DNA REPAIR 7 (2008) 497–506

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Specificity of mutations induced by incorporation of oxidized dNTPs into DNA by human DNA polymerase η

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ARTICLE INFO

Article history:
Received 8 August 2007
Received in revised form
9 December 2007
Accepted 11 December 2007
Published on line 31 January 2008

Keywords:
Oxidative mutagenesis
Nucleotide pool
8-Hydroxy-dGTP
2-Hydroxy-dATP
Human DNA polymerase η

ABSTRACT

Aberrant oxidation is a property of many tumor cells. Oxidation of DNA precursors, i.e., deoxynucleotide triphosphates (dNTPs), as well as DNA is a major cause of genome instability. Here, we report that human DNA polymerase η (h Polη) incorporates oxidized dNTPs, i.e., 2-hydroxy-2′-deoxyadenosine 5′-triphosphate (2-OH-dATP) and 8-hydroxy-2′-deoxyguanosine 5′-triphosphate (8-OH-dGTP), into DNA in an erroneous and efficient manner, thereby inducing various types of mutations during in vitro gap-filling DNA synthesis. When 2-OH-dATP was present at a concentration equal to those of the four normal dNTPs in the reaction mixture, DNA synthesis by h Polη enhanced the frequency of G-to-T transversions eight-fold higher than that of the transversions in control where only the normal dNTPs were present. When 8-OH-dGTP was present at an equimolar concentration to the normal dNTPs, it enhanced the frequency of A-to-C transversions 17-fold higher than the control. It also increased the frequency of C-to-A transversions about two-fold. These results suggest that h Polη incorporates 2-OH-dATP opposite template G and incorporates 8-OH-dGTP opposite template A and slightly opposite template C during DNA synthesis. Besides base substitutions, h Polη enhanced the frequency of single-base frameshifts and deletions with the size of more than 100 base pairs when 8-OH-dGTP was present in the reaction mixture. Since h Polη is present in replication foci even without exogenous DNA damage, we suggest that h Polη may be involved in induction of various types of mutations through the erroneous and efficient incorporation of oxidized dNTPs into DNA in human cells.

1. Introduction

Reactive oxygen species (ROS) are produced by normal cellular respiration, cell injury or by exposure to environmental carcinogens and radiation. ROS generate a variety of altered purines and pyrimidines in DNA [1,2], thereby playing important roles in mutagenesis, carcinogenesis and aging [3,4]. It should be emphasized, however, that oxidized bases in DNA are introduced not only by direct oxidation of DNA but also by incorporation of oxidized deoxynucleotide triphosphates (dNTPs) into DNA by DNA polymerases [5–7]. Indeed, the frequency of A-to-C transversion mutations increases more

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doi:10.1016/j.dnarep.2007.12.005

than a 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) [8,9]. 8-OH-dGTP leads to A-to-C 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 [6,10]. 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 [11]. Another oxidized nucleotide, i.e., 1,2-dihydro-2-oxo-dATP (2-hydroxy-dATP, 2-OH-dATP), can induce G-to-T transversions when it is incorporated opposite guanine (G) in the template [12,13]. 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 [14,15].

Oxidized dNTPs also cause genome instability in mammalian cells. Spontaneous tumorigenesis in the 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 [16,17]. Recent studies with mismatch repair (MMR)-defective cells suggest that the majority of mutations in human cells that are deficient in MMR functions do not arise from spontaneous replication errors, but from the incorporation of oxidized dNTPs [18,19]. Thus, it is of great interest in the mechanisms as to how these oxidized dNTPs induce genome instability and oxidative mutagenesis, which lead to carcinogenesis.

The roles of oxidized dNTPs in mutagenesis have been questioned, however, because oxidized dNTPs are in general poor substrates for DNA polymerases (Pols) [20]. 8-OH-dGTP is poorly incorporated into DNA by Pol δ, T7 Pol exo$^-$, HIV reverse transcriptase, *E. coli* Pol II and Klenow exo$^-$ [21,22]. 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 to incorporate it opposite template A [23]. 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 [24].

The Y-family DNA Pols are recently recognized Pols that comprise proteins from different species, including members of eukarya, archaea and bacteria [25]. The most distinct feature of this family of enzymes is their ability to bypass various lesions, such as ultraviolet light photoproducts, in template DNA [26–28]. Some bypass reactions, i.e., translesion DNA synthesis (TLS), catalyzed by these enzymes are error prone while others are error free [29]. Thus, this family of Pols seems to be involved in mutagenesis and DNA-damage tolerance [30]. Interestingly, some of Y-family Pols have been shown to incorporate oxidized dNTPs into DNA in an efficient and erroneous manner. In humans, Polη (h Polη) incorporates 8-OH-dGTP opposite template A at almost the same efficiency as incorporation of normal dTTP, and incorporates 2-OH-dATP opposite template T, G and C at substantial rates [31]. Archaeal Y-family Pols of *Sulfolobus* sp. and a Y-family Pol of *E. coli*, i.e., DNA Pol IV (DinB), almost exclusively incorporate 8-OH-dGTP opposite template A, and 2-OH-dATP opposite template G and thymine (T) [32,33]. In fact, both Y-family DNA Pols of *E. coli*, i.e., Pol IV and Pol V (UmuD'C), are shown to be involved in oxidative mutagenesis in *sod/fur* mutants where the mutator effects are caused by oxidation of dNTPs instead of DNA [33]. Collectively, these results suggest that some of Y-family Pols may be involved in mutagenesis through the erroneous incorporation of oxidized dNTPs into DNA.

In this study, we characterized mutations induced by incorporation of 8-OH-dGTP or 2-OH-dATP into DNA by h Polη using in vitro gap-filling assay with M13mp2 phage DNA [34]. The assay allows us to analyze not only base substitutions but also frameshifts and deletions, which are generated during DNA synthesis to fill in a gap of 407 base pairs (bps) in M13 DNA. We show here that h Polη incorporates 8-OH-dGTP into DNA, thereby inducing single-base frameshifts and large deletions as well as base substitutions including A-to-C transversions. Addition of a quite small amount of 8-OH-dGTP, i.e., 1/1000 amount of the four normal dNTPs, into the reaction mixture enhanced mutagenesis significantly. h Polη also incorporates 2-OH-dATP opposite template G, thereby inducing G-to-T transversions. Because h Polη is present in replication factories in cells not deliberately exposed to DNA damaging agents [35,36], we suggest that h Polη may be involved in a variety of oxidative mutagenesis through the incorrect and efficient incorporation of oxidized dNTPs during DNA synthesis in human cells.

2. Materials and methods

2.1. Materials

h Polη was prepared as described [37]. 8-OH-dGTP was purchased from Trilink Bio Tech, and 2-OH-dATP was purified as described [24]. Normal dNTP mixture was purchased from TAKARA Bio Inc. (Kyoto, Japan). Restriction enzyme *Pvu*II was purchased from New England Biolabs (Massachusetts, USA). Oligonucleotides were from Japan Bio Services Co., Ltd. (Saitama, Japan).

The *E. coli* strain MC1061 and CSH50 were used for electroporation and phage growth, respectively, and M13mp2 bacteriophage was for construction of gapped DNA substrate [34]. These are gifts from Dr. Thomas A. Kunkel, National Institute of Environmental Health Sciences (NIEHS), USA.

2.2. *Preparation of gapped M13 DNA and gap-filling reactions by h Polη*

The gapped M13mp2 DNA was constructed in way that a 407-bp single-stranded gap contains the *lacZ* α-complementation target sequence [34]. As the first transcribed nucleotide of the *lacZ* gene is numbered +1, the single-stranded gap was numbered nucleotide +191 to −216 and the 5′ end of the *lacZ* gene-84.

Reaction mixtures (15 μL) contained 40 mM tris–HCl (pH 8.0), 10 mM DTT, 3.75 μg BSA, 60 mM KCl, 2.5% glycerol, 10 mM $MgCl_2$, 1.4 nM gapped M13mp2 DNA, 72 nM h Polη and 200 μM of the four normal dNTPs. An equimolar concentration (200 μM) of oxidized dNTP, i.e., 2-OH-dATP or 8-OH-dGTP, was added to the reaction mixtures, if necessary. In this study, we

Table 1 – Mutant frequency induced by oxidized dNTPs

Condition	Total plaques counted	Mutant plaques counted	Mutant frequency	Mean ± S.D.
Control	5308	2076	0.39	0.40 ± 0.01
	6382	2580	0.40	
	8207	3443	0.41	
Plus 2-OH-dATP	3900	1180	0.30	0.36 ± 0.05
	1767	706	0.40	
	8100	3020	0.37	
Plus 8-OH-dGTP	1200	1012	0.84	0.85 ± 0.01*
	950	819	0.86	
	1392	1195	0.86	

The four normal dNTPs (200 μM) were included in the reaction mixture containing h Polη (72 nM). In plus 2-OH-dATP and plus 8-OH-dGTP conditions, 200 μM of 2-OH-dATP or 8-OH-dGTP, respectively, was additionally included in the reaction mixture. Asterisk indicates a significant difference ($P < 0.05$ versus control).

refer to these conditions as "plus 2-OH-dATP" or "plus 8-OH-dGTP" conditions, respectively. To examine the mutagenicity of 8-OH-dGTP at lower concentrations, reactions were conducted with lower concentrations (0.02, 0.2, 2.0 and 20 μM) of 8-OH-dGTP in the presence of the four normal dNTPs (200 μM) and h Polη (72 nM). The gap-filling reactions were carried out at 37 °C for 2 h and terminated by adding EDTA to the final concentration of 15 mM. Completion of the gap-filling reaction was confirmed by agarose gel electrophoresis of the reaction products [34]. Mutagenesis experiments were conducted only when the completion of the reactions was confirmed.

2.3. *Gap-filling assay and determination of mutant frequencies (MFs)*

The gap-filled DNAs were introduced into *E. coli* MC1061 by electroporation at 1.8 kV with Micro pulser set (Bio-Rad) and proliferated in *E. coli* strain CSH50 for α-complementation of β-galactosidase as described [34]. MC1061 was used for electroporation due to its higher efficiency of DNA incorporation than CSH50. Wild-type plaques were dark blue while mutant plaques were light blue or colorless. The experiments were repeated three times in each condition, i.e., control, plus 2-OH-dATP and plus 8-OH-dGTP. MFs were calculated by dividing the numbers of mutant plaques by the total numbers of plaques, and the mean ± standard deviation (S.D.) of three experiments were calculated.

2.4. *Determination of numbers of mutations per DNA molecule*

To determine the number of mutations per DNA molecule, we isolated DNA from independent *lacZ* mutants and sequenced nucleotides +191 through −84 using an ABI PRISM Dye Terminator Cycle Sequencing Kit and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems Japan, Tokyo). When deletions were detected beyond position −84, we expanded the sequence analysis through −216 to determine the length and start/end points of the deletions. We then calculated the average numbers of each type of mutations per DNA molecule ± S.D. of three experiments. In the calculation, we estimated the numbers of DNA molecules by dividing the numbers of sequenced mutant plaques by the MF. In other words, the numbers of mutations in sequenced mutant plaques was divided by the total number of mutant and corresponding wild-type plaques. The statistical significance of the results was examined by the Student's t-test.

3. Results

3.1. *Mutations induced by incorporation of oxidized dNTPs into DNA by h Polη*

We began this study by determining the MFs in the *lacZ* gene when the gap of M13mp2 DNA was filled by h Polη with or without oxidized dNTPs. In agreement with the previous report [38], the MF was high of 40% even in the control condition where only the four normal dNTPs were present in the reaction mixture (Table 1). The MF in plus 2-OH-dATP condition was similar to that in the control. In contrast, inclusion of 8-OH-dGTP at a concentration equal to that of normal dNTPs yielded about two-fold increase in MF, i.e., 85% ($P = 0.00015$).

