substitutions substantially (Fig. 3B). Introduction of plasmid pYG787 carrying *polB* had almost no effects on the mutability. Unlike ENNG-induced mutagenesis, −2 frameshift, −1 frameshift and base substitutions induced by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) were not enhanced by the introduction of either plasmid pYG768 or pKM101 (data not shown). These results suggest that both DNA polymerase IV and DNA polymerase RI bypass ethyl, but not methyl, adducts in DNA leading to −2 and −1 frameshifts and base substitutions.

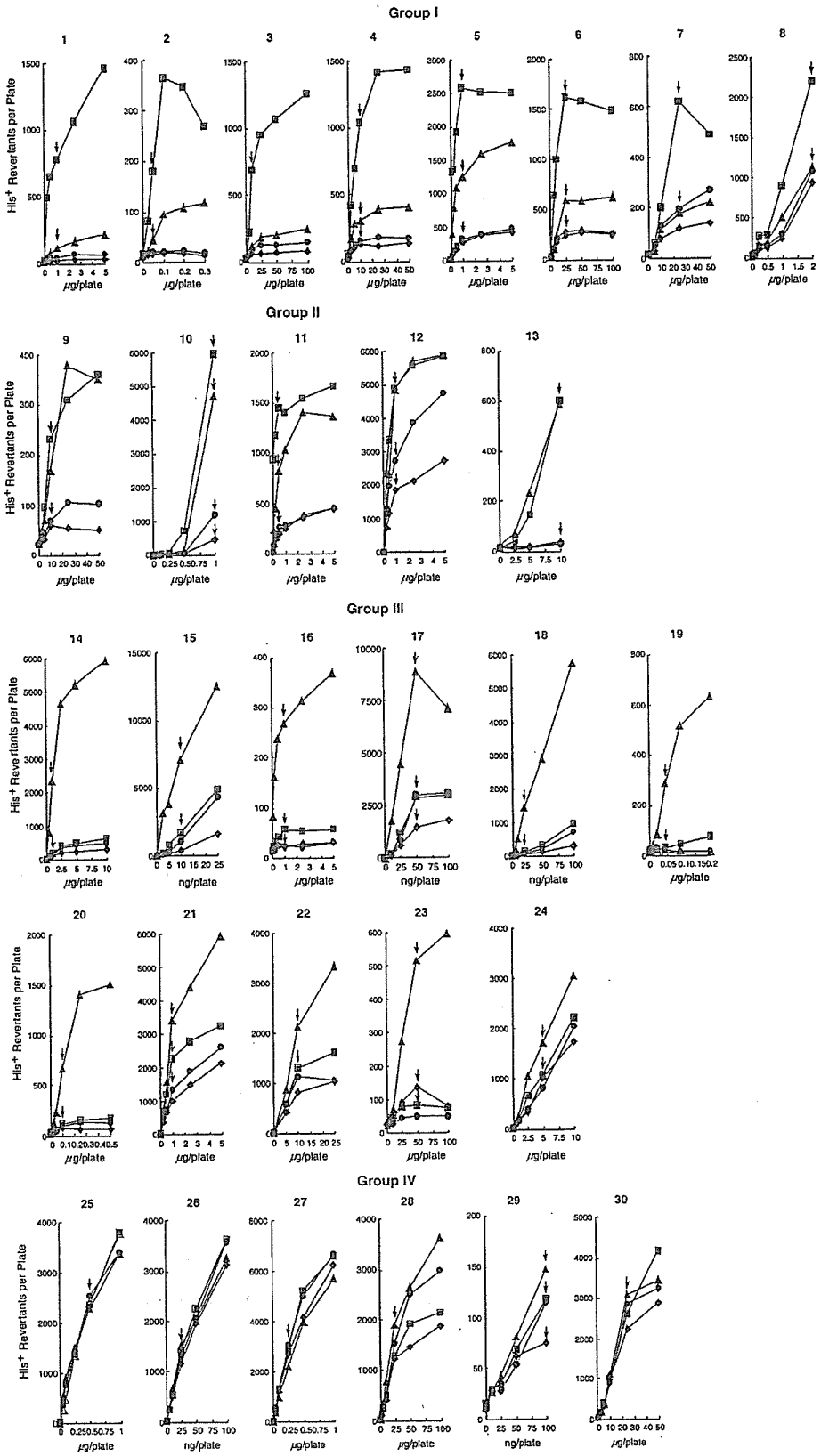

Table 3 – Mutagenicity of 30 chemicals in *S. typhimurium* strains harboring plasmids carrying genes encoding *E. coli* SOS DNA polymerases

Number	Chemical	Group	S9	TA1538 no plasmid	TA98 pKM101 (DNA pol RI)	YG5160 pYG787 (DNA pol II)	YG5161 pYG768 (DNA pol IV)
1	Benzo[a]pyrene-7,8-dihydroepoxide	I	–	44 (1.0)	113 (2.6)	22 (0.5)	776 (17.6)
2	Benzo[a]pyrene diol epoxide	I	–	420 (1.0)	920 (2.2)	320 (0.8)	3620 (8.6)
3	10-Azabenz[a]pyrene	I	+	9 (1.0)	12 (1.3)	6 (0.7)	68 (7.6)
4	Benzo[a]pyrene	I	+	15 (1.0)	30 (2.0)	13 (0.9)	104 (6.9)
5	3-Nitro-benzo[a]pyrene	I	–	316 (1.0)	1244 (3.9)	277 (0.9)	2580 (8.2)
6	3-Methylcholanthrene	I	+	10 (1.0)	23 (2.3)	11 (1.1)	65 (6.5)
7	1-Aminoanthracene	I	+	8 (1.0)	7 (0.9)	5 (0.6)	25 (3.1)
8	2-Aminoanthracene	I	+	540 (1.0)	564 (1.0)	468 (0.9)	1102 (2.0)
9	DMBA	II	+	7 (1.0)	17 (2.4)	6 (0.9)	23 (3.3)
10	6-Aminochrysene	II	+	1200 (1.0)	4693 (3.9)	461 (0.4)	5955 (5.0)
11	1-Nitro-benzo[a]pyrene	II	–	524 (1.0)	1640 (3.1)	400 (0.8)	2896 (5.5)
12	Benzo[a]pyrene-4,5-dihydroepoxide	II	–	2724 (1.0)	4836 (1.8)	1856 (0.7)	4900 (1.8)
13	ENNG	II	–	3 (1.0)	58 (19.3)	4 (1.3)	60 (20)
14	1-Nitropyrene	III	–	154 (1.0)	2354 (15.3)	112 (0.7)	194 (1.3)
15	1,8-Dinitropyrene	III	–	110100 (1.0)	708300 (6.4)	39300 (0.4)	171600 (1.6)
16	6-Nitro-benzo[a]pyrene	III	–	24 (1.0)	268 (11.2)	21 (0.9)	58 (2.4)
17	1-Nitro-6-azabenzo[a]pyrene	III	–	60500 (1.0)	178180 (2.9)	29700 (0.5)	58020 (1.0)
18	3-Nitro-6-azabenzo[a]pyrene	III	–	3560 (1.0)	57640 (16.2)	2480 (0.7)	5240 (1.5)
19	Fulylfuramide	III	–	480 (1.0)	5760 (12.0)	220 (0.5)	620 (1.3)
20	Aflatoxin B1	III	+	990 (1.0)	6680 (6.7)	750 (0.8)	1160 (1.2)
21	Benzo[a]pyrene-7,8-tetrahydroepoxide	III	–	1332 (1.0)	3404 (2.6)	1000 (0.8)	2252 (1.7)
22	Acridine orange	III	+	113 (1.0)	234 (2.1)	83 (0.7)	131 (1.2)
23	Benz[a]anthracene	III	+	1 (1.0)	10 (10.0)	3 (3.0)	2 (2.0)
24	2-Nitrofluorene	III	–	162 (1.0)	341 (2.1)	203 (1.3)	215 (1.3)
25	PBTA-1	IV	+	5074 (1.0)	4568 (0.9)	4768 (0.9)	4720 (0.9)
26	Glu-P-1	IV	+	52800 (1.0)	61120 (1.2)	46480 (0.9)	56640 (1.1)
27	Aminophenylnorharman	IV	+	12352 (1.0)	8880 (0.7)	10688 (0.9)	11456 (0.9)
28	N-OH-AAF	IV	–	62 (1.0)	76 (1.2)	49 (0.8)	52 (0.8)
29	4-NQO	IV	–	1150 (1.0)	1470 (1.3)	750 (0.7)	1180 (1.0)
30	2-Acetylaminofluorene	IV	+	114 (1.0)	124 (1.1)	89 (0.8)	105 (0.9)

