Ames [30]
TA1538	*hisD3052, gal,* Δ *(chl, uvrB, bio), rfa*	Maron and Ames [30]
TA98	As TA1538 but harbors plasmid pKM101	Maron and Ames [30]
YG5160	As TA1538 but harbors plasmid pYG787	This study
YG5161	As TA1538 but harbors plasmid pYG768	This study
YG6215	As TA1538 but Δ*umuDC*$_{ST}$::Km^r, Δ*samAB*::Cm^r, Δ*dinB*$_{ST}$::Sp^r, Δ*polB*$_{ST}$::Tc^r	Kokubo et al. [34]
Plasmids		
pKM101	Plasmid carrying the *mucAB* genes	Maron and Ames [30]
pYG768	Derivative of pWSK29 with the *E. coli dinB* gene	Kim et al. [36]
pYG787	Derivative of pWKS30 with the *E. coli polB* gene	Kokubo et al. [34]
pIMA-1	Derivative of pWKS30 with the *E. coli polA* gene	Imai and Yamamoto (unpublished)

by introduction of plasmid pYG787 carrying *polB* and plasmid pYG768 carrying *dinB*, respectively, into strain TA1538 [34,36]. Plasmid pIMA-1 carrying *E. coli polA* [9] was constructed by the insertion of a 3.5-kb fragment of the *polA* gene between EcoRI and *Sal*I sites of plasmid pWKS30 (Imai and Yamamoto, unpublished). The direction of transcription of the *polA* gene in the plasmid is opposite to that of the *lacZ* gene. The plasmid could complement the killing sensitivity of a *polA* strain of *E. coli* to ultraviolet light and methyl methanesulfonate. Transformation was conducted by electroporation [37].

2.2. *Chemicals*

The names, abbreviations, CAS registry numbers and sources of the chemicals used in this study are listed in Table 2. The chemical structures are presented in Fig. 1.

2.3. *Media*

Luria–Bertani broth and agar were used for bacterial culture [38]. Vogel–Bonner minimal agar plates and top agar were prepared as previously described, and used for the His$^+$ reversion assay with *S. typhimurium* [30]. Nutrient broth (Difco, MI, USA) with ampicillin (AP, 25 μg/ml) was used for pre-cultures of the strains for the reversion assay.

2.4. *Mutagenicity assay*

The mutagenicity assay was carried out with a pre-incubation procedure [30]. Briefly, 0.1 ml overnight culture was incubated with the chemicals dissolved in 0.1 ml solvent and 0.5 ml S9 mix for 20 min at 37 °C. When S9 mix is not required, 0.5 ml of 1/15 M phosphate buffer pH 7.4 was added. The mixture was then poured onto agar plates with soft agar and incubated for 2 days at 37 °C. Each chemical was assayed with four to seven doses on duplicate plates with four strains, i.e., strain TA1538, TA98, YG5160 and YG5161, in parallel. In the series of experiments, we regarded the effects of introduction of plasmids, i.e., pYG787, pYG768 or pKM101, on the mutability of strain T1538 as significant when the transformed strains displayed more than and including 50% higher or lower mutability, compared to the parent strain TA1538.

3. Results

3.1. *Specificity of SOS-inducible DNA polymerases in frameshift induced by 30 chemicals*

To assign the role of SOS-inducible DNA polymerases in bypass of DNA lesions, we introduced plasmids carrying *E. coli polB*, *dinB* or *mucAB* encoding different SOS-inducible DNA polymerases to strain TA1538 and examined their mutability to 30 chemicals. The dose–response curves are presented in Fig. 2, and the numbers of revertants per microgram per plate of each chemical and strain are summarized in Table 3. To make the comparison easier, we also calculated the relative mutability of each derivative by assigning the number of revertants per microgram in strain TA1538 as 1.0. According to the mutagenicity, we classified the 30 chemicals into four groups as follows.

Group I includes benzo[a]pyrene and other seven chemicals. The mutagenicity of these compounds was highest in strain YG5161 harboring plasmid pYG768 carrying *dinB* encoding DNA polymerase IV, followed by strain TA98 harboring plasmid pKM101 carrying *mucAB* encoding DNA polymerase RI. The mutagenicity of the chemicals to strain YG5160 harboring plasmid pYG787 carrying *polB* encoding DNA polymerase II was very similar to the parent strain TA1538 except for benzo[a]pyrene-7,8-dihydroepoxide and 1-aminoanthracene where introduction of plasmid pYG787 appeared to alleviate the mutagenicity by 50% and 40%, respectively. For benzo[a]pyrene, the ratio of the mutability of strain YG5161, TA98, YG5160 and TA1538 was 7:2:1:1. The compounds in this group are derivatives of benzo[a]pyrene except for 3-methylcholanthrene, 1-aminoanthracene and 2-aminoanthracene.

Group II includes ENNG and other four chemicals. The mutagenicity of these compounds was almost equally high in strain YG5161 and strain TA98. The introduction of plasmid pYG787 carrying *polB* did not enhance the mutagenicity. Rather, plasmid pYG787 seemed to reduce the mutagenicity of 6-aminochrysene by 60%. The ratio of the mutability of strain YG5161, TA98, YG5160 and TA1538 was 20:19:1:1 for ENNG. The compounds in this group are PAHs and the derivative except for ENNG.