3.2. *Spectra of mutations induced by 2-OH-dATP and 8-OH-dGTP*

To determine what types of mutations were induced by incorporation of oxidized dNTPs, we isolated DNA from 33, 34 and 67 mutant plaques from triplicate experiments of control, plus 2-OH-dATP and plus 8-OH-dGTP conditions, respectively, and sequenced them in the nucleotides +191 through −84. The mutants contained multiple sequence changes per DNA molecule, consistent with the colorless phenotype of most of the plaques (Table 2). Even in the control reactions, the mutants had more than five mutations per molecules (5.70 ± 0.74) and about 85% of the mutations were base substitutions (398/457 mutations, Supplement Table 1). Of the base substitutions, more than 90% were single-base substitutions (362/398) and tandem-base substitutions were rare (18/398). Similar distribution of mutations was observed in plus 2-OH-dATP condition. The mutants contained more than five mutations per DNA molecule (5.70 ± 1.57) and more than 90% of the mutations were base substitutions (485/540 mutations). About 90% of the base substitutions were single-base substitutions (423/485) and tandem-base

Table 2 – Classification of mutations induced by oxidized dNTPs

Mutation	Number of mutations per DNA molecule[a]		
	Control	Plus 2-OH-dATP	Plus 8-OH-dGTP
Base substitution	4.91 ± 0.67	5.12 ± 1.43	16.49 ± 3.92[b]
Deletion	0.50 ± 0.04	0.37 ± 0.10	1.18 ± 0.27[b]
Addition	0.17 ± 0.05	0.14 ± 0.04	0.44 ± 0.26
Complex	0.11 ± 0.07	0.07 ± 0.04	0.25 ± 0.11
Total mutation	5.70 ± 0.74	5.70 ± 1.57	18.35 ± 3.84[b]

[a] The mean ± S.D. of three experiments. The number of DNA molecule was estimated by dividing the number of sequenced mutant plaques by the MF shown in Table 1.
[b] A significant difference with $P < 0.05$ versus control.

substitutions were rare (30/485). However, the percentage of transversions (48% = 233/485) was slightly higher compared to that of transversions in the control (41% = 163/398). In contrast, the number of base substitutions per DNA was about three-fold higher in plus 8-OH-dGTP condition than in the control (16.49 ± 3.92 versus 4.91 ± 0.67, $P = 0.040$). Besides base substitutions, the number of deletions per DNA molecule also increased compared to the control (1.18 ± 0.27 versus 0.50 ± 0.04, $P = 0.041$).

3.2.1. *Base substitutions induced by 2-OH-dATP and 8-OH-dGTP*

To examine what types of base substitutions were induced in plus 2-OH-dATP and plus 8-OH-dGTP conditions, we compared the numbers of specific base substitution mutations per DNA molecule (Fig. 1, Supplement Table 2). In plus 2-OH-dATP condition, the number of G-to-T transversions increased about eight-fold compared to the control (0.79 ± 0.23 versus 0.10 ± 0.08, $P = 0.031$). No other types of base substitutions were increased significantly. In plus 8-OH-dGTP condition, the number of A-to-C transversions per DNA increased 17-fold over the control (8.52 ± 2.20 versus 0.50 ± 0.18, $P = 0.022$). The number of transitions of T-to-C also increased about two-fold (4.26 ± 1.30 versus 2.03 ± 0.35, $P = 0.13$), but the increase was not statistically significant because of the high T-to-C transitions in the control (Fig. 1, Supplement Table 2) [39]. Instead, the number of C-to-A transversions increased significantly although the numbers of C-to-A mutations per DNA were relatively low (0.21 ± 0.03 versus 0.11 ± 0.03, $P = 0.013$). No other base substitutions increased significantly in plus 8-OH-dGTP condition. Since the frequencies of base substitutions were high even in the control, no obvious hotspot sequences were identified in plus 2-OH-dATP and plus 8-OH-dGTP conditions (Supplement Fig. 1).

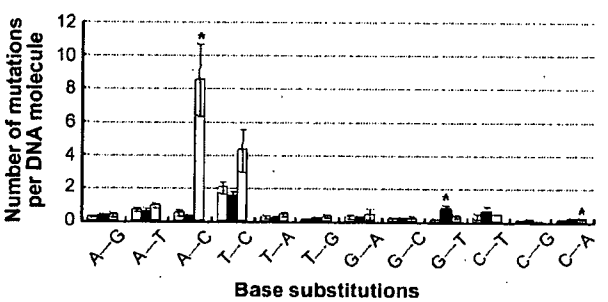

Fig. 1 – Twelve base substitutions induced by oxidized dNTPs. Frequencies of base substitutions are represented as numbers of base substitutions per DNA molecule. Asterisks indicate a significant difference versus control with $P < 0.05$. Each group of bars consists of the results from control (in white), a plus 8-OH-dGTP condition (in gray), and a plus 2-OH-dATP condition (in black). Exact values are presented in Supplement Table 2.

Table 3 – Classification of deletions induced by 8-OH-dGTP

Deletion		Number of mutations per DNA molecule[a]	
		Control	Plus 8-OH-dGTP
One base deletion	ΔA	0.16 ± 0.09	0.10 ± 0.12
	ΔG	0.05 ± 0.06	0.14 ± 0.12
	ΔC	0.02 ± 0.04	0.19 ± 0.05[b]
	ΔT	0.06 ± 0.04	0.33 ± 0.05[b]
	Total	0.29 ± 0.04	0.76[b] ± 0.16
2-99 bases deletion		0.23 ± 0.04	0.30 ± 0.08
>100 bases deletion		<0.01	0.13[b] ± 0.09

[a] The mean ± S.D. of three experiments. The number of DNA molecule was estimated by dividing the number of sequenced mutant plaques by the MF shown in Table 1.
[b] A significant increase with $P < 0.05$ versus control.

3.2.2. *Single-base frameshifts and large deletions induced by 8-OH-dGTP*

To examine what types of deletions were induced in plus 8-OH-dGTP condition, we compared the numbers of deletion mutations per DNA molecule between control and plus 8-OH-dGTP conditions (Table 3, Fig. 2). The numbers of single-base deletions at template C and T increased more than eight-fold and five-fold, respectively, compared to the control condition (0.19 ± 0.05 versus 0.02 ± 0.04 for ΔC $P = 0.031$, and 0.33 ± 0.05 versus 0.06 ± 0.04 for ΔT $P = 0.030$). When we looked for characteristic sequences around the deleted bases, we noticed that G was disfavored at 5′-side of the deleted bases. Only five out of 62 single-base deletions occurred in a sequence of 5′-GX-3′ where X represents the deleted bases, i.e., A, T, G or C. Among other three bases, C was slightly favored at the 5′-side (27/62) while A and T were present evenly (14/62 and 16/62). Besides one-base deletions, large deletions with the size of more than 100 bps increased more than 10-fold compared to the controls

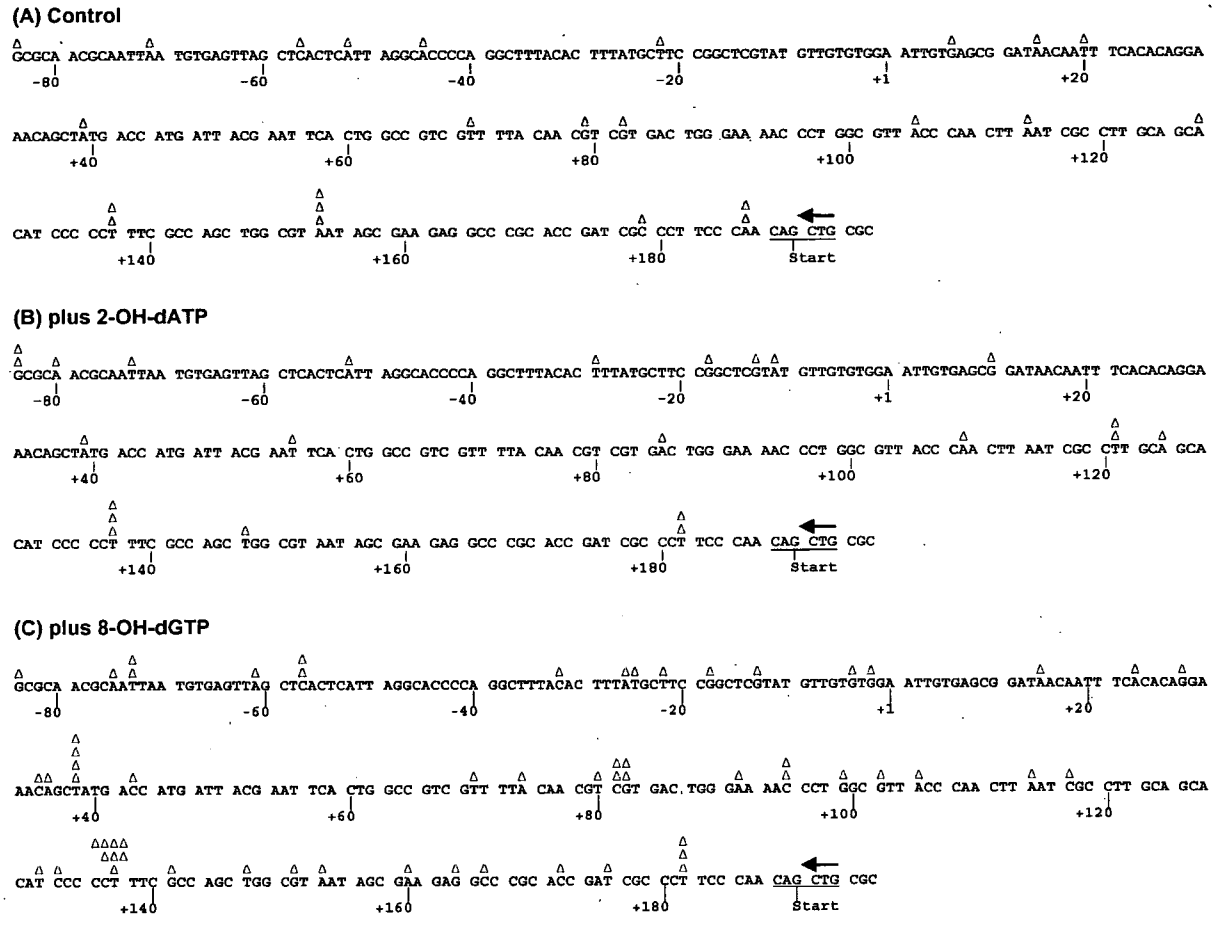

Fig. 2 – Spectra of one-base deletions by h Polη. (A) Control, (B) plus 2-OH-dATP and (C) plus 8-OH-dGTP conditions. One-base deletions are shown as triangles above the template sequence of nucleotide −84 to +194 of the *lacZ* gene in M13mp2 DNA. Nucleotide +1 is the first transcribed nucleotide. DNA synthesis starts at nucleotide +191 and the direction is shown with an arrow.

(0.13 ± 0.09 versus < 0.012). In fact, there were no such large deletions in the mutant plaques from the control and only one in plus 2-OH-dATP condition. The large deletions in plus 8-OH-dGTP mostly occurred between two GC-rich regions, i.e., one-region nucleotides −150 and −140 and the other from +165 to +169 in the *lacZ* gene (Fig. 3).

3.3. *Significant increase in MFs at low concentrations of 8-OH-dGTP*

To examine whether low concentrations of 8-OH-dGTP can induce mutations under the experimental conditions, we lowered the concentrations of 8-OH-dGTP from 200 to 0.02 μM in the reaction mixture where the concentrations of the four normal dNTPs were hold at 200 μM (Fig. 4, Supplement Table 3). The MFs were 0.802 ± 0.022 at 200 μM, 0.565 ± 0.020 at 20 μM, 0.406 ± 0.022 at 2 μM, 0.391 ± 0.008 at 0.2 μM and 0.352 ± 0.008 at 0.02 μM. Statistically significant increases in MFs over the concurrent control MF, i.e., 0.350 ± 0.004, were observed at concentrations of 200, 20, 2 and 0.2 μM of 8-OH-dGTP but not at a concentration of 0.02 μM. To further analyze the mutations at low concentrations of 8-OH-dGTP, we conducted sequence analysis of the mutants and calculated the specific MFs of A-to-C mutations per DNA molecule. The specific MFs were 8.83 ± 0.99 at 200 μM, 0.51 ± 0.20 at 2 μM, 0.43 ± 0.08 at 0.2 μM, 0.50 ± 0.19 at 0.02 μM and 0.35 ± 0.15 at 0 μM (Supplement Table 4).