Each chemical was assayed with four to seven doses on duplicate plates with four strains in parallel. The assays with chemical nos. 2, 4, 7, 8, 10, 12, 15, 17, 23 and 29 were repeated to confirm the initial results. The numbers of His$^+$ revertants per plate per microgram of each strain are calculated at the doses indicated with arrows in Fig. 2. The numbers in parentheses represent the values relative to the numbers of His$^+$ revertants per microgram in TA1538 (no plasmid). Difference of the relative mutability two-fold or more was regarded as significant effects of the introduction of plasmids on the mutability.

Group I: the chemicals whose mutagenicity was highest in strain YG5161 harboring plasmid pYG768 carrying *dinB* (DNA pol IV).

Group II: the chemicals whose mutagenicity was equally high in both strain YG5161and strain TA98 harboring plasmid pKM101 carrying *mucAB* (DNA pol RI).

Group III: the chemicals whose mutagenicity was highest in strain TA98.

Group IV: the chemicals whose mutagenicity was not substantially modulated by the introduction of any of the plasmids.

Fig. 2 – Responses of *S. typhimurium* tester strains to 30 chemical mutagens. The chemicals are: benzo[a]pyrene-7,8-dihydroepoxide (1); benzo[a]pyrene diol epoxide (2); 10-azabenzo[a]pyrene (3); benzo[a]pyrene (4); 3-nitro-benzo[a]pyrene (5); 3-methylcholanthrene (6); 1-aminoanthracene (7); 2-aminoanthracene (8); DMBA (9); 6-aminochrysene (10); 1-nitro-benzo[a]pyrene (11); benzo[a]pyrene-4,5-dihydroepoxide (12); ENNG (13); 1-nitropyrene (14); 1,8-dinitropyrene (15); 6-nitro-benzo[a]pyrene (16); 1-nitro-6-azabenzo[a]pyrene (17); 3-nitro-6-azabenzo[a]pyrene (18); furylfuramide (19); aflatoxin B1 (20); benzo[a]pyrene-7,8-tetrahydroepoxide (21); acridine orange (22); benz[a]anthracene (23); 2-nitrofluorene (24); PBTA-1 (25); Glu-P-1 (26); aminophenylnorharman (27); N-OH-AAF (28); 4-NQO (29); 2-acetylaminofluorene (30). The strains used are: TA1538 (circles ●); YG5160 (diamonds ◆); YG5161 (squares ■); TA98 (triangles ▲). The arrow indicates the dose that was used for the calculation of His$^+$ revertants per microgram per plate in Table 3.

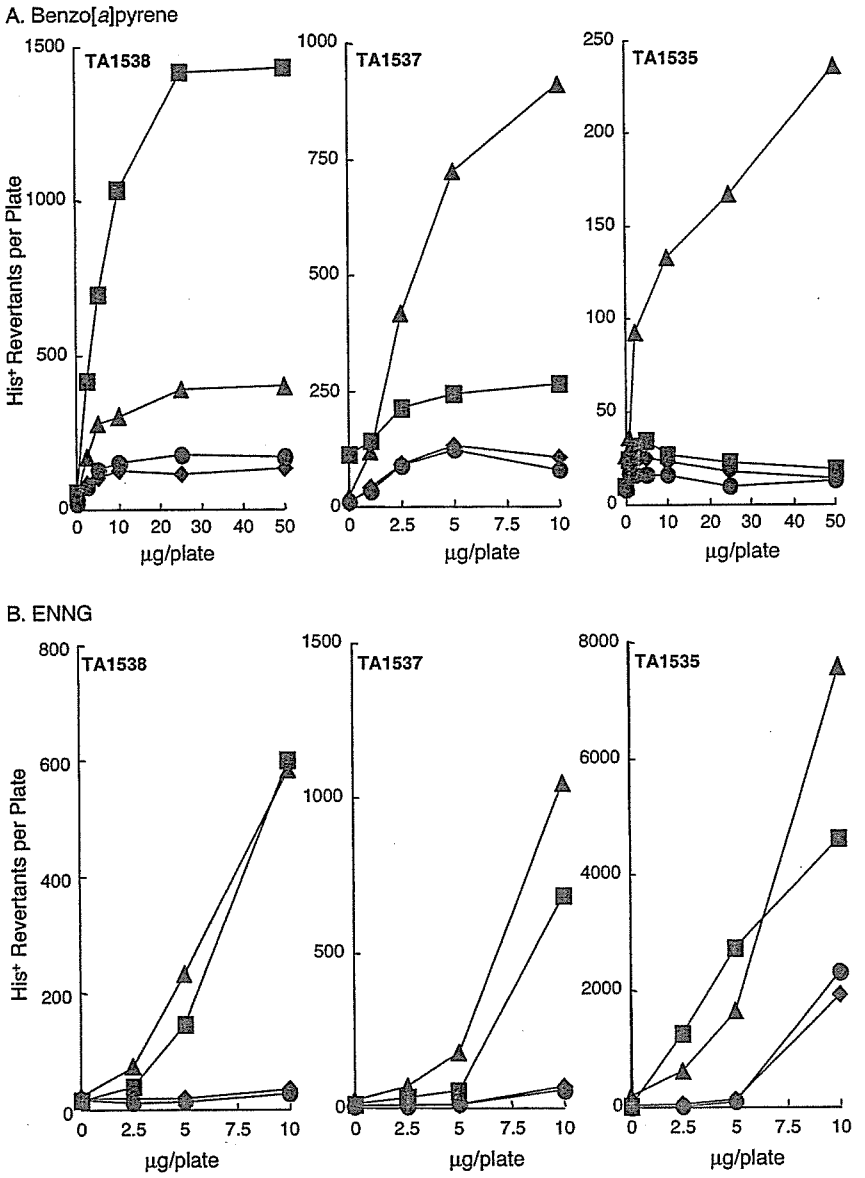

Fig. 3 – Responses of S. *typhimurium* tester strains to benzo[a]pyrene and ENNG. Benzo[a]pyrene plus S9 mix (A) or ENNG (B) was assayed with four to six doses on duplicate plates with four strains, i.e., the parent strain without plasmids (circles ●), the derivative harboring plasmid pYG787 carrying *polB* (diamonds ♦), the derivative harboring plasmid pYG768 carrying *dinB* (squares ■) and the derivative harboring plasmid pKM101 carrying *mucAB* (triangles ▲) in parallel. The parent strains were S. *typhimurium* TA1538, TA1537 and TA1535 for the detection of −2 frameshift, −1 frameshift and base substitutions, respectively, and they were transformed with one of three plasmids, i.e., pYG787, pYG768 and pKM101.