Table 2 – Names, abbreviations, CAS registry numbers and sources of the chemicals

Chem. no.	Chemical	CAS registry numbers	Sources[a]
1	Benzo[a]pyrene-7,8-dihydroepoxide	36504-65-1	Mi
2	Benzo[a]pyrene diol epoxide	58917-67-2	Mi
3	10-Azabenzo[a]pyrene	189-92-4	1
4	Benzo[a]pyrene	50-32-8	W
5	3-Nitro-benzo[a]pyrene	70021-98-6	4
6	3-Methylcholanthrene	56-49-5	S
7	1-Aminoanthracene	610-49-1	S
8	2-Aminoanthracene	613-13-8	W
9	7,12-Dimethylbenz[a]anthracene (DMBA)	57-97-6	W
10	6-Aminochrysene	2642-98-0	S
11	1-Nitro-benzo[a]pyrene	70021-99-7	4
12	Benzo[a]pyrene-4,5-dihydroepoxide	64437-52-1	Mi
13	N-Ethyl-N′-nitro-N-nitrosoguanidine (ENNG)	4245-77-6	5
14	1-Nitropyrene	5522-43-0	T
15	1,8-Dinitropyrene	42397-65-9	T
16	6-Nitro-benzo[a]pyrene	63041-90-7	4
17	1-Nitro-6-azabenzo[a]pyrene	138835-35-5	4
18	3-Nitro-6-azabenzo[a]pyrene	138835-36-6	4
19	Furylfuramide	3688-53-7	W
20	Aflatoxin B1	1162-65-8	S
21	Benzo[a]pyrene-7,8-tetrahydroepoxide	36504-67-3	Mi
22	Acridine orange	65-61-2	Me
23	Benz[a]anthracene	56-55-3	S
24	2-Nitrofluorene	607-57-8	T
25	2-[2-(Acetylamino)-4-[bis-(2-methoxy-ethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2H-benzotriazole (PBTA-1)	194590-84-6	3
26	2-Amino-6-methyldipyrido[1,2-a:3′,2′-d]imidazole (Glu-P-1)	67730-11-4	W
27	Aminophenylnorharman	219959-86-1	3
28	N-Hydroxyacetylaminofluorene (N-OH-AAF)	53-95-2	Mi
29	4-Nitroquinoline-1-oxide (4-NQO)	56-57-5	T
30	2-Acetylaminofluorene	53-96-3	T

[a] The chemicals were purchased from the following sources at the highest grade of purity: Wako Pure Chemical (W); Tokyo Kasei Kogyo (T); Sigma–Aldrich (S); Merck (Me); Midwest Research Institute (Mi); Nacalai Tesque (N). Commercially unavailable chemicals were provided by the follwing persons: Dr. Ken-ichi Saeki, Nagoya City University, Japan (1); Dr. Takeji Takamura-Enya, National Cancer Center Research Institute, Tokyo, Japan (2); Dr. Yukari Totsuka, National Cancer Center Research Institute, Tokyo, Japan (3); Dr. Kiyoshi Fukuhara, National Institute of Health Sciences, Tokyo, Japan (4); laboratory stock (5).

Group III includes 1-nitropyrene and other 10 chemicals. The mutagenicity of these compounds was highest in strain TA98. Introduction of plasmid pYG768 carrying *dinB* displayed moderate (less than three-fold) enhancing effects on the mutagenicity of this group of chemicals. The introduction of plasmid pYG787 carrying *polB* enhanced the mutagenicity of benz[a]anthracene three-fold reproducibly, although it had no enhancing effects on other chemicals. For the mutagenicity of 1,8-dinitropyrene, 1-nitro-6-azabenzo[a]pyrene and furylfuramide, plasmid pYG787 reduced the mutagenicity by half. The ratio of the mutability of strain TA98, YG5161, YG5160 and TA1538 was 16:1:1:1 for 1-nitropyrene. The compounds in this group include structurally unrelated compounds such as furylfuramide, aflatoxin B1 and acridine orange.

Group IV includes 2-acetylaminofluorene and other five compounds. The characteristic of this group was that the mutagenicity was not enhanced by the introduction of any of the plasmids encoding SOS-inducible DNA polymerases. The ratio of the mutability of strain YG5161, TA98, YG5160 and TA1538 was 1:1:1:1 for 2-acetylaminofluorene. The compounds in this group are aromatic amines except for 4-NQO.