4. Discussion

Although h Polη was initially recognized as an error-free bypass Pol across a *cis-syn* thymine–thymine dimer in DNA [26,27], recent evidence suggests that it plays roles in several important DNA transactions besides TLS [40]. Polη may be involved in homologous recombination by interacting with RAD51 [41,42] and may participate in somatic hypermutation of immunoglobulin genes [43]. In addition, Polη is co-localized with replication factories in cells not deliberately exposed to DNA damaging agents [35,36]. Thus, it may constitutively gain access to the genomic DNA and contribute to mutagenesis and/or damage avoidance even without external DNA damage. Interestingly, both Y-family DNA Pols in *E. coli*, i.e., Pol IV (DinB) and Pol V (UmuD'C), have been shown to partici-

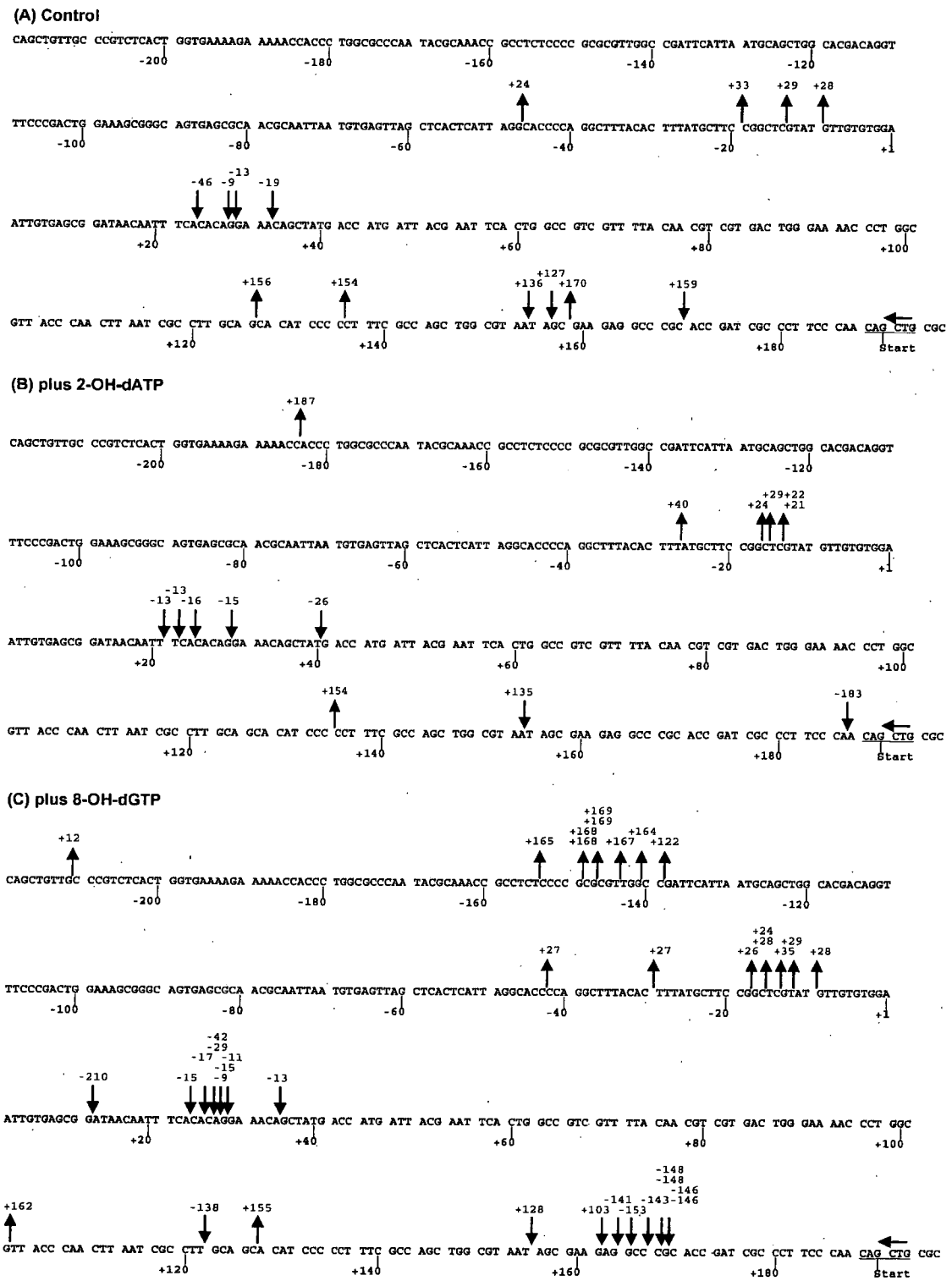

Fig. 3 – Spectra of deletions with the size of more than 10 bps by h Polη. (A) Control, (B) plus 200 μM of 2-OH-dATP and (C) plus 200 μM of 8-OH-dGTP conditions. Each deletion is defined by two arrows; one point down and the other point up. Each arrow is associated with a number indicating the nucleotide position at which the other endpoint for that deletion is located, and each arrow indicates the border between a deleted base and an undeleted base. Nucleotide +1 is the first transcribed nucleotide of the *lacZ* gene in M13mp2 DNA. DNA synthesis starts at nucleotide +191 marked with an arrow, which indicates the direction of DNA synthesis.

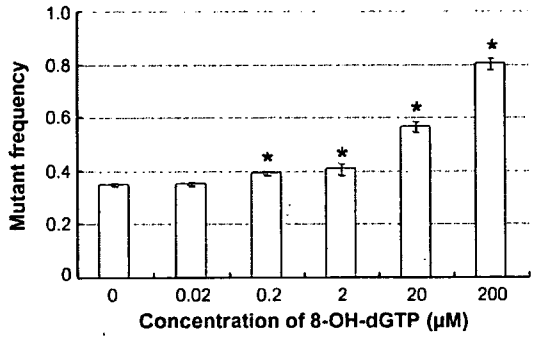

Fig. 4 – Mutant frequencies by h Polη in the presence of various concentrations of 8-OH-dGTP. Asterisks indicate a significant difference versus control, that is, the reaction only with the four normal dNTPs ($P < 0.05$). Exact values are presented in Supplement Table 3.

pate in mutagenesis through the erroneous incorporation of oxidized dNTPs into DNA when oxidation of the nucleotide pool is increased by *sod/fur* mutations [33]. h Polη and the *E. coli* Y-family Pols are involved in the chromosome replication when the cells are treated with hydroxyurea, which does not induce DNA damage but depletes the nucleotide pool [44,45]. h Polη can compete with replicative Pols during in vitro DNA replication [46]. We envisage, therefore, that h Polη might be involved in mutagenesis through misincorporation of oxidized dNTPs when the nucleotide pool is heavily oxidized. In fact, Kamiya et al. observed that knockdown of the expression of h Polη reduced the mutagenicity of 8-OH-dGTP incorporated into human cells (Ph.D. thesis of Dr. K. Satou in Hokkaido University, 2007).

To better understand the roles of h Polη in oxidative mutagenesis, we characterized the mutations induced by incorporation of 2-OH-dATP and 8-OH-dGTP into DNA by h Polη without lesions in template DNA in vitro, and compared the characteristic mutations to those observed in oxidative mutagenesis in mouse and human cells. When 2-OH-dATP was present at an equimolar concentration to the four normal dNTPs during DNA synthesis, incorporation of 2-OH-dATP by h Polη enhanced G-to-T transversions about eight-fold compared to the control where only the normal dNTPs were present (Fig. 1, Supplement Table 2). Because G-to-T transversions are rare, i.e., less than 2% of total mutations in control and plus 2-OH-dATP conditions, the total MF and the numbers of total base substitutions per DNA molecule were not enhanced by addition of 2-OH-dATP in the reaction mixture (Tables 1 and 2). Nevertheless, the significant increase in the frequency of G-to-T transversions in plus 2-OH-dATP condition suggests that h Polη misincorporates 2-OH-dATP opposite template G during the gap-filling reactions in vitro (Fig. 5A). This is consistent with the results of the kinetics analysis that h Polη tends to incorporate 2-OH-dATP opposite T, G and C in this order [31].

Intriguingly, Russo et al. [19] report that over-expression of hMTH1, which hydrolyzes both 2-OH-dATP and 8-OH-dGTP, substantially reduces the mutation rates in MMR-defective mouse and human cells and conclude that high spontaneous mutation rates in MMR-defective cells are largely due to incorporation of oxidized dNTPs into DNA. In particular, the mutation rates of G:C-to-T:A transversions are reduced more than 30-fold by the over-expression. 2-OH-dATP is in general a poor substrate for mammalian DNA Pols [24]. Thus, the results shown in Fig. 1 together with the report by Russo et al. suggest that Polη may be involved in misincorporation of 2-OH-dATP opposite template G during DNA replication, thereby inducing G:C-to-T:A transversions in the MMR-defective cells. The incorporated 2-OH-A in DNA opposite template G may be recognized and removed by MMR. In fact, Egashira et al. [47] report that the frequency of G:C-to-T:A transversions in the spleen of *Mth1*$^{-/-}$ *Msh2*$^{-/-}$ mice is increased more than 30-fold compared to *Mth1*$^{-/-}$ mice. Thus, Polη may contribute to the induction of G:C-to-T:A transversions via incorporation of 2-OH-dATP opposite template G in the mouse and human cells.

In plus 8-OH-dGTP condition, addition of the oxidized dGTP increased the frequency of A-to-C transversions 17-fold during DNA synthesis (Fig. 1, Supplement Table 2). It indicates that h Polη inserts 8-OH-dGTP opposite template A with high efficiency (Fig. 5B). This is consistent with the kinetic data that suggest that h Polη incorporates 8-OH-dGTP opposite template A with almost as the incorporation of dTTP [31]. However, in the MMR-deficient cells, only three-fold reduction in the frequency of A:T-to-C:G transversions is observed associated with the over-expression of hMTH1 [19]. The frequency of A:T-to-C:G transversions in the *Mth1*$^{-/-}$ *Msh2*$^{-/-}$ mice was also increased about three-fold as compared to *Mth1*$^{-/-}$ mice [47]. Thus, the activity of Polη incorporating 8-OH-dGTP opposite template A may be attenuated in the mouse cells. The incorporated 8-OH-dGMP opposite template A might be excised by proofreading activities of other Pols [48].

Besides base substitutions, single-base frameshifts and large deletions with the size more than 100 bps were induced in plus 8-OH-dGTP condition (Figs. 2 and 3). This is in contrast to the spectra of mutations induced by the incorporation of 8-OH-dGTP into DNA by other Pols such as exo$^-$ *E. coli* Pol I, T4 and *Thermus thermophilus* Pols where only base substitutions are observed [48,49]. In the MMR-defective cells, hMTH1 expression also dramatically reduces single-base and dozens-base deletions, which indicate such frameshifts would be caused by misincorporation of oxidized purine dNTPs into DNA [19]. However, the reduction in single-base deletions by hMTH1 expression is mainly in a run of G whereas the increase in single-base deletions was in non-run sequences in the present study. Hence the contribution of Polη to the induction of the single-base deletions in the MMR-defective cells seems limited. In addition, no large deletions are observed in the MMR-defective cells and in the spleen of *Mth1*$^{-/-}$ *Msh2*$^{-/-}$ mice [47]. Thus, at present, relevance of frameshifts and large deletions observed in this study remains to be elucidated.