3.3. *Effects of the introduction of plasmid carrying polA encoding E. coli DNA polymerase I on the mutability of strain TA1538*

The mutagenicity of the compounds of group IV was not influenced by introduction of any of the plasmids carrying genes encoding SOS-inducible DNA polymerases (Fig. 2). This suggests the involvement of replicative DNA polymerases, i.e., DNA polymerase I and/or DNA polymerase III, in the mutagenesis. To examine the possible involvement of DNA polymerase I, we introduced plasmid pIMA-1 carrying *polA* encoding DNA polymerase I to strain TA1538 and its derivative YG6215, which lacks all the genes encoding SOS-inducible DNA polymerases [34], and compared the mutability to the group IV compounds, i.e., PBTA-1, Glu-P-1, aminophenylnorharman, 4-NQO and 2-acetylaminofluorene (Fig. 4). We also examined the mutagenicity of 2-aminofluorene, a derivative of 2-acetylaminofluorene. The introduction of plasmid pIMA-1 did not affect the mutability of strain TA1538 and YG6215 to the group IV compounds and 2-aminofluorene. We also examined the mutability of strain TA1538 and YG6215 harboring plasmid pIMA-1 carrying *polA* to other chemicals belong to group I, i.e., benzo[a]pyrene, 10-azabenzo[a]pyrene, 3-methylcholanthrene and 1-aminoanthracene, group II,

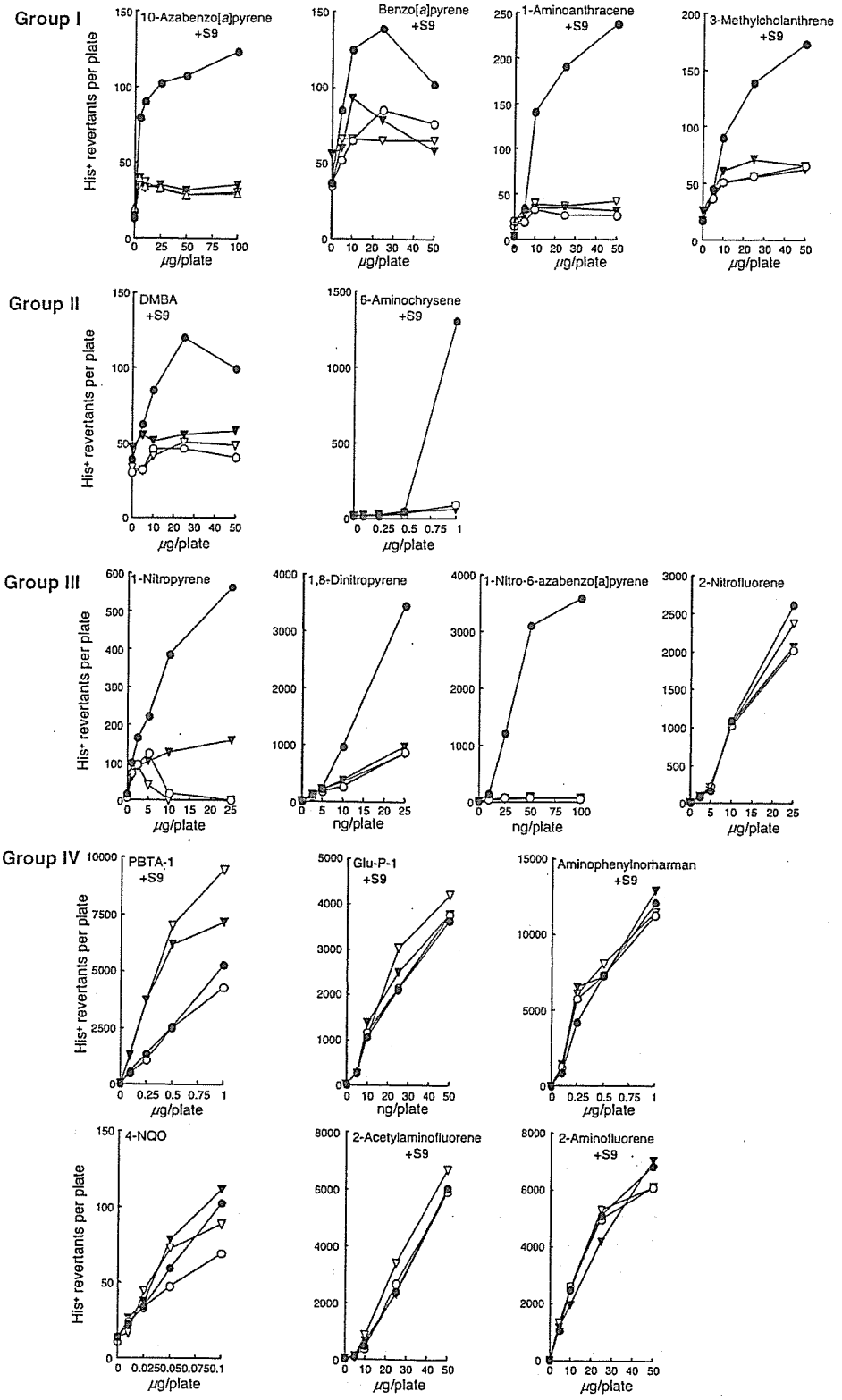

Fig. 4 – Effects of introduction of plasmid pIMA-1 carrying *polA* on the mutability of strain TA1538 and YG6215 to chemicals. The strains used were *S. typhimurium* TA1538 harboring the vector pWKS30 (closed circles ●), TA1538 harboring plasmid pIMA-1 (open circles ○), YG6215 harboring the vector pWKS30 (closed triangles ▼) and YG6215 harboring plasmid pIMA-1 (open triangles ▽). Each chemical was assayed with five to six doses on triplicate plates with four strains in parallel. The dose response curves of PBTA-1 plus S9 was unique in that strain YG6215 lacking all SOS-inducible DNA polymerases displayed higher mutability than strain TA1538 [34].

i.e., DMBA and 6-aminochrysene and group III, i.e., 1-nitropyrene, 1,8-dinitropyrene, 1-nitro-6-azabenzo[a]pyrene and 2-nitrofluorene. Surprisingly, the introduction of plasmid pIMA-1 reduced the mutability of strain TA1538 to the level of strain YG6215 harboring the vector plasmid (Fig. 4). Only exception was 2-nitrofluorene where the mutability of strain TA1538 and YG6215 was not affected by the introduction of plasmid pIMA-1 as in the case of group IV compounds. The dose–response curves of strain YA1538 harboring plasmid pIMA-1 almost completely overlapped those of strain YG6215 harboring the vector plasmid. The mutability of strain YG6215 was not affected by the introduction of plasmid pIMA-1 carrying *polA* except for 1-nitropyrene where strain YG6215 as well as strain TA1538 exhibited an enhanced killing sensitivity to the chemical when the *polA* plasmid was introduced.