3.2. −1 *Frameshit and base substitutions by benzo[a]pyrene and ENNG promoted by DNA polymerase IV and DNA polymerase RI*

Since DNA polymerase IV encoded by *dinB* appeared to promote −2 frameshift induced by benzo[a]pyrene (group I chemical) and ENNG (group II chemical), we examined the possibility whether the polymerase also promotes other types of mutations, i.e., −1 frameshift and base substitutions, by the chemicals. To this end, we took advantage of other *S. typhimurium* strains, i.e., TA1537 and TA1535, which detects mutagens that cause −1 frameshift in CCC sequence in the *hisC* gene and base substitutions in GGG sequence in the *hisG* gene, respectively [30]. For benzo[a]pyrene-induced mutagenesis, the introduction of plasmid pYG768 carrying *dinB* into strain TA1537 slightly enhanced the mutagenicity, but the effect of enhancing mutagenesis was much lower compared to the effect of plasmid pKM101 carrying *mucAB* encoding DNA polymerase RI (Fig. 3A). For base substitutions, DNA polymerase IV seemed inactive and virtually no enhancement was observed in strain TA1535 with plasmid pYG768. In contrast, DNA polymerase RI actively promoted the base substitution mutations. As has been observed

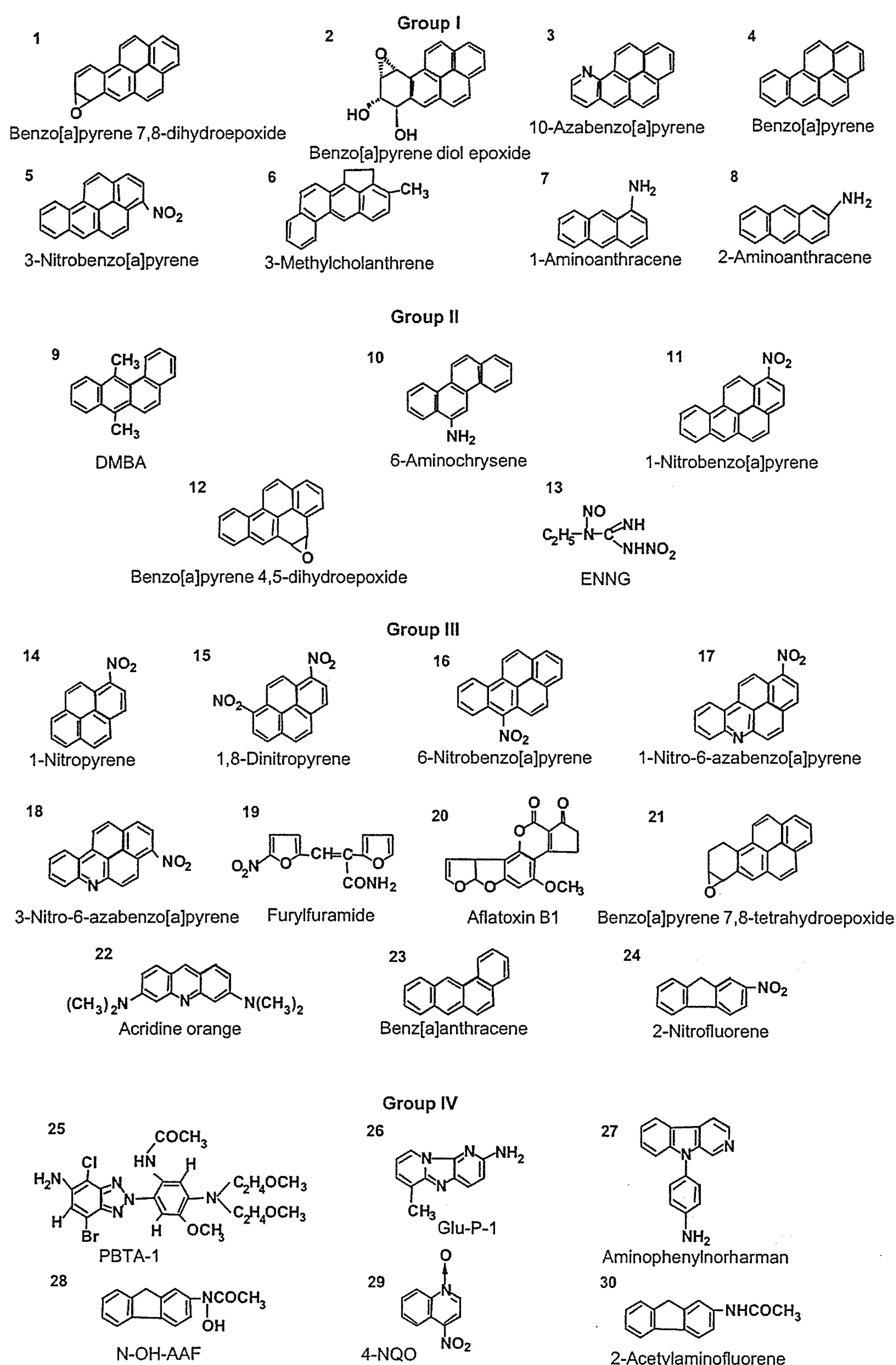

Fig. 1 – Structures of the chemical mutagens used in this study.

in −2 frameshift, introduction of plasmid pYG787 carrying *polB* had almost no effects on any types of mutations induced by benzo[a]pyrene. These results suggest that the efficiency of error-prone bypass across lesions by DNA polymerase IV strongly depends on the types of mutations and the sequence context surrounding the lesions. For ENNG-induced mutagenesis, both DNA polymerase IV and DNA polymerase RI appeared to promote −1 frameshift and base