How are the single-base frameshifts and large deletions induced by incorporation of 8-OH-dGTP into DNA? We envisage that preference of h Polη incorporating 8-OH-dGTP into DNA could, at least in part, account for the mechanisms by which single-base frameshifts and large deletions are induced. This speculation is based on the facts that pyrimidines, i.e., T and C, in the template DNA were more frequently deleted than purines in the single-base frameshifts and that G was disfavored at 5′-side of the deleted bases (Fig. 2C, Table 3). The

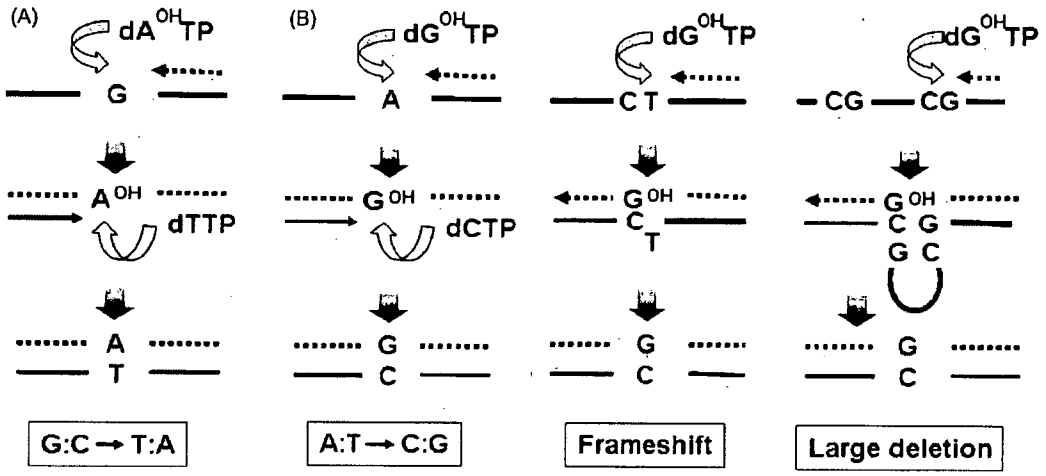

Fig. 5 – Proposed mechanisms by which various mutations are induced by incorporation of oxidized dNTPs into DNA by h Polη. (A) If 2-OH-dATP is incorporated opposite template G, and T is incorporated opposite 2-OH-A in the next round of DNA replication, G-to-T transversions will be induced (G:C → T:A). (B) If 8-OH-dGTP is incorporated opposite template A, and C is incorporated opposite 8-OH-G in the next round of DNA replication, A-to-C transversions will be induced (A:T → C:G). If 8-OH-dGTP is incorporated opposite template next C instead of correct T in 5′-CT-3′ sequences, one-bp deletion will be induced in the next round of DNA replication (frameshift). If h Polη stalls in GC-rich regions, i.e., nucleotides +165 to +169, and 8-OH-dGTP is incorporated opposite template C more than 100 bps downstream from the stalling site, large deletions will be induced in the next round of DNA replication (large deletion). Although this is not shown in the figure, if 8-OH-dGTP is incorporated opposite template C, and A is incorporated opposite 8-OH-G in the next round of DNA replication, C-to-A transversions will be induced (G:C → T:A).

hotspot of single-base frameshifts in plus 8-OH-dGTP condition is found in a sequence of 5′-CT-3′ where T was deleted eight times at nucleotide 137. Two moderate hotspots were also found in the sequence of 5′-CT-3′ where T was deleted four times at nucleotide 38 and three times at nucleotide 182. Thus, it seems that h Polη tends to incorporate 8-OH-dGTP opposite the adjacent C rather than T at the correct position, thereby inducing one-bp frameshifts (Fig. 5B). Since 5′-GX-3′ where X represents the deleted base was rare in the frameshifts, it probably disfavors incorporating 8-OH-dGTP opposite next G. This is basically consistent with the results of enzyme kinetics which indicate that h Polη poorly incorporates 8-OH-dGTP opposite template T and G [31]. The poor incorporation opposite template G might also contribute to the induction of large deletions because the regions where large deletions start and end, i.e., nucleotides −150 and −140 and nucleotides +165 to +169, respectively, are mostly GC-rich regions. We speculate that the h Polη may stall when it proceeds in the region nucleotides −150 and −140, which provides a chance to form secondary structures, i.e., more than 100 bps bulge in the template strand between the two regions. h Polη may skip over the bulge structures and restart DNA synthesis more than 100 bps downstream from the stalling site, thereby inducing deletions. The large active site of Polη that can accommodate bulky lesions might be the physical basis for the induction of frameshifts and large deletions associated with incorporation of 8-OH-dGTP into DNA [28].

Finally, we observed significant increases in MF compared to the control in the presence of low concentrations 8-OH-dGTP (Fig. 4, Supplement Table 3). The experiments were conducted because Tassotto and Mathews [50] failed to demonstrate the mutagenicity of 8-OH-dGTP at a concentration of 0.34 μM with HeLa cell extracts using the in vitro replication error detection system and raise the question of the role of 8-OH-dGTP in oxidative mutagenesis. Although we used purified Pol instead of cell extracts, which might contain less amounts of h Polη, the lowest concentration of 8-OH-dGTP where a significant increase in MF was observed was 0.2 μM, which was 1/1000 of that of the four normal dNTPs in the reaction mixture. To examine the significance of the increase in MFs at low concentrations of 8-OH-dGTP, we determined sequence changes of the mutants and calculated specific MFs of A-to-C transversions. However, the increases in specific MFs over the control level were not statistically significant. This may be due to the high background of mutations generated by Polη even without 8-OH-dGTP. Estimated concentration of normal dGTP in human cells is about 10 μM, and tumor cells have usually 5–10-fold higher concentrations of dGTP over normal cells [51]. Nonetheless, the results shown here are those obtained with in vitro experiments and thus additional work in vivo is required to estimate the role of Polη in induction of mutations at low concentrations of oxidized dNTPs.

In summary, we analyzed the mutations induced by incorporation of 2-OH-dATP and 8-OH-dGTP by h Polη using in vitro gap-filling assay. h Polη promotes G-to-T and A-to-C transversions in the presence of 2-OH-dATP and 8-OH-dGTP, respectively, and also promotes C-to-A transversions, one-base frameshifts and deletions with the size of more than 100 bps when 8-OH-dGTP was included in the reaction mixture. Since some of the mutations, e.g., G:C-to-T:A transversions, are observed in cells where oxidized dNTPs play important roles in mutagenesis [19,47], we suggest that h Polη may be

involved in a variety of mutations in human cells by efficient and erroneous incorporation of oxidized dNTPs into DNA. It is reported, however, that extracts of XP-V cells deficient in h Polη exhibit similar capacity to induce mutations with 8-OH-dGTP and 2-OH-dATP in an in vitro DNA replication system to those of HeLa cells [52]. Other Pols also have a potential to induce A-to-C transversions by incorporation of 8-OH-dGTP into DNA in vitro [48]. Thus, further work is needed to examine the cellular roles of h Polη to induce oxidative mutations by incorporation of oxidized dNTPs into DNA in human cells.

Acknowledgements

We thank Dr. Thomas A. Kunkel, NIEHS, USA, for providing bacteriophage and E. coli. This work is supported by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (MEXT), the Ministry of Health, Labour and Welfare, Japan (MHLW) and the Japan Health Science Foundation (JHSF). This work was also supported by grants-in-aid for international collaborative research (SH34407) from JHSF, cancer research from MHLW and nuclear research from MEXT, based on the screening and counseling by the Atomic Energy Commission.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dnarep.2007.12.005.

REFERENCES

[1] S. Bjelland, E. Seeberg, Mutagenicity, toxicity and repair of DNA base damage induced by oxidation, Mutat. Res. 531 (2003) 37–80.
[2] H. Kamiya, Mutagenic potentials of damaged nucleic acids produced by reactive oxygen/nitrogen species: approaches using synthetic oligonucleotides and nucleotides: survey and summary, Nucleic Acids Res. 31 (2003) 517–531.
[3] B.N. Ames, Dietary carcinogens and anticarcinogens. Oxygen radicals and degenerative diseases, Science 221 (1983) 1256–1264.
[4] A.L. Jackson, L.A. Loeb, The contribution of endogenous sources of DNA damage to the multiple mutations in cancer, Mutat. Res. 477 (2001) 7–21.
[5] M. Sekiguchi, T. Tsuzuki, Oxidative nucleotide damage: consequences and prevention, Oncogene 21 (2002) 8895–8904.
[6] M.L. Michaels, J.H. Miller, The GO system protects organisms from the mutagenic effect of the spontaneous lesion 8-hydroxyguanine (7,8-dihydro-8-oxoguanine), J. Bacteriol. 174 (1992) 6321–6325.
[7] Y. Nakabeppu, K. Sakumi, K. Sakamoto, D. Tsuchimoto, T. Tsuzuki, Y. Nakatsu, Mutagenesis and carcinogenesis caused by the oxidation of nucleic acids, Biol. Chem. 387 (2006) 373–379.
[8] H. Maki, M. Sekiguchi, MutT protein specifically hydrolyses a potent mutagenic substrate for DNA synthesis, Nature 355 (1992) 273–275.
[9] C. Yanofsky, E.C. Cox, V. Horn, The unusual mutagenic specificity of an E. Coli mutator gene, Proc. Natl. Acad. Sci. U. S. A 55 (1966) 274–281.
[10] H. Kasai, Chemistry-based studies on oxidative DNA damage: formation, repair, and mutagenesis, Free Radic. Biol. Med. 33 (2002) 450–456.
[11] A. Sakai, M. Nakanishi, K. Yoshiyama, H. Maki, Impact of reactive oxygen species on spontaneous mutagenesis in *Escherichia coli*, Genes Cells 11 (2006) 767–778.
[12] M. Inoue, H. Kamiya, K. Fujikawa, Y. Ootsuyama, N. Murata-Kamiya, T. Osaki, K. Yasumoto, H. Kasai, Induction of chromosomal gene mutations in *Escherichia coli* by direct incorporation of oxidatively damaged nucleotides. New evaluation method for mutagenesis by damaged DNA precursors in vivo, J. Biol. Chem. 273 (1998) 11069–11074.
[13] H. Kamiya, H. Kasai, 2-Hydroxy-dATP is incorporated opposite G by *Escherichia coli* DNA polymerase III resulting in high mutagenicity, Nucleic Acids Res. 28 (2000) 1640–1646.
[14] H. Kamiya, E. Iida, N. Murata-Kamiya, Y. Yamamoto, T. Miki, H. Harashima, Suppression of spontaneous and hydrogen peroxide-induced mutations by a MutT-type nucleotide pool sanitization enzyme, the *Escherichia coli* Orf135 protein, Genes Cells 8 (2003) 941–950.
[15] H. Kamiya, N. Murata-Kamiya, E. Iida, H. Harashima, Hydrolysis of oxidized nucleotides by the *Escherichia coli* Orf135 protein, Biochem. Biophys. Res. Commun. 288 (2001) 499–502.
[16] T. Tsuzuki, A. Egashira, H. Igarashi, T. Iwakuma, Y. Nakatsuru, Y. Tominaga, H. Kawate, K. Nakao, K. Nakamura, F. Ide, S. Kura, Y. Nakabeppu, M. Katsuki, T. Ishikawa, M. Sekiguchi, Spontaneous tumorigenesis in mice defective in the MTH1 gene encoding 8-oxo-dGTPase, Proc. Natl. Acad. Sci. U. S. A 98 (2001) 11456–11461.
[17] K. Fujikawa, H. Kamiya, H. Yakushiji, Y. Fujii, Y. Nakabeppu, H. Kasai, The oxidized forms of dATP are substrates for the human MutT homologue, the hMTH1 protein, J. Biol. Chem. 274 (1999) 18201–18205.
[18] C. Colussi, E. Parlanti, P. Degan, G. Aquilina, D. Barnes, P. Macpherson, P. Karran, M. Crescenzi, E. Dogliotti, M. Bignami, The mammalian mismatch repair pathway removes DNA 8-oxodGMP incorporated from the oxidized dNTP pool, Curr. Biol. 12 (2002) 912–918.
[19] M.T. Russo, M.F. Blasi, F. Chiera, P. Fortini, P. Degan, P. Macpherson, M. Furuichi, Y. Nakabeppu, P. Karran, G. Aquilina, M. Bignami, The oxidized deoxynucleoside triphosphate pool is a significant contributor to genetic instability in mismatch repair-deficient cells, Mol. Cell Biol. 24 (2004) 465–474.
[20] T. Nohmi, S.R. Kim, M. Yamada, Modulation of oxidative mutagenesis and carcinogenesis by polymorphic forms of human DNA repair enzymes, Mutat. Res. 591 (2005) 60–73.
[21] H.J. Einolf, F.P. Guengerich, Fidelity of nucleotide insertion at 8-oxo-7,8-dihydroguanine by mammalian DNA polymerase delta. Steady-state and pre-steady-state kinetic analysis, J. Biol. Chem. 276 (2001) 3764–3771.
[22] H.J. Einolf, N. Schnetz-Boutaud, F.P. Guengerich, Steady-state and pre-steady-state kinetic analysis of 8-oxo-7,8-dihydroguanosine triphosphate incorporation and extension by replicative and repair DNA polymerases, Biochemistry 37 (1998) 13300–13312.
[23] H. Miller, R. Prasad, S.H. Wilson, F. Johnson, A.P. Grollman, 8-oxodGTP incorporation by DNA polymerase beta is modified by active-site residue Asn279, Biochemistry 39 (2000) 1029–1033.
[24] H. Kamiya, H. Kasai, Formation of 2-hydroxydeoxyadenosine triphosphate, an oxidatively damaged nucleotide, and its incorporation by DNA polymerases. Steady-state kinetics of the incorporation, J. Biol. Chem. 270 (1995) 19446–19450.
[25] H. Ohmori, E.C. Friedberg, R.P. Fuchs, M.F. Goodman, F. Hanaoka, D. Hinkle, T.A. Kunkel, C.W. Lawrence, Z. Livneh, T. Nohmi, L. Prakash, S. Prakash, T. Todo, G.C. Walker, Z. Wang,