4. Discussion

DNA polymerase III holoenzyme replicates the chromosome of *E. coli* with high fidelity but its progress is occasionally blocked by DNA lesions, and DNA polymerase V encoded by *umuDC* bypasses the lesions to assist the chromosome replication, which mostly results in base substitutions [10,28]. Less is known, however, about the mechanisms of translesion bypass leading to frameshift [39–41]. To assign the role of each SOS-inducible DNA polymerases of *E. coli* in TLS leading to frameshift, we have introduced plasmids carrying the genes encoding SOS-inducible DNA polymerases to strain TA1538 possessing CGCGCGCG sequence in the *hisD* gene as a −2 frameshift hot spot [31], and examined the mutability to 30 chemical mutagens. The *mucB* gene is expressed 13,000 molecules per cell from plasmid pKM101 when LexA repressor is inactivated [42], and the expression level is much higher than the chromosomal expression level of the *umuC* gene, i.e., about 200 molecules per cell upon SOS induction [43]. Similarly, the *dinB* gene is expressed 25,000–50,000 molecules per cell from plasmid pYG768 in the presence of DNA damage, which is 10–20 times higher than the expression level of *dinB* from the chromosome upon SOS induction [25]. The exact expression levels of DNA polymerase I and DNA polymerase II from plasmid pIMA-1 and pYG787, respectively, are unclear, but they could be at least two to three times higher than those from the chromosome because both plasmids are derivatives of low-copy-number plasmid pWKS30 [44]. DNA polymerase I and DNA polymerase II encoded by *polA* and *polB*, respectively, are expressed 400 and 30–50 molecules per cell from the chromosome and the expression of *polB* is enhanced about seven-fold by DNA damage [10,45]. Thus, we assume the expression levels are about 1000 for DNA polymerase I, 500–1000 for DNA polymerase II, 10–20 for DNA polymerase III [10], 25,000–50,000 for DNA polymerase IV and 13,000 for DNA polymerase RI per cell when the chromosome DNA is damaged by chemicals. Because of the high levels of expression of these DNA polymerases from the plasmids, we could observe distinct enhancing effects on the mutability of strain TA1538 to 30 chemicals (Fig. 2, Table 3). Based on the mutagenicity, we classified the chemicals into four groups as follows.

Group I includes eight chemicals that exhibited highest mutagenicity in strain YG5161 harboring plasmid pYG768 carrying *dinB*. The introduction of plasmid pKM101 also enhanced the mutagenicity of some compounds, but the extent of the enhancement was much less compared to the effects of plasmid pYG768. In fact, the introduction of plasmid pKM101 into strain TA1538 did not enhance the mutagenicity of 1-aminoanthracene and 2-aminoanthracene while plasmid pYG768 enhanced the mutagenicity more than two times. These results suggest that DNA polymerase IV encoded by *dinB* efficiently bypasses DNA lesions induced by group I compounds in CGCGCGCG sequence leading to −2 frameshift. This is consistent with our previous results that deletion of endogenous *dinB* gene of *S. typhimurium*, i.e., $dinB_{ST}$, significantly reduced the mutability of strain TA1538 to 10-azabenzo[a]pyrene, benzo[a]pyrene, 1-aminoanthracene and 2-aminoanthracene [34]. DNA polymerase IV could have specificity to bypass across guanine bases modified with the polycyclic aromatics, leading to −2 frameshift. In contrast to −2 frameshift, the introduction of plasmid pYG768 did not enhance base substitutions induced by benzo[a]pyrene (Fig. 3A). *E. coli* DNA polymerase IV is reported to bypass N^2-guanine adducts of benzo[a]pyrene diol epoxide in vitro with higher efficiency and high fidelity compared to DNA polymerase II and DNA polymerase V [46]. Thus, we suggest that DNA polymerase IV correctly incorporate cytosine opposite the adducted guanine in the GGG sequence in the *hisG* gene, thereby leading to no base substitutions. Correct insertion of cytosine opposite the adducted guanine in the CGCGCGCG sequence in *hisD*, however, may induce a two-bp slippage leading to −2 frameshift [47].

Group II includes five chemicals that displayed equally high mutagenicity to strain YG5161 and strain TA98. They are derivatives of PAHs, i.e., DMBA and benzo[a]pyrene-4,5-dihydroepoxide, an aromatic amine, i.e., 6-aminochrysene, a nitroaromatic, i.e., 1-nitrobenzo[a]pyrene and an alkylating agent, i.e., ENNG. The results that strains YG5161 and TA98 exhibited similar mutability to the compounds suggest that lesions induced by group II compounds can be bypassed by either DNA polymerase IV or DNA polymerase RI at similar efficiency. In fact, the previous study indicates that deletion of either $dinB_{ST}$ or $umuDC_{ST}$ reduced the mutagenicity of DMBA and 6-aminochrysene [34]. These compounds appear to require the presence of at least two specialized DNA polymerases to bypass the lesions. Of group II compounds, ENNG is exceptional because it is a simple alkylating agent that induces a mutagenic guanine base, i.e., O^6-ethylguanine [48]. Since the lesion in DNA directs the incorporation of thymine as well as cytosine during DNA synthesis, it was expected that this compound enhanced base substitutions in strain TA1535 (Fig. 3 B). It was a big surprise, however, that the simple alkylating agent was also capable of inducing −1 and −2 frameshifts in the repetitive sequences in strains TA1537 and TA1538, respectively, and that the bypass reactions leading to frameshifts appeared to be mediated by DNA polymerase IV or DNA polymerase RI. We also observed that the introduction of plasmids pYG768 and pKM101 enhanced the mutability of strain TA1538 against ethylnitrosourea (ENU), which induces O^6-ethylguanine in DNA (unpublished results). Eckert and Hile reported that frameshift errors are generated during in vitro DNA synthesis of ENU-treated template single-stranded DNA by mammalian DNA alpha-primase and DNA polymerase beta

[49]. Since humans possess Y-family DNA polymerases, i.e., DNA polymerase eta, iota, kappa and REV1 [8], it seems worth examining the abilities to bypass O^6-ethylguanine in repetitive sequences leading to frameshifts.

Since the newly established strain YG5161 harboring plasmid pYG768 exhibited higher sensitivity to groups I and II compounds than the standard Ames tester strain TA98 harboring plasmid pKM101, strain YG5161 could be useful for the sensitive detection of environmental mutagens and carcinogens such as benzo[a]pyrene and its derivatives. Actually, 10-azabenzo[a]pyrene, benzo[a]pyrene and 3-nitrobenzo[a]pyrene are present in polluted air and soot of combustion of coal [50,51], and some of them are identified in cigarette smoke [52].

Group III includes 1-nitropyrene and other 10 compounds. They include structurally unrelated compounds such as furylfuramide, aflatoxin B1 and acridine orange. The previous study indicated that deletion of $umuDC_{ST}$ significantly reduced the mutagenicity of 1-nitropyrene, 1,8-dinitropyrene, 1-nitro-6-azabenzo[a]pyrene and 3-nitro-6-azabenzo[a]pyrene [34], which are all included in this group. DNA adducts induced by group III compounds appeared to be more efficiently bypassed by DNA polymerase RI leading to −2 frameshift, compared to DNA polymerase IV or DNA polymerase II. Of the compounds, the mutagenicity of 3-nitro-6-azabenzo[a]pyrene is reduced by more than 95% by the deletion of $umuDC_{ST}$ [34]. Since the endogenous DNA polymerase V encoded by $umuDC_{ST}$ is capable to bypass DNA adduct(s) induced by this compound, the exogenous expression of DNA polymerase RI from plasmid pKM101 merely enhanced the mutagenicity by less than three-fold. Although DNA polymerase II encoded by *polB* had virtually no enhancing effects or rather suppressing effects on the mutagenicity of the chemicals examined, the introduction of plasmid pYG787 carrying *polB* specifically and repeatedly enhanced the mutagenicity of benz[a]anthracene, which is a potent carcinogen (Table 3). This compound induces adducts in guanine N^2 and adenine N6 atoms upon metabolic activation [53,54]. Thus, guanine N^2-adducts by the active metabolites of benz[a]anthracene in the CG repetitive sequence could be bypassed by DNA polymerase II leading to −2 frameshift. Since DNA polymerase II is a member of B-family DNA polymerase, its mammalian counterpart such as DNA polymerase delta may have an ability to bypass the adducts in the repetitive sequences.

Group IV includes PBTA-1 and other five compounds. PBTA-1 is a potent aromatic amine mutagen in a polluted river [55]. The characteristic of this group of compounds is that the introduction of any of the plasmids encoding SOS-inducible DNA polymerase had no enhancing effects on the mutagenicity. In the previous study, we reported that the mutagenicity of PBTA-1, Glu-P-1, aminophenylnorharman, N-OH-AAF, 4-NQO and 2-acetylaminofluorene, which are all belong to group IV in this study, are not reduced by the deletions of any of *S. typhimurium* genes encoding SOS-inducible DNA polymerases [34]. Thus, we suggested that the replicative DNA polymerase, i.e., DNA polymerase III holoenzyme, is responsible for the translesion bypass across DNA adducts induced by the chemicals in the CG repetitive sequence leading to −2 frameshift [34]. Although we cannot strictly rule out the possibility that DNA polymerase I is involved in the translesion, we prefer the possibility that DNA polymerase III holoenzyme is responsible for the bypass reactions because the introduction of plasmid pIMA-1 carrying *polA* did not enhance the mutagenicity (Fig. 4). The group IV compounds are all aromatic amines except for 4-NQO and some of them are proved to induce guanine C8 adducts in DNA [56–58]. Thus, we suggest that DNA polymerase III holoenzyme efficiently skips over guanine C8 adducts by aromatic amines in certain sequence context such as CGCGCGCG, thereby inducing −2 frameshift. Involvement of the replicative DNA polymerase may make this repetitive sequence a hot spot for frameshift mutagenesis.

In the previous study, we systematically disrupted one or all the genes of *S. typhimurium* strain TA1538 encoding SOS-inducible DNA polymerases and examined the mutability to chemical mutagens [34]. It is in contrast with the present study where the expression of SOS-inducible DNA polymerases is enhanced. As expected, most of the chemicals exhibited contrastive responses. They displayed enhanced mutagenicity in the presence of enhanced expression of the DNA polymerase, and diminished mutagenicity in the absence of the gene encoding the polymerase. Curiously, some compounds exhibited unexpected mutagenicity in the plasmid-bearing strains and the deletion strains. For example, 3-nitrobenzo[a]pyrene, 1-nitrobenzo[a]pyrene and 2-nitrofluorene were classified into groups I, II and III, respectively (Table 3, Fig. 1). The mutagenicity was enhanced by the introduction of plasmid pYG768 carrying *dinB* encoding DNA polymerase IV and/or pKM101 carrying *mucAB* encoding DNA polymerase RI. However, the mutagenicity of these compounds was not reduced by deletion of any of the genes encoding SOS-inducible DNA polymerases (class IV compounds in the previous study [34]). These results suggest that DNA polymerase III holoenzyme is responsible for the translesion bypass across the lesions induced by the chemicals in physiological conditions, but the Y-family DNA polymerases can take over the translesion reactions when the expression levels are enhanced. In other words, DNA polymerase III holoenzyme, DNA polymerase IV and DNA polymerase RI share, at least in part, the specificity to bypass the lesions and the polymerase actually involved in the translesion depends upon the cellular expression levels or the concentrations of the DNA polymerase in the replication complex.

To examine the possible involvement of DNA polymerase I in the frameshift mutagenesis, we introduced plasmid pIMA-1 carrying *polA* to strain TA1538 and strain YG6215, in which all the genes encoding SOS-inducible DNA polymerase are deleted [34]. Strikingly, the introduction of plasmid pIMA-1 sharply reduced the mutability of strain TA1538 against groups I, II and III compounds to that of strain YG6215 (Fig. 4). The dose–response curves of strain TA1538 harboring plasmid pIMA-1 with benzo[a]pyrene, 10-azabenzo[a]pyrene, 1-aminoanthracene, 3-methylcholanthrene (group I), DMBA, 6-aminochrysene (group II), 1,8-DNP and 1-nitro-6-azabenzo[a]pyrene (group III) almost overlapped those of strain YG6215 harboring plasmid pIMA-1 or the vector plasmid pWKS30. Because the mutability of strain TA1538 harboring plasmid pIMA-1 appears to be similar to that of strain YG6215 harboring the vector, we suggest that the enhanced expression of DNA polymerase I prevents the access of the Y-family DNA polymerases, i.e., DNA polymerase IV and DNA polymerase V, to the replication complex

where translesion bypass actually occurs. It is known that all five DNA polymerases in *E. coli* interact with the beta-subunit of DNA polymerase III holoenzyme [59–61]. Thus, there should be some competition for the polymerases to interact with the beta clamp. The beta-subunit assembles in a donut-like shape as a dimer and tethers DNA polymerase to a template/primer DNA, thereby preventing a falling off of polymerase from template DNA [62]. We speculate the order of the affinity of each DNA polymerase to the beta clamp or the replication complex may be DNA polymerase III > DNA polymerase I > DNA polymerase II > DNA polymerase IV = DNA polymerase V. This assumption is based on the observation that the introduction of plasmid pIMA-1 reduced the mutagenicity of compounds of groups I, II and III, which require the presence of the Y-family DNA polymerases for the maximum mutagenesis, but not group IV, whose mutagenicity is depended upon DNA polymerase III holoenzyme (Fig. 4). In addition, Foster suggested that DNA polymerase II may be dominant over DNA polymerase IV in the replication complex because the *dinB* mutator effects are more pronounced in the stationary-phase mutagenesis when the *polB* gene is deleted [63]. At present, we do not know which of DNA polymerase I or DNA polymerase II has a higher affinity to the replication complex. We prefer the possibility, however, that DNA polymerase I is dominant over DNA polymerase II because it is involved in lagging strand DNA synthesis during the chromosome replication. An alternative explanation for the suppressive effects of plasmid pIMA-1 (Fig. 4) is that DNA polymerase I expressed from the plasmid bypasses the lesions induced by the chemicals of groups I–III in an error-free manner, thereby reducing the mutagenicity. However, we think it less likely because the suppressive effects would vary with chemicals or lesions if DNA polymerase I mediated the error-free TLS. Each DNA polymerase including DNA polymerase I should have specificity to bypass the lesions. Hence, the introduction of pIMA-1 would suppress the mutagenicity of some compounds efficiently but not others. In fact, the strong suppressive effects were observed with almost all the compounds of groups I–III we examined. This is in contrast with the suppressive effects of DNA polymerase II expressed from plasmid pYG787, which reduced the mutagenicity of some of the compounds of groups I–III with various efficiencies (see below more detail). Thus, we prefer the possibility that DNA polymerase I expressed from the plasmid inhibits the access of the Y-family DNA polymerases to the replication complex. Nevertheless, it is important to examine whether a catalytically dead mutant of DNA polymerase I exhibits the suppressive effects on the mutagenicity of groups I–III chemicals to distinguish the possibilities.