R. Woodgate, The Y-family of DNA polymerases, Mol. Cell 8 (2001) 7–8.
[26] C. Masutani, R. Kusumoto, A. Yamada, N. Dohmae, M. Yokoi, M. Yuasa, M. Araki, S. Iwai, K. Takio, F. Hanaoka, The XPV (xeroderma pigmentosum variant) gene encodes human DNA polymerase η, Nature 399 (1999) 700–704.
[27] R.E. Johnson, S. Prakash, L. Prakash, Efficient bypass of a thymine–thymine dimer by yeast DNA polymerase, Polη, Science 283 (1999) 1001–1004.
[28] S. Prakash, R.E. Johnson, L. Prakash, Eukaryotic translesion synthesis DNA polymerases: specificity of structure and function, Ann. Rev. Biochem. 74 (2005) 317–353.
[29] E.C. Friedberg, Suffering in silence: the tolerance of DNA damage, Nat. Rev. Mol. Cell Biol. 6 (2005) 943–953.
[30] T. Nohmi, Environmental stress and lesion-bypass DNA polymerases, Ann. Rev. Microbiol. 60 (2006) 231–253.
[31] M. Shimizu, P. Gruz, H. Kamiya, C. Masutani, Y. Xu, Y. Usui, H. Sugiyama, H. Harashima, F. Hanaoka, T. Nohmi, Efficient and erroneous incorporation of oxidized DNA precursors by human DNA polymerase eta, Biochemistry 46 (2007) 5515–5522.
[32] M. Shimizu, P. Gruz, H. Kamiya, S.R. Kim, F.M. Pisani, C. Masutani, Y. Kanke, H. Harashima, F. Hanaoka, T. Nohmi, Erroneous incorporation of oxidized DNA precursors by Y-family DNA polymerases, EMBO Rep. 4 (2003) 269–273.
[33] M. Yamada, T. Nunoshiba, M. Shimizu, P. Gruz, H. Kamiya, H. Harashima, T. Nohmi, Involvement of Y-family DNA polymerases in mutagenesis caused by oxidized nucleotides in *Escherichia coli*, J. Bacteriol. 188 (2006) 4992–4995.
[34] K. Bebenek, T.A. Kunkel, Analyzing fidelity of DNA polymerases, Methods Enzymol. 262 (1995) 217–232.
[35] P. Kannouche, B.C. Broughton, M. Volker, F. Hanaoka, L.H. Mullenders, A.R. Lehmann, Domain structure, localization, and function of DNA polymerase η, defective in xeroderma pigmentosum variant cells, Genes Dev. 15 (2001) 158–172.
[36] A. Tissier, P. Kannouche, M.P. Reck, A.R. Lehmann, R.P. Fuchs, A. Cordonnier, Co-localization in replication foci and interaction of human Y-family members, DNA polymerase polη and REV1 protein, DNA Repair (Amst.) 3 (2004) 1503–1514.
[37] C. Masutani, R. Kusumoto, S. Iwai, F. Hanaoka, Mechanisms of accurate translesion synthesis by human DNA polymerase eta, EMBO J. 19 (2000) 3100–3109.
[38] T. Matsuda, K. Bebenek, C. Masutani, F. Hanaoka, T.A. Kunkel, Low fidelity DNA synthesis by human DNA polymerase-eta, Nature 404 (2000) 1011–1013.
[39] T. Matsuda, K. Bebenek, C. Masutani, I.B. Rogozin, F. Hanaoka, T.A. Kunkel, Error rate and specificity of human and murine DNA polymerase eta, J. Mol. Biol. 312 (2001) 335–346.
[40] A.R. Lehmann, New functions for Y family polymerases, Mol. Cell 24 (2006) 493–495.
[41] M.J. McIlwraith, A. Vaisman, Y. Liu, E. Fanning, R. Woodgate, S.C. West, Human DNA polymerase eta promotes DNA synthesis from strand invasion intermediates of homologous recombination, Mol. Cell 20 (2005) 783–792.
[42] T. Kawamoto, K. Araki, E. Sonoda, Y.M. Yamashita, K. Harada, K. Kikuchi, C. Masutani, F. Hanaoka, K. Nozaki, N. Hashimoto, S. Takeda, Dual roles for DNA polymerase eta in homologous DNA recombination and translesion DNA synthesis, Mol. Cell 20 (2005) 793–799.
[43] V.I. Mayorov, I.B. Rogozin, L.R. Adkison, P.J. Gearhart, DNA polymerase eta contributes to strand bias of mutations of A versus T in immunoglobulin genes, J. Immunol. 174 (2005) 7781–7786.
[44] V.G. Godoy, D.F. Jarosz, F.L. Walker, L.A. Simmons, G.C. Walker, Y-family DNA polymerases respond to DNA damage-independent inhibition of replication fork progression, EMBO J. 25 (2006) 868–879.
[45] S. de Feraudy, C.L. Limoli, E. Giedzinski, D. Karentz, T.M. Marti, L. Feeney, J.E. Cleaver, Pol eta is required for DNA replication during nucleotide deprivation by hydroxyurea, Oncogene 26 (2007) 5713–5721.
[46] K. Bebenek, T. Matsuda, C. Masutani, F. Hanaoka, T.A. Kunkel, Proofreading of DNA polymerase eta-dependent replication errors, J. Biol. Chem. 276 (2001) 2317–2320.
[47] A. Egashira, K. Yamauchi, K. Yoshiyama, H. Kawate, M. Katsuki, M. Sekiguchi, K. Sugimachi, H. Maki, T. Tsuzuki, Mutational specificity of mice defective in the MTH1 and/or the MSH2 genes, DNA Repair (Amst.) 1 (2002) 881–893.
[48] Y.I. Pavlov, D.T. Minnick, S. Izuta, T.A. Kunkel, DNA replication fidelity with 8-oxodeoxyguanosine triphosphate, Biochemistry 33 (1994) 4695–4701.
[49] K.C. Cheng, D.S. Cahill, H. Kasai, S. Nishimura, L.A. Loeb, 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G to T and A to C substitutions, J. Biol. Chem. 267 (1992) 166–172.
[50] M.L. Tassotto, C.K. Mathews, Assessing the metabolic function of the MutT 8-oxodeoxyguanosine triphosphatase in Escherichia coli by nucleotide pool analysis, J. Biol. Chem. 277 (2002) 15807–15812.
[51] T.W. Traut, Physiological concentrations of purines and pyrimidines, Mol. Cell Biochem. 140 (1994) 1–22.
[52] K. Satou, H. Kasai, C. Masutani, F. Hanaoka, H. Harashima, H. Kamiya, 2-Hydroxy-2′-deoxyadenosine 5′-triphosphate enhances A·T–>C·G mutations caused by 8-hydroxy-2′-deoxyguanosine 5′-triphosphate by suppressing its degradation upon replication in a HeLa extract, Biochemistry 46 (2007) 6639–6646.

DNA Repair and DNA Damage Tolerance in Archaeal Bacteria: Extreme Environments and Genome Integrity

7

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Abstract

Maintenance of genome integrity is a mechanism central to cellular life. Many Archaeal species live in harsh habitats that are extreme challenges to genome stability. In the habitat at high ambient temperatures, deamination, oxidation and depurination are greatly accelerated and various lesions are supposed to accumulate in the genomic DNA. Thus, the organisms living in such extreme conditions seem to evolve novel strategies for repairing DNA damage and avoiding mutations caused by the lesions. In this chapter, we review mechanisms of DNA repair in archaeal Bacteria and unique properties of archaeal DNA polymerases (Pols) to tolerate DNA damage. In general, archaeal DNA repair proteins are eukaryote-like although many counterparts are missing in the genome sequences. Archaeal B-family DNA Pols, such as *Sso* DNA Pol B1 or *Pfu*, halt DNA replication several basepairs before template uracil or hypoxanthine, deamination products of cytosine or adenine in template DNA, respectively, thereby apparently avoiding mutations (read-ahead mechanism). Fifteen out of 38 archaeal species whose genome sequences have been completely determined seem to possess Y-family DNA Pols, which are specialized to bypass lesions in DNA. Collectively, these molecular features warrant the future investigation on how Archaea accommodate DNA damage inevitably occurring in the extreme harsh environments.

Introduction

Archaea, the third domain of life, is thought to possess pathways involved in DNA replication, repair, recombination and transcription, similar to those of Eukarya, although its morphology is more prokaryotic-like (Olsen and Woese, 1996). Many eukaryotic repair proteins have close homologues in Archaea and thus archaeal proteins have been utilized as structural and biochemical models to reveal the mechanisms of action of the eukaryotic counterparts because of their structural stability and ease of purification. However, DNA-repair pathways in Archaea themselves are not yet well characterized, and several important counterparts are apparently missing in the Archaeal genome, suggesting that Archaea may possess unique mechanisms to protect the genome. This may not be surprising given the environmental challenges faced by hyperthermophilic Archaea living in habitats at extremely high temperatures. Chemical reactions such as hydrolytic deamination are greatly accelerated at high temperatures so that the hyperthermophilic organisms are supposed to be exposed to massive DNA damages. In addition, most of proteins and nucleic acids are denatured at high temperatures. Nevertheless, the mutation frequencies in hyperthermophilic Archaea, i.e. *Sulfolobus acidocaldarius*, are comparable with or lower than those of other microorganisms such as *Escherichia coli* (Grogan *et al.*, 2001). Thus, Archaea seem to have evolved unique and efficient mechanisms to protect their genome from harsh environmental conditions.

In addition to DNA repair, B-family DNA polymerases (DNA Pols) in the hyperthermophic Archaea have a unique property that they halt replication once they encounter uracil (U) or hypoxanthine (HX), which are

deamination products of cytosine and adenine, respectively, in the template genome (Fogg *et al.*, 2002; Gruz *et al.*, 2003). Because they stall several basepairs before the lesions, it is called 'read-ahead mechanism' (Greagg *et al.*, 1999). This is regarded as an error avoidance mechanism in hyperthermophilic Archaea because the stalling of DNA replication provides a chance to remove the deaminated bases from the template DNA strands. The molecular feature is unique in hyperthermophic Archaea since DNA Pols from thermophilic eubacteria such as *Taq* DNA Pol do not share similar properties. Besides B-family DNA Pols, which are responsible for chromosome replication, 15 out of 38 archaeal species whose genome sequences have been completed seem to possess Y-family DNA Pols (Ohmori *et al.*, 2001). The Y-family represents a novel family of DNA Pols found in Archaea, Bacteria and Eukarya, and the most remarkable feature is that they can bypass a variety of DNA lesions efficiently (Prakash *et al.*, 2005; Nohmi, 2006). Thus, they are involved in tolerance to DNA damage induced by exogenous and endogenous genotoxic agents. In this chapter, we review unique mechanisms of DNA repair and damage tolerance in Archaea and discuss the future directions.

DNA repair

DNA repair is the molecular mechanism that removes various damages in DNA, thereby ensuring the genome integrity. In fact, all organisms from virus to humans including Archaea are equipped with multiple DNA repair machineries to counteract the genotoxic damage. DNA lesions are induced by exposure to hazardous environmental factors, e.g. sunlight, and endogenous mutagens produced during oxygen consumption and nutritional metabolism, e.g. reactive oxygen species and alkylating agents. Deficit of DNA repair leads to hypersensitivity to toxic and mutagenic effects of genotoxic hazards as has been exemplified by the presence of human genetic diseases such as xeroderma pigmentosum (XP) (Tanaka *et al.*, 1990). The XP patients are sensitive to sun light-induced skin cancer because of the deficit of the ability to remove cyclobutane pyrimidine dimers (CPD) from DNA. In the following section, we view six most important pathways of DNA repair: alkylation repair; photorepair; nucleotide excision repair (NER); base excision repair (BER); mismatch repair (MMR) and homologous recombination (HR) in Archaea (Fig. 7.1, Table 7.1). Of these, alkylation repair and photorepair directly reverse the damages while NER, BER and MMR require multiple steps to remove the damaged or mismatched bases, followed by resynthesis of DNA to fill in the gaps. HR as well as translesion DNA synthesis (TLS), which will be described in detail below, does not remove DNA damage but rescues the stalled DNA replication to complete the duplication of chromosome DNA (damage avoidance). Most Archaea as well as Bacteria and Eukarya have multiple, functionally redundant pathways to combat a variety of damages in DNA induced by environmental stress.