In contrast to the clear suppressive effects by plasmid pIMA-1 carrying *polA*, the suppressive effects of plasmid pYG787 carrying *polB* on strain TA1538 were moderate. The introduction of plasmid pYG787 reduced the mutagenicity of benzo[a]pyrene-7,8-dihydroepoxide and 1-aminoanthracene (group I), 6-aminochrysene (group II), 1,8-dinitropyrene, 1-nitro-6-azabenzo[a]pyrene and furylfuramide (group III) by 40–60% (Table 3). DNA polymerase II may mediate the error-free translesion across DNA adducts induced by these compounds. In the previous study, deletion of $polB_{ST}$ reduced the −2 frameshift mutations induced by benzo[a]pyrene-7,8-tetrahydroepoxide, 3-methycholanthrene, 1-nitropyrene, 1,8-nitropyrene, 1-nitro-6-azabenzo[a]pyrene and 3-nitro-6-azabenzo[a]pyrene by 30–60% [34]. Thus, it seems that the enhanced expression as well as the lack of expression diminished the mutagenicity of 1,8-dinitropyrene and 1-nitro-6-azabenzo[a]pyrene. In other words, DNA polymerase II could have an optimal cellular concentration to enhance the translesion DNA synthesis leading to −2 frameshift. This is contrast to DNA polymerase IV and DNA polymerase RI, which enhance the mutagenesis when the levels of their expression are elevated. *E. coli* DNA polymerase II is reported to be involved in the immediate recovery of DNA synthesis after UV irradiation [13,14]. It is tempting to speculate that it might be required to re-synthesize the primer strand to reach the lesion when the primer strand was degraded. This degradation might occur when DNA polymerase III holoenzyme encountered the lesion and stopped the replication. If the expression level of DNA polymerase II was enhanced, it might promote error-free bypass reactions across the lesions while the lack of DNA polymerase II might lead to poor translesion DNA synthesis by DNA polymerase IV or DNA polymerase V.

In summary, our results suggest that DNA polymerase IV and DNA polymerase RI possess distinct but partly overlapping specificity to bypass lesions leading to −2 frameshift, and also that the replicative DNA polymerase, i.e., DNA polymerase III holoenzyme, participates in the bypass reactions in the CG repetitive sequence. Although DNA polymerase III holoenzyme is responsible for the translesion, the Y-family DNA polymerase may take over the primer termini, thereby enhancing the bypass reactions, when the expression of the polymerases is enhanced. Based on the suppressive effects of plasmid pIMA-1, we speculate that the order of DNA polymerases in *E. coli* to access to the replication complex could be DNA polymerase III > DNA polymerase I > DNA polymerase II > DNA polymerase IV = DNA polymerase V. Our results also raise an interesting possibility that strain YG5161 harboring plasmid pYG768 is a sensitive tester strain to identify the mutagenicity of environmental PAHs.

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Visualization of the interaction between archaeal DNA polymerase and uracil-containing DNA by atomic force microscopy

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Deamination of cytosine to uracil is a hydrolytic reaction that is greatly accelerated at high temperatures. The resulting uracil pairs with adenine during DNA replication, thereby inducing G:C to A:T transitions in the progeny. Interestingly, B-family DNA polymerases from hyperthermophilic Archaea recognize the presence of uracil in DNA and stall DNA synthesis. To better understand the recognition mechanism, the binding modes of DNA polymerase B1 of *Sulfolobus solfataricus* (Pol B1) to uracil-containing DNA were examined by gel mobility shift assays and atomic force microscopy. Although PolB1 per se specifically binds to uracil-containing single-stranded DNA, the binding efficiency was substantially enhanced by the initiation of DNA synthesis. Analysis by the atomic force microscopy showed a number of double-stranded DNA (dsDNA) in the products of DNA synthesis. The generation of ds DNA was significantly inhibited, however, by the presence of template uracil, and intermediates where monomeric forms of Pol B1 appeared to bind to uracil-containing DNA were observed. These results suggest that Pol B1 more efficiently recognizes uracil in DNA during DNA synthesis rather than during random diffusion in solution, and that single molecules of Pol B1 bind to template uracil and stall DNA synthesis.

Introduction

Deamination of exocyclic amino groups of DNA bases such as deamination of the amino group of cytosine poses a common genotoxic risk in all organisms (Lindahl 1993; Pearl 2000). 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. Since the rate of the hydrolytic reaction is greatly accelerated at high temperatures (Lindahl & Nyberg 1974), hyperthermophilic organisms, which live in habitats at more than 80 °C, are supposed to be exposed to massive DNA damages by the deamination. However, the spontaneous mutation rate in hyperthermophilic archaea *Sulfolobus acidocaldarius* is reported to be similar to that of *Escherichia coli* (Grogan *et al.* 2001). Thus, hyperthermophilic archaea appear to possess mechanisms to protect a stability of the genomic DNA from the mutagenic threat of deaminated bases generated at high temperatures.

Interestingly, B-family DNA polymerases from hyperthermophilic archaea such as *Sulfolobus solfataricus* DNA polymerase B1 (Pol B1) or *Pyrococcus furiosus* DNA polymerase (Pfu) recognize the presence of uracil in DNA and tightly bind to uracil-containing oligonucleotides (Lasken *et al.* 1996; Greagg *et al.* 1999). Pol B1 is likely to play an important role in DNA replication in *S. solfataricus* because the activity is highly stimulated by PCNA-like and RFC-like factors in *in vitro* (De Felice *et al.* 1999). Pol B1 and Pfu stall DNA polymerization

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three to four base pairs (bps) before template uracil. The recognition and stalling mechanisms by the polymerases appear to contribute to the genome integrity of hyperthermophilic archaea because they may prevent the misincorporation of adenine opposite the template uracil. More surprisingly, a recent study suggests that the enzymes also bind to the deamination product of adenine, i.e. hypoxanthine, in a template DNA strand and stall DNA synthesis upstream of the lesion (Gruz *et al.* 2003). Hypoxanthine can pair with cytosine, thereby inducing A:T to G:C transitions if not repaired (Lindahl 1993). Thus, the recognition mechanisms of deaminated bases by archaeal B family DNA polymerases may play more important roles in maintaining the genome stability than previously thought. The recognition mechanism seems unique to archaeal B family DNA polymerases because viral B family DNA polymerases such as T4 DNA polymerase or DNA polymerases from hyperthermophilic eubacteria such as *Thermus aquaticus* (Taq) do not stall DNA synthesis when the template DNA has uracil or hypoxanthine (Greagg *et al.* 1999; Gruz *et al.* 2003). There are no specific reports on the recognition of uracil in DNA by eukaryotic B-family DNA polymerases as far as we know. Structural analysis for uracil recognition by archaeal B family DNA polymerases indicates a pocket in the N-terminal domains interacting with a template strand is responsible for the discrimination of uracil from normal DNA bases (Fogg *et al.* 2002).

Since Pol B1 is abundantly expressed in the cell, i.e. 1500 molecules per *S. solfataricus* cell, it may bind to the deaminated bases in the chromosome DNA even without DNA synthesis. In fact, it binds to uracil- or hypoxanthine-containing oligonucleotides without primers or dNTPs necessary for DNA synthesis (Gruz *et al.* 2003). If such a binding occurs *in vivo*, it may interfere with normal repair of the deaminated bases by DNA glycosylases (Sartori *et al.* 2002). Thus, there appear to be mechanisms that prevent non-productive binding of Pol B1 to the deaminated bases in non-replicating chromosomes. For further insights into the binding mechanisms of archaeal B family DNA polymerases to the deaminated bases in DNA, we compared the binding efficiencies of Pol B1 to uracil in DNA with or without DNA synthesis. For this purpose, we employed atomic force microscopy, which is suitable for the analysis of the behavior of individual molecules, as well as gel mobility shift assays (Engel & Muller 2000; Murakami *et al.* 2000). The results indicated the binding of Pol B1 to uracil in DNA is greatly accelerated by DNA synthesis and suggested that Pol B1 is targeted to the deaminated bases in replicating DNA. In addition, analyses with atomic force microscope (AFM) suggested that Pol B1 binds to uracil-containing DNA as a monomer, which directly supports the "read-ahead" mechanism where single molecules of DNA polymerase bind to template uracil and halt DNA replication (Greagg *et al.* 1999).

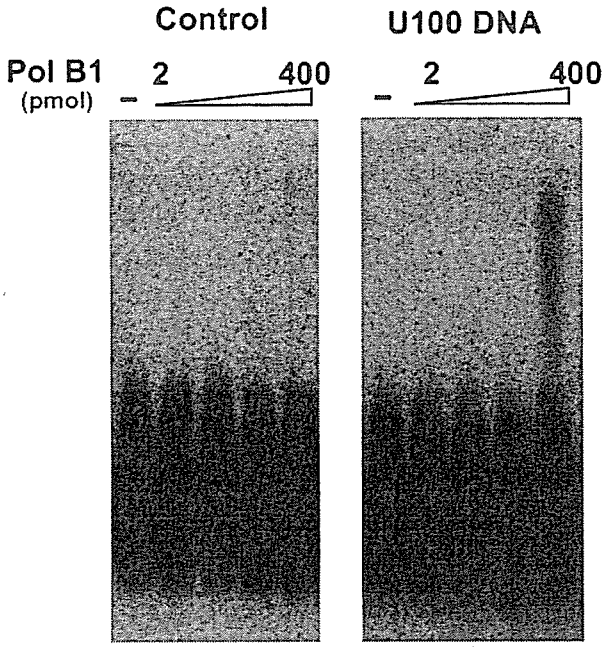

Figure 1 Binding of Pol B1 with template uracil without DNA synthesis. The reaction mixture (20 μL) contained Tris/KCl/$MgCl_2$, ssDNA (control or U100 DNA, 2 pmol) and Pol B1 (2, 20, 200 or 400 pmol), and the mixture was incubated for 15 min at 55 °C. The products were analyzed by 1% agarose gel electrophoresis, followed by Southern hybridization. The bands were visualized with ChemiDoc.—no Pol B1 was added in the reaction mixture.

Results

Binding of Pol B1 to uracil-containing DNA is accelerated by DNA synthesis

To examine the binding of Pol B1 to uracil in single-stranded DNA (ssDNA), control or U100 DNA, which contains uracil instead of thymine in DNA, was incubated with various amounts of Pol B1, and the migration of DNA bands was analyzed by gel mobility shift assays (Fig. 1). A clear band shift was observed when U100 DNA was incubated with Pol B1. A smear band appeared in the upper part of the gel while the original DNA bands remained in the lower part. In these experiments, primers and dNTPs were omitted from the reaction mixtures. Thus, it seems that Pol B1 can bind uracil-containing ssDNA even without DNA synthesis. This is consistent

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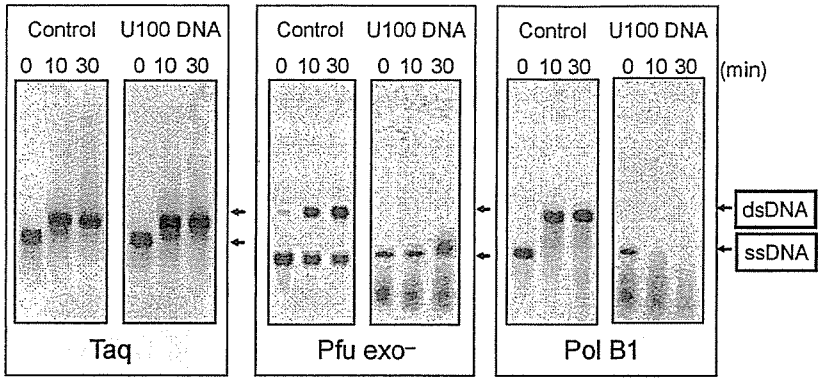

Figure 2 Template uracil inhibits DNA synthesis by Pol B1 and Pfu exo⁻ but not by Taq DNA polymerase. The reaction mixture (20 μL) contained Tris/KCl/$MgCl_2$, F1 primer (10 pmol), four dNTPs (4 nmol each), ssDNA (control or U100 DNA, 0.1 pmol) and Pol B1 (20 pmol). Taq DNA polymerase (1 unit) and Pfu exo⁻ DNA polymerase (2.5 units) were used as controls. The reaction was carried out for 10 or 30 min at 55 °C and was terminated by the addition of EDTA. The products were analyzed by Southern hybridization and visualized with ChemiDoc. Taq, Taq DNA polymerase; Pfu exo⁻, Pfu DNA polymerase exo⁻; Pol B1, DNA polymerase B1.

with our previous results that the K_d values of Pol B1 to primed DNA, ssDNA without uracil and ssDNA with uracil are 81, 55 and 4 nM, respectively (Gruz *et al.* 2003). It should be noted, however, that the binding was not efficient: it needed a large amount of Pol B1, i.e. 400 pmol, and no band shift was observed with lower amounts of the protein. The binding appears specific to uracil-containing DNA because no clear band shift was observed in control DNA.

Next, we examined the inhibitory effects of template uracil on DNA synthesis with U100 DNA (Fig. 2). DNA synthesis by Pol B1 was severely inhibited by the presence of uracil in template DNA. ssDNA containing uracil (U100 DNA) was hardly converted to double-stranded DNA (dsDNA), while control ssDNA was almost completely converted to dsDNA within 10 min. Most of ssDNA bands of U100 diminished during 10- or 30-min incubation without formation of dsDNA. Since Pol B1 possesses an exonuclease proofreading activity (Pisani & Rossi 1994), uracil-containing ssDNA might be partially digested by the exonuclease during the incubation. Like DNA synthesis by Pol B1, DNA synthesis by another archaeal B-family DNA polymerase, i.e. Pfu exo⁻, was severely inhibited by the presence of uracil in template DNA. In this case, however, ssDNA bands were observable even after 30-min incubation. The lack of exonuclease activity in Pfu exo⁻ DNA polymerase might account for the persistence of the ssDNA bands. In contrast to the archaeal enzymes, DNA synthesis by hyperthermophilic eubacterial DNA polymerase, i.e. Taq, was not inhibited by the presence of template uracil.