Repair of alkylation damage

DNA repair protein O^6-alkylguanine-DNA alkyltransferase (AGT) is considered to play central roles in the mechanism of cellular resistance to the toxic and mutagenic effects of DNA damage induced by monofunctional alkylating agents (Sedgwick, 2004; Lindahl *et al.*, 1988). AGT operates by the transfer of the offending alkyl groups from the O^6 position of guanine and the O^4 position of thymine in DNA to a cysteine residue at the active site of the protein. This is an irreversible process that results in the stoichiometric inactivation of the protein, the repair of other lesions requiring additional active molecules. The alkylation damage to DNA occurs under a variety of living conditions because alkylating agents including *N*-nitroso compounds are present in the environment and can be generated even endogenously (Taverna and Sedgwick, 1996). The AGT proteins share a highly conserved -PCHRV- amino acid sequence in their active site (Margison *et al.*, 2003). The presence of an AGT gene product or a putative gene has been reported so far in c.a. 100 different species from the three domains of life.

It is shown that extracts of two Crenarchaeota, i.e. *Sulfolobus acidocaldarius* and *Pyrobaculum islandicum*, and two Euryarchaota, i.e. *Pyrococcus furiosus* and *Thermococcus litoralis*, contain the AGT activities (Skorvaga *et al.*,

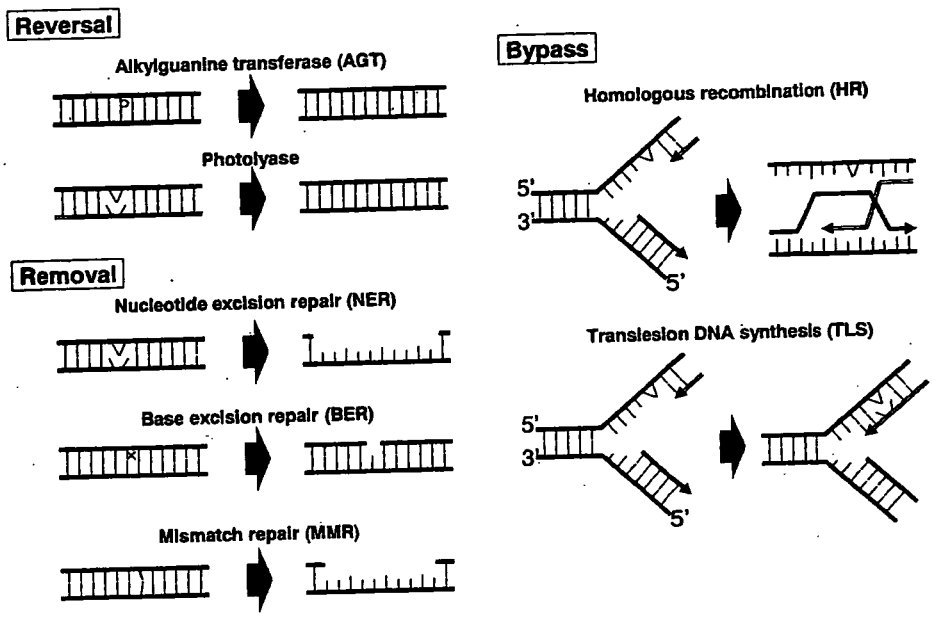

Figure 7.1 Schematic representation of mechanisms of DNA repair. Alkylguanine transferase (AGT) and photolyase directly reverse the damaged bases to correct ones. Nucleotide excision repair (NER), DNA glycosylase involved in base excision repair (BER) and mismatch repair (MMR) remove damaged bases or DNA sequeces containing damaged bases, followed by DNA re-synthesis to fill in the gaps. Homologous recombination (HR) and TLS do not remove damaged bases but assist to resume stalled DNA replication at the damaged sites.

1998). The enzymes seem to be similar to a human AGT because they lack the alkylphosphotriester-DNA alkyltransferase activity seen in some prokaryotes. More over, *P. islandicum* and *Pyrococcus furiosus* have maximal AGT activity at approximately 100°C. These enzymes would thus appear to be able to act on ssDNA (Skorvaga *et al.*, 1998).

AGT in other Euryarchaeota were cloned from *Pyrococcus* sp. KOD1(Leclere *et al.*, 1998), *Archaeoglobus fulgidus* (*Af*-AGT) (Kanugula and Pegg, 2003) and *Ferroplasma acidarmanus* (*Fa*-AGT) (Kanugula *et al.*, 2005). The AGT from KOD1 displays the activity at 90°C for at least 30 min. The enzyme exhibits considerable similarity to the corresponding mammalian, yeast and bacterial enzymes. *Af*-AGT is active in repairing O^6-methylguanine and, at a lower rate, O^4-methylthymine in DNA, and is readily inactivated by the low-molecular-mass inhibitor O^6-benzylguanine. *Fa*-AGT consists of a fusion of the C-terminal active site domain of AGT with an endonuclease V (Endo V) domain. The purified protein repairs O^6-meG in DNA and also cleaves the deaminated bases, i.e. U, HX or xanthine, in a similar manner to *E. coli* Endo V. In this case, two distinct but functional DNA repair proteins co-exist in a single polypeptide chain.

Photoreactivation

Photoreactivation is an efficient and direct repair mechanism for CPDs, which are the major DNA photoproducts induced by the UV component of solar radiation. This mechanism was discovered by the finding that survival of Bacteria irradiated with UV increased after exposure to visible light (Kelner, 1949). The DNA repair process is catalysed by a single enzyme, i.e. photolyase, which monomerizes UV-induced dimers by transfer of blue light energy absorbed by the chromophores (Sancar, 1990; Sancar, 1994). Most photolyases repair CPDs but some repair 6–4 photoproducts (6–4 PPs). Photoreactivation is widely distributed among species, ranging from Bacteria to plants and mammals. The ubiquity and efficient use of visible light emitted by the sun, the same source that induces DNA lesions, indicate that photolyase is an ancient protein that may have played an important role in evolution.

Table 7.1 DNA repair pathways in Archaea

	AGT	PHT	NER	UDG	MIG	AP	OGG	FEN-1	MutS	HR
Crenarchaeota										
Aeropyrum pernix	++	++	+++	++	+++	++	++	+++	++	++
Pyrobaculum aerophilum	++	++	++	+++	+++	++	++	++		++
Pyrobaculum islandicum	+++	++	+	+++		++				+++
Sulfolobus acidocaldarius	+++	+++	++	+++		++	++	++		++
Sulfolobus solfataricus	++	+++	+++	+++		+++	++	++		++
Sulfolobus shibatae				+++						++
Euryarchaeota										
Archaoglobus fulgidus	+++	++	+++	+++		++	+++	+++		++
Ferroplasma acidarmanus	+++		+	+		++	++		++	+
Halobacterium halobium		+++	++	++	++	+++	++	++	++	++
Haloferax		+	++							+++
Methanococcus jannaschii	++		++	+++		++	+++	+++		++
Methanothermobacter thermautotrophicus	++	+++	+++		+++	+++	++	++		++
Pyrococcus furiosus	+++	++	++	+++	+	++	++	+++	++	+++
Pyrocuccus horikoshii	++	++	++	++		++	++	+++	++	++
Pyrocuccus sp. KOD1	+++	++	++	++		++	++	++	++	++
Thermococcus litoralis	+++			+++						

The abbreviations are as follows: AGT – O^6-MeG methyltransferase, PHT – photolyase, NER – nucleotide excision repair (XPF, XPB, XPD), UDG – uracil DNA glycosylase, MIG – mismatch glycosylase, AP – AP endonuclease, OGG – 8-oxoG glycosylase, FEN-1 – flap endonuclease, MutS – mutS, HR – recA. The enzymes characterized biochemically are indicated as +++, enzymes with sequence homology as described in the NCBI GeneBank protein sequence database of March, 2007 are indicated as ++ while proteins with only limited homology are labelled as +.

Among Archaea, photolyases are not common, although the photoreactivation or photolyases are identified in several archaeal species. These include Crenarchaeota, i.e. *Sulfolobus acidocaldarius* (Wood *et al.*, 1997) and *Sulfolobus tokodaii* (Fujihashi *et al.*, 2007), and Euryarchaeota, i.e. *Halobacterium* sp. NRC-1 (McCready and Marcello, 2003) and *Methanothermobacter thermoautotrophicus* (Kiener *et al.*, 1989; Yasui *et al.*, 1994). In addition, several hypothetical genes encoding photolyases are identified in *Haloarcula marismortui* (Baliga *et al.*, 2004c), *Methanopyrus kandleri* (Slesarev *et al.*, 2002) and *Methanosarcina acetivorans* (Galagan *et al.*, 2002). Sso7d and Sac7d are two small (~7 kDa), but abundant, nucleoproteins in *Sulfolobus solfataricus* and *S. acidocaldarius*, respectively (Gao *et al.*, 1998; Robinson *et al.*, 1998). Recently it was demonstrated that Sso7d efficiently repairs the thymine dimer under irradiation conditions, suggesting that Sso7d and Sac7d may be involved in the photoreactivation in the organisms (Tashiro *et al.*, 2006). Two photolyase-like genes, *phr1* and *phr2*, are identified in *Halobacterium NRC-1*, and the *phr2* gene is shown to encode a CPD photolyase (McCready and Marcello, 2003). The photolyase from the *Methanothermobacter thermoautotrophicum* exhibits 37% homology to those of higher eukaryotes and only 15% to microbial photolyases(Yasui *et al.*, 1994)

Nucleotide excision repair

NER is a highly conserved DNA repair pathway able to detect and remove a variety of bulky DNA lesions caused by UV light and environmental mutagens and thereby contributes to the genomic integrity of an organism (Sancar, 1996; Lindahl and Wood, 1999). NER is uni-

versal in Bacteria and Eukarya although other mechanisms, i.e. photoreactivation enzymes (see Photoreactivation), DNA glycosylase (see Base excision repair) and endonuclease known as UVDE (Yasui and McCready, 1998), also operate to counteract UV-induced DNA damage in a number of organisms. NER in higher eukaryotes involves the coordinated assembly of a large number of proteins, including the core NER factors XPC-HR23B, TFIIH, XPA, replication protein A (RPA) and two endonucleases XPG and ERCC1-XPF (Aboussekhra *et al.*, 1995). These proteins cooperate to recognize, unravel and excise a 24–32-mer oligonucleotide bearing the DNA lesion prior to filling in the missing gap (Araujo and Wood, 1999). Although Bacteria have a similar overall repair strategy, a much simpler and structurally unrelated multiprotein complex known as UvrABC carries out the same task (Petit and Sancar, 1999). The availability of fully sequenced archaeal genomes has revealed that many Archaea have proteins related to eukaryotic NER factors rather than to the UvrABC bacterial repair system (Aravind *et al.*, 1999; White, 2003; Grogan, 2000). Only certain Euryarchaeota, i.e. *Halobacterium sp.* NRC-1 (Ng *et al.*, 2000), *Methanothermobacter thermoautotrophicus* (Ogrunc *et al.*, 1998), *Haloarcula marismortui* (Baliga *et al.*, 2004a), *Methanosarcina acetivorans* (Galagan *et al.*, 2002) and *Methanosarcina mazei* (Deppenmeier *et al.*, 2002), have Bacteria-type repair proteins, although all of them have eukaryotic-type NER proteins too. It is interesting that Archaea lack damage recognition proteins such as XPA and XPC, which are essential for eukaryotic NER, while they have other eukaryotic-type components, such as XPF/ERCC1, Flap endonuclease, and XPB and XPD helicases (White, 2003). It is worth noting that Crenarchaea have a 'short' form of XPF that lacks the helicase-like domain while Euryarchaeota have a long form of XPF, which includes a helicase-like domain at the N-terminal (Roberts *et al.*, 2003).

In a few species, actual NER activities have been demonstrated. In Crenarchaeota, UV-induced CPDs are efficiently repaired in vivo in the dark in *Sulfolobus solfataricus* (Salerno *et al.*, 2003). In Euryarchaeota, *Methanobacterium thermoautotrophicum* is shown to remove UV-induced 6–4 PPs in the form of 10- to 11-mers by incising 3′- and 5′-sides of the DNA damage (Ogrunc *et al.*, 1998). In addition two structural analyses provide more precise information to this field; XPF homologue in *Aeropyrum pernix* (Newman *et al.*, 2005), and XPB homologue in *Archaeoglobus fulgidus* (AfXPB) (Fan *et al.*, 2006). Interestingly, the DNA damage recognition domain and the flexible thumb motif of AfXPB appear structurally as well as functionally analogous to the MutS mismatch recognition and DNA polymerase thumb domains.

Base excision repair

BER pathway involves the initial recognition of specific types of base damage and/or damage associated basepairing mismatches by a BER DNA glycosylase. An estimated rate of 10^4 damaging events/mammalian cell/day underscores the importance of the BER pathway (Nakamura *et al.*, 1998; Lindahl and Wood, 1999; Lindahl, 1993). After damage recognition, the glycosylase cleaves the damaged or misincorporated base from the DNA backbone, leaving an apurinic/apyrimidinic (AP) site (Lu *et al.*, 2001). These AP sites are cleaved by the major 5′ AP endonuclease, APE1, to leave a 5′ dRp terminus that is then removed by DNA Pol β producing a single nucleotide gap. DNA glycosylases/AP lyases or bifunctional glycosylases, such as endonuclease III (NTH1) and 8-oxoguanine DNA glycosylase (OGG1), besides base excision, cleave DNA at the resultant AP site by β elimination generating a 3′ terminal sugar phosphate that is then removed by APE1 yielding a gap with a 3′ OH (Demple and Harrison, 1994). Ligation will continue either via short-patch (SP) (for one nucleotide) (Dianov *et al.*, 1992) or long-patch (LP) (two or more nucleotides) repair (Frosina *et al.*, 1996; Klungland and Lindahl, 1997). Pol β is the repair-synthesis polymerase of the SP-repair. Pol β is also likely to incorporate the first nucleotide in the LP-repair (Podlutsky *et al.*, 2001) but the elongation step in this pathway is carried out by replicative DNA Pols. Proliferating cell nuclear antigen (PCNA), the sliding clamp for Pol β and flap endonuclease-1 (FEN-1), a structure specific nuclease that excises the displaced oligonucleotide (Pascucci *et al.*, 1999). The ligation step is then operated by the X-ray repair cross

complementing 1 (XRCC1)/DNA ligase IIIα complex in the SP-repair. The XRCC1/DNA ligase IIIα complex is dispensable for LP-repair implying that in this pathway the sealing step is performed by DNA ligase I (Cappelli *et al.*, 1997; Sleeth *et al.*, 2004).

Of various BER glycosylases, uracil DNA glycosylase (UDG) have been studied most extensively (Lindahl and Nyberg, 1974; Percival *et al.*, 1989). They are extremely efficient enzymes, which recognize U in ss DNA, in A/U pairs that arise when dUMP is incorporated opposite A during DNA replication, or in G/U mispairs arising through cytosine deamination. Thermostable UDG activities have been identified in Crenarchaeota, i.e. *Sulfolobus solfataricus, S. shibatae, S. acidocaldarius, Thermococcus litoralis, Pyrobaculum islandicum* and P. aerophilum, and Euryarcheota, i.e. *Pyrococcus furiosus, Methanococcus jannaschii* and *Archaeoglobus fulgidus* (Sandigursky and Franklin, 2000; Koulis *et al.*, 1996; Hinks *et al.*, 2002; Chung *et al.*, 2003b). In addition, novel types of UDGs have been identifed in hyperthermophilic Archaea (Pearl, 2000; Sartori *et al.*, 2002; Chung *et al.*, 2003b). One such example is *Pa*-UDGb in *Pyrobaculum aerophilum*, which lacks the polar amino acid at the active site (motif A) and removes HX as well as U in DNA. This enzyme is a functional component of in vitro BER system that consists of *Pa*-UDGb, AP endonuclease IV, DNA Pol B2 and DNA ligase (Sartori and Jiricny, 2003). Besides *Pa*-UDGb, *P. aerophilum* possesses two other UDGs, i.e. *Pa*-UDGa and *Pa*-MIG (mismatch specific DNA glycosylase). *Pa*-UDGa has glutamate at the active site and is more active than *Pa*-UDGb. *Pa*-UDGa appears to be the major UDG in this organism and *Pa*-UDGb is probably responsible for the removal of HX or hydroxymethylU in DNA. *Pa*-MIG can remove T as well as U paired with G so that it may contribute to mismatch correction (Sartori *et al.*, 2002; Yang *et al.*, 2000). MIG activites are also identified in *Aeropyrum pernix* and *Methanobacterium thermoautotrophicum* (Yang *et al.*, 2000; Horst and Fritz, 1996; Fondufe-Mittendorf *et al.*, 2002).

Hypothetical counterparts of OGG1, which removes 8-oxoguanine (8-oxo-G) in DNA, have been identified in the genome of Crenarchaeota, i.e. *Sulfolobus solfataricus* (She *et al.*, 2001) and Euryarchaeota, i.e. *Methanococcus jannaschii* (Gogos and Clarke, 1999), *Archaeoglobus fulgidus* (Chung *et al.*, 2001), *Thermoplasma acidophilum* (Ruepp *et al.*, 2000) and *Thermoplasma volcanicum* (Kawashima *et al.*, 2000). Among them, enzymatic activities against 8-oxo-G in DNA have been demonstrated in *M. jannaschii* and *A. fulgidus* (Gogos and Clarke, 1999; Chung *et al.*, 2001).

FEN-1 has important roles in DNA repair as well as DNA replication and recombination. The enzyme cleaves 5′ flap structure endonucleolytically and has double-strand-specific 5′–3′-exonuclease activity. When working in BER, FEN-1 removes damaged nucleotides after AP endonuclease has incised the 5′ side of the AP site in LP-repair (Klungland and Lindahl, 1997; Kim *et al.*, 1998; Gary *et al.*, 1999). It recognizes a specific DNA structure, independent of the DNA sequence. FEN-1 seems highly conserved in Archaea and Eukaryotes (Matsui *et al.*, 1999; Hosfield *et al.*, 1998; Kaiser *et al.*, 1999; Rao *et al.*, 1998) and the crystal structures have been solved in Euryarchaeota, i.e. *Methanococcus janaschii* (Hwang *et al.*, 1998; Sayers and Artymiuk, 1998; Bae *et al.*, 1999), *Pyrococcus furiosus* (Hosfield *et al.*, 1998), *Archaeoglobus fulgidus* (Chapados *et al.*, 2004) and *Pyrococcus horikoshii* (Matsui *et al.*, 2002) and in Crenarchaeota, i.e. *Aeropyrum pernix* (Collins *et al.*, 2004).

Mismatch repair

The ability to recognize and repair mismatches in DNA after replication has been well documented in many species (Modrich and Lahue, 1996). The most extensively studied general MMR system is the one in the bacterium *E. coli*. It functions with the MutSLH, UvrD, and RecJ proteins through a d(GATC)-specific methylase (dam) methylation-directed pathway and is initiated by the mismatch-stimulated d(GATC) endonuclease activity of MutH, which takes advantage of transient undermethylation of the DNA during replication, and specifically nicks the newly synthesized, that is, unmethylated daughter strand. Subsequently, the RecJ exonuclease selectively degrades the nicked strand, and the mutations are corrected by the action of a DNA Pol, a helicase, and a ligase (Marti *et al.*, 2002).

Hypothetical MutS-like proteins were found in several species of Euryarchaeota, but not in ones of Crenarchaeota, such as *Sulfolobus solfataricus* (She *et al.*, 2001). The MutS-like proteins that have ATPase domain may be divided into two subfamilies, i.e. MutS-I lineage related to MMR and MutS-II lineage related to chromosome aberrations and meiotic crossing-over (Eisen, 1998). The MutS-I lineage includes bacterial MutS and eukaryotic MSH2/6, and the MutS-II includes eukaryotic MSH4 and MSH5. Phylogenetic analysis also shows that the proteins in the MutS-II subfamily are distant and distinct from those involved in MMR (Kawarabayasi *et al.*, 1998; Smith *et al.*, 1997; Robb *et al.*, 2001; Cohen *et al.*, 2003). *Halobacterium* NRC-1 and *Methanosarcina activorans* have both types of MutS homologues as well as a MutL-like protein (Ng *et al.*, 2000).

Unlike MutS and MutL, which are absolutely necessary for MMR, and therefore evolutionarily highly conserved, MutH is significantly less conserved. In fact, no MutH homologue has been identified in either the Eukaryotes or the Archaea. Since it has been revealed that Eukaryotic MMR protein complex, MutLα, is an endonuclease (Kadyrov *et al.*, 2006), it is not surprising that Eukaryote does not possess MutH endonuclease. Although *Halobacterium* NRC-1 does not have MutH endonuclease or a *dam* methylase, it has d(CTAG) methylase (Zim), MutS/L, four RecJ-like exonucleases and the UvrD repair helicase (Ng *et al.*, 2000). However, a d(CTAG)-specific endonuclease was not detected using either sequence- or structure-based annotation methods (Baliga *et al.*, 2004b). *P. furiosus* has detectable enzymatic activities of MutS2 protein, an ATPase activity and a non-mismatch specific DNA-binding activity (Vijayvargia and Biswas, 2002).

Spontaneous mutation frequency in *P. aerophilum* is high, especially in mononucleotide repeats (Fitz-Gibbon *et al.*, 2002). Although this is consistent with the absence of orthologues of MMR proteins, *P. aerophilum* may possess a distinct MMR system. Taken together, it has been, so far, unclear how mismatches generated during DNA replication are corrected in Archaea. It is possible that a novel system that manages mismatch errors operates in Archaea such as *S. solfataricus*, where no MutS/L homologues are identified.

Recombinational repair

DNA double-strand breaks (DSBs) are highly cytotoxic and mutagenic, thus the repair of DSBs in DNA is an essential process in all organisms. DSBs are predominantly repaired by two pathways, i.e. non-homologous end joining and HR. HR conserves genetic identity by using an undamaged chromosome as a template to replace missing or damaged nucleotides. It also serves to underpin genome replication by providing the means to resume replication when the replication machinery is blocked (Seigneur *et al.*, 1998; McGlynn and Lloyd, 2000). Mre11/Rad50 complex plays a key role in DSB repair. The complex participates in the generation of the 3′ ssDNA tails possibly by bridging DNA ends or sister chromatids in the very early steps of HR. RecA family of recombinases entails the reciprocal exchange of the single strands between homologous DNA duplexes by utilizing conserved DNA-binding modules and a common core ATPase domain to form a four-way branched intermediate commonly referred to as the Holliday junction (HJ). In the late step of HR, the HJ is resolved by dual-strand incision across the branch point to release nicked duplexes, then whole procedure completes with sealing by DNA ligase. Enzymes of HR have been unearthed in all three domains of life, i.e. Bacteria, Archaea and Eukarya.

Hyperthermophilic Archaea are reported to be more resistant to ionizing radiation than hyperthermophilic Bacteria or *E. coli* (DiRuggiero *et al.*, 1997). A γ-ray dose allowing 75% survival of *Pyrococcus furiosus* was shown initially to fragment the chromosome into the lengths of about 30 kb, yet full-length chromosomes eventually reappeared *in vivo* after prolonged incubation at 95°C. There is evidence that RadA, the archaeal RecA protein, is induced by DNA damage in thermophilic and mesophilic Archaea (Reich *et al.*, 2001). Although the results imply that effective DSB repair mechanisms, which could be HR, exist in Archaea, very little is known about the enzymes for homologous exchange in Archaea, with the exception of RecA-like strand exchange enzymes (Seitz *et al.*, 1998) and en-

zymes that cleave HJs *in vitro* found in *P. furiosus* (Komori *et al.*, 1999), or *Sulfolobus solfataricus* (Kvaratskhelia and White, 2000a).

Structural analyses of a Rad50 homologue from *P. furiosus* illuminate the catalytic core of the enzyme, an ATP-binding domain related to the ABC transporter family of ATPases (Hopfner *et al.*, 2000; Hopfner *et al.*, 2001). Its S793R mutation prevents ATP binding and dimerization, and disrupts the communication among the other ATP-binding loops (Moncalian *et al.*, 2004). Crystal structure of Mre11 and Rad50 of *P. furiosus* reveal a dual functional complex consisting of $(Mre11)_2/(Rad50)_2$ heterotetrameric DNA (Hopfner *et al.*, 2001). Putative *recA* and *RAD51* homologues in three archaeans were identified by PCR and referred to as *radA* (Sandler *et al.*, 1996). The same research group amplified fragments of *radA* genes from 11 archaeal species and analysed the phylogenetic relationships (Sandler *et al.*, 1999). RadA proteins that are functional *in vivo* are found in Euryarchaeota, i.e. *Haloferax volcanii* (Woods and Dyall-Smith, 1997) and *P. furiosus* (Komori *et al.*, 2000b; Komori *et al.*, 2000a; Hayashi *et al.*, 1999), and Crenarchaeota, i.e. *Pyrobaculum islandicum* (Spies *et al.*, 2000). Crystal structure is presented for a RadA from *Methanococcus voltae* (Qian *et al.*, 2005). These enzymes share a higher level of sequence and more structural similarity with eukaryotic Rad51 than bacterial RecA (Uemori *et al.*, 1997; DiRuggiero *et al.*, 1997). RadB was also identified as a Rad51-like protein on the basis of conserved ATPase sequences (Rashid *et al.*, 1996). However, RadB from *P. furiosus* is not a recombinase. It does not catalyse strand exchange and does not turn over ATP efficiently (Komori *et al.*, 2000b). Despite the lack of ATPase activities, an ATP-binding activity seems required for RadB to promote survival of *Haloferax volcanii* after UV irradiation. ATP induces pronounced conformational change in RadB (Akiba *et al.*, 2005), which seems to be important for maintaining genome stability in Archaea. RPA is found in *P. furiosus* and is composed of three subunits, i.e. RPA41, RPA14, and RPA32, like the eukaryotic RPA (p70-p14-p32). RPA clearly stimulates a RadA-mediated strand exchange reaction (Komori and Ishino, 2001).

Regarding the late stage, in which the HJ intermediates are processed, the first archaeal HJ resolvase (Hjc, HJ cleavage) is discovered in *P. furiosus* (Komori *et al.*, 1999). Similar resolvases have been purified from *S. solfataricus* (Kvaratskhelia and White, 2000b) and *Methanobacterium thermoautotrophicum* (Bolt *et al.*, 2001). Although Hjc does not resemble any of the known HJ resolvases, it does share significant similarity to restriction endonucleases and exonuclease (Aravind *et al.*, 2000; Kvaratskhelia *et al.*, 2000). Thus, Hjc is an archaeal-specific HJ resolvase. Neither its sequence nor its three-dimensional structure is similar to other known HJ resolvases (Nishino *et al.*, 2001; Komori *et al.*, 2000c). HJ resolvases with distinct properties have been characterized from bacteriophages (T4 endo VII, T7 endo I, RusA and Rap), Bacteria (RuvC), Archaea (Hjc and Hje), yeast (CCE1) and poxviruses (A22R). Thus, the HJ resolvase is quite interesting, from an evolutional point of view (Lilley and White, 2000). The Hjc enzymes target junctions with high specificity but, unlike RuvC and RuvA, they show no sequence-preference for strand cleavage. Hjc from *P. furiosus* has been found to interact with the archaeal equivalent of PCNA and with RadB (Komori *et al.*, 1999; Komori *et al.*, 2000c). The Hje (HJ endonuclease) protein has similar properties to Hjc, including a preference for four-way junction DNA and the ability to resolve both static and mobile HJs (Kvaratskhelia and White, 2000a). Its crystal structure serves more precise information for understanding its function (Nishino *et al.*, 2005). The gene, *hjm* (HJ migration), encodes a protein composed of 720 amino acids. A homology search revealed that Hjm shares sequence similarity with the human PolQ, HEL308, and *Drosophila* Mus308 proteins, which are involved in a DNA repair, whereas no similar sequences were found in Bacteria and yeast (Fujikane *et al.*, 2005).

DNA damage tolerance by archaeal DNA polymerases

Life represents a succession of biological information stored in DNA from ancestors to descendants. Thriving in harsh habitats such as high temperatures, therefore, requires organisms to evolve unique mechanisms to maintain the

genome integrity. Temperatures more than 80°C where thermophilic Bacteria optimally grow pose challenges to the genome to be denatured or decomposed. Although denaturation may not be a serious problem for DNA as long as it is covalently closed, heat-induced depurination, oxidation and deamination could have deleterious effects. Depurination leads to AP site, a major cytotoxic lesion, and oxidation generates a variety of DNA lesions such as 8-oxo-G in DNA (Lindahl, 1993). Hydrolytic deamination of exocyclic amino groups of cytosine leads to the formation of U in DNA, and G:U basepairs result in G:C to A:T transitions in a half of the progeny if not repaired (Lindahl and Nyberg, 1974; Pearl, 2000). Although most of the lesions can be removed by DNA repair mechanisms as has been reviewed above, some escape from the repair mechanisms and persist in the template DNA. These residual lesions can block DNA replication and induce mutations or chromosome breaks. To counteract the lesions, cells evolved the mechanisms called DNA damage tolerance to accomplish the chromosome replication. Unlike DNA repair, the damage tolerance mechanism does not remove DNA damage but instead allows DNA Pols proceed beyond the damage and permits the accomplishment of chromosome replication. The mechanisms may also include the behaviour of DNA Pols that stop replication when they encounter spontaneous DNA lesions, thereby giving a chance to cells to remove the lesions before Pols incorporate incorrect dNMPs opposite the lesions. Although DNA damage tolerance mechanisms may include recombination repair in theory, we focus on the mechanisms associated with DNA Pols of Archaea, i.e. read-ahead mechanisms and translesion bypass replication (Lasken *et al.*, 1996; Nohmi, 2006; Fogg *et al.*, 2002). The physiological significance of damage tolerance mechanisms in Archaea is still open to question, but they may be critical to survive in harsh environments and maintain the genome stability. Since Pols involved in damage tolerance are also involved in induction of mutations by incorporation of incorrect dNMP opposite template lesions, they could play a role in evolution or genetic flexibility of archaeal Bacteria living in variable harsh environments. In the following sections, we will see how B-family and Y-family DNA Pols contribute to DNA damage tolerance in Archaea.

Read-ahead mechanism

Deamination of bases in DNA such as cytosine may be the most common genotoxic threat to all living organisms. Deamination of cytosine to U leads to C to T transitions unless repaired and disrupts the succession of genetic information stored in the genome. To counteract the mutagenic potential of U in DNA, Eukarya, Bacteria and plants have a BER pathway (see above) utilizing a UDG or a G:U/T mismatch specific DNA glycosylase. In addition, cells in Bacteria, Eukarya and Archaea possess dUTPase, which converts dUTP to dUMP and pyrophosphate (PPi), to minimize the levels of dUTP in the nucleotide pool (Hogrefe *et al.*, 2002). dUTP is a major source of U in DNA because of their ease of incorporation into DNA by Pols. In fact, it is estimated that 10^4 dUTP is incorporated per DNA replication based on a genome size of 10^{10} basepairs (Savva *et al.*, 1995). In Bacteria and yeast, cells lacking dUTPase exhibit DNA fragmentation and cell death because of the excess excision of U in DNA by the action of UDGs and the following steps of BER (Gadsden *et al.*, 1993). Despite the presence of UDG and dUTPase, elevated temperatures should pose an increase risk of DNA damage in hyperthermophilic Archaea because hydrolytic deamination of U greatly accelerated at high temperatures. However, the spontaneous mutation rate in hyperthermophilic Archaea *Sulfolobus acidocardarium* is reported to be similar to that of *Escherichia coli* (Grogan *et al.*, 2001). Thus, hyperpethermophilic Archaea appear to possess additional mechanisms to protect a stability of the genome from the mutagenic threat of deaminated bases generated at high temperatures.

Interestingly, B-family DNA Pols from hyperthomophilic Archaea such as *Sulfolobus solfataricus* DNA Pol B1 (Pol B1), *Pyrococcus furiosus* DNA Pol (Pfu) and *Thermococcus litoralis* DNA Pol (Vent and Vent exo-), recognize the presence of U in DNA and tightly bind to U-containing oligonucleotides (Lasken *et al.*, 1996). These Pols stall DNA replication several basepairs before template U and thus the recognition and stalling mechanisms seem to contribute to counteract the

mutagenic potential of U in DNA because the stalling mechanisms may reduce the chance of misincorporation of adenine opposite template U (Greagg *et al.*, 1999). More interestingly, both Pol B1 and Pfu exo- bind not only to template U but also to template HX, a deamination product of adenine (Gruz *et al.*, 2003). Pfu exo- stalls three to four basepairs before HX as well as U in the template strand, and Pol B1 displays a similar stalling pattern. HX pairs with cytosine during DNA replication and induces A to G transitions if not corrected (Lindahl, 1993). Thus, the stalling behaviour seems to suppress the incorporation of cytosine opposite template HX, thereby reducing the mutagenic potential of HX.

The recognition and stalling mechanism seems unique to archaeal B family DNA Pols because viral B family Pols such as T4 Pol or Pols from hyperthermophilic eubacteria such as *Thermus aquaticus* (Taq) do not stall when the template DNA has U or HX (Greagg *et al.*, 1999). Eukaryotic B-family Pol, i.e. human Pol alpha, does not tightly bind to a U-containing oligonucleotide (Lasken *et al.*, 1996). Several lines of evidence suggest that single molecules of the archaeal Pols scan the template DNA and stall when they encounter U several basepairs ahead of the progress (Asami *et al.*, 2006; Shuttleworth *et al.*, 2004). Thus, this is called 'read-ahead mechanism'. Structural analysis of archaeal B-family Pols suggest that they comprise five domains, i.e. the N-terminal domain, the exonuclease domain and three polymerase active site domains, and also that a pocket in the N-terminal domain is responsible for the discrimination of U from normal DNA bases (Fogg *et al.*, 2002). The pocket seems highly conserved in the N-terminal domains of 15 archaeal B-family Pols including seven euryarchaeal and eight crenarchaeal Pols. It remains to be seen how this pocket accommodates not only U but also HX in template DNA (Savino *et al.*, 2004).

Since archaeal B-family DNA Pols such as *Pfu* or *Vent* are frequently used as PCR enzymes, the inhibitory effects of U are practical problems to achieve long and efficient PCR amplification of DNA. To circumvent the problems, dUTPase is included in the reaction mixture to reduce the levels of dUTP, which are generated during PCR through deamination of dCTP and limit the efficiency of PCR achieved by archaeal B-family DNA Pols (Hogrefe *et al.*, 2002). Pfu dUTPase, which converts dUTP to dUMP and PPi, improves the yield of products amplified by *Pfu* DNA Pol by preventing dUTP incorporation and subsequent inhibition of the Pol by U-containing DNA. It is suggested that archaeal dUTPase may play an important role in preventing dUTP incorporation and inhibition of DNA synthesis by B-family DNA Pols in vivo.

Although it seems plausible that the read-ahead mechanism contributes to the genome stability in hyperthermophilic Archaea, it is an open question of what will happen after the Pols bind to the template U or HX (Fig. 7.2). A hint is that the archaeal Pol binds to UDG (Connolly *et al.*, 2003). It is tempting to speculate, therefore, that HR occurs after the stalling and double-strand DNA is formed where one strand possesses U and the other possesses guanine. UDG excises U from one strand and the resulting single base gap is filled by Pol using a guanine base in the opposite strand as a template. If these events actually occurred, the correct base, i.e. cytosine, would be restored in the original site. Another hint is the interaction between Pol B1 from *S. solfataricus* and Pol Y1 (Dpo4) from the same species (De Felice *et al.*, 2007). Pol Y1 bypasses a variety of DNA lesions including U (Gruz *et al.*, 2001; Boudsocq *et al.*, 2004). It does not stall even when it encounters U or HX during in vitro DNA synthesis (Gruz *et al.*, 2003). Thus, translesion bypass across U or HX by Y-family Pols may occur to continue the DNA replication after removal of stalling Pols at the lesions. The deficit of the bypass pathway is that the translesion reactions induce mutations of C to T as an expense of continuation of chromosome replication. PCNA may be involved in these events because it is a molecular scaffold to recruit several proteins involving in the processing of DNA damage (Gruz *et al.*, 2001; Dionne *et al.*, 2003). In either case, further work is needed to clearly establish the molecular mechanisms after stalling Pols at the lesions in hyperthermophilic Archaea.

DNA lesion tolerance

The need of high fidelity in chromosomal replication imposes significant constrains on the