Since template uracil strongly inhibited DNA synthesis by Pol B1, the binding of Pol B1 during DNA synthesis might be stronger than that of Pol B1 without DNA synthesis. To examine the possibility, we analyzed the binding during DNA synthesis by the gel shift assay (Fig. 3). When Pol B1 and U100 DNA were incubated

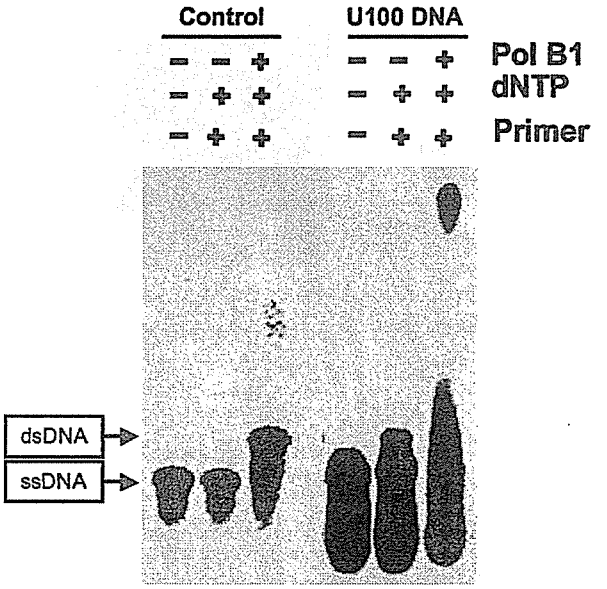

Figure 3 Specific binding of Pol B1 with template uracil during DNA synthesis. The reaction mixture (20 μL) contained Tris/KCl/$MgCl_2$, F1 primer (10 pmol), four dNTPs (4 nmol), ssDNA (control or U100 DNA, 2 pmol) and Pol B1 (20 pmol). The mixtures were incubated for 10 min at 55 °C, and the reactions were terminated by the addition of EDTA. The products were analyzed by Southern hybridization and visualized with ChemiDoc.

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(A)

Name	Sample	Image	Height (nm)	Width or Length (nm)
Form I	ssDNA		1.2 ~ 2.0	70 ~ 100
Form II	dsDNA		0.2 ~ 0.5	500
Form III	Pol B1		0.5 ~ 1.0	50
Form IV	Other Forms (DNA + Pol B1)	200 nm	1.5 ~ 2.0	100 ~ 450

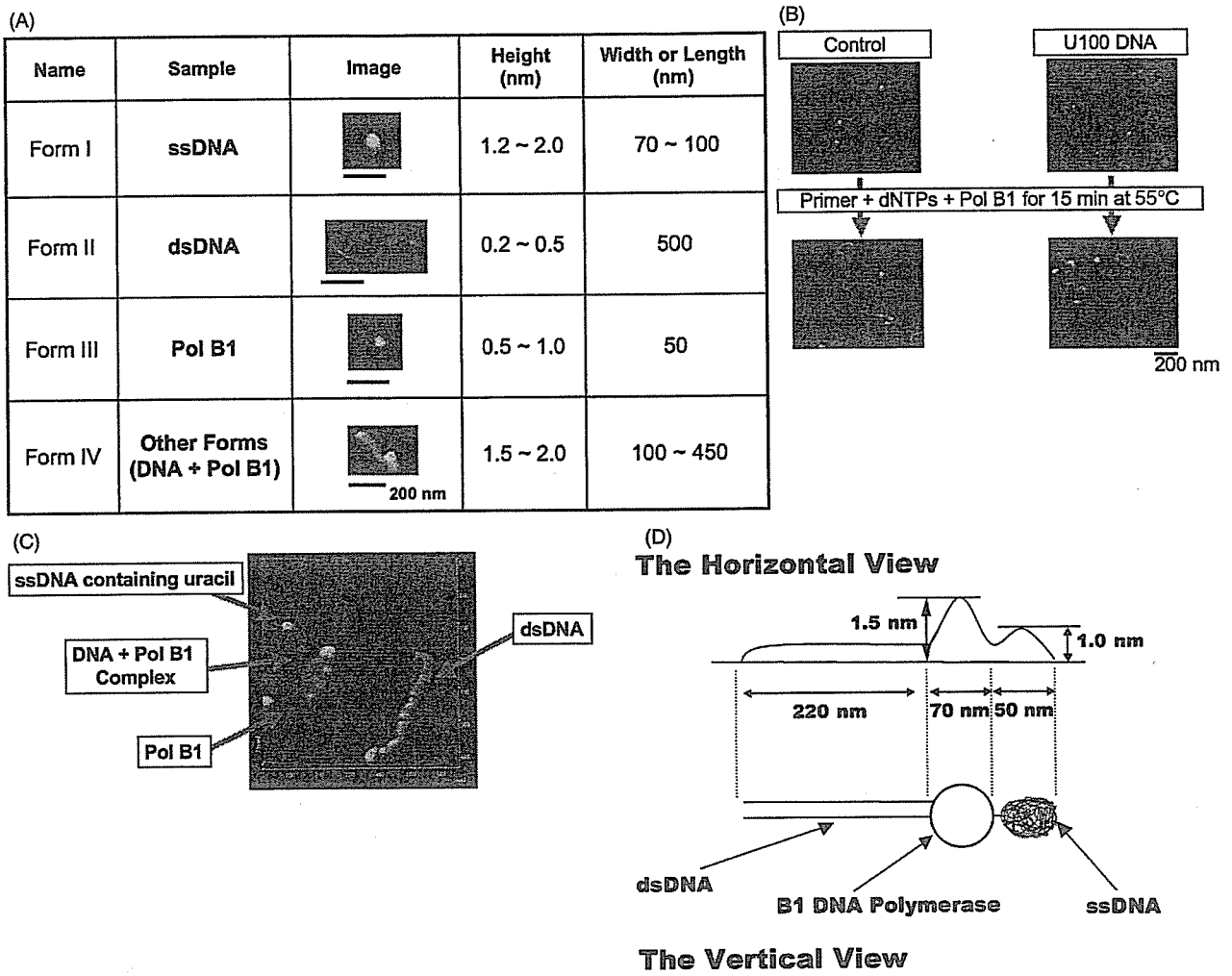

Figure 4 AFM images of DNA, Pol B1 and intermediates where Pol B1 binds with uracil-containing DNA. (A) Classification of the images of ssDNA (Form I), dsDNA (Form II), Pol B1 (Form III) and other forms including the intermediates (Form IV). (B) AFM images of the products of DNA synthesis with Pol B1 plus control DNA or U100 DNA. The reaction mixtures before and after incubation were analyzed by AFM. (C) Typical images of ssDNA, dsDNA, Pol B1 and the intermediates. (D) Possible explanation for the AFM image of the intermediate Form IV.

with primer and dNTPs, a smear band appeared on the gel. The band intensity was much stronger with U100 DNA than with control DNA. When we exposed the gel for shorter period of time, it became evident that substantial amounts of U100 DNA disappeared during the incubation (data not shown). It should be emphasized that the amount of Pol B1 (20 pmol, Fig. 3) required for binding with U100 DNA was 1/20 of that required for the binding to U100 DNA without primer and dNTPs in the reaction mixture (400 pmol, Fig. 1). These results suggest that the binding efficiency of Pol B1 with template uracil is substantially enhanced by DNA synthesis.

Pol B1 binds to uracil-containing DNA as a monomer

Atomic force microscopy is a powerful and convenient tool to visually analyze the behavior of individual DNA and protein molecules (Argaman *et al.* 1997; Murakami *et al.* 2001). The AFM method was employed to directly characterize the features of ssDNA, dsDNA and Pol B1 at the single molecular level (Fig. 4A). In the image analysis, ssDNA and dsDNA appeared as spherical and linear forms, respectively (Forms I and II). Pol B1 appeared as smaller spherical forms (Form III). The observed length of dsDNA, i.e. 500 nm, was consistent with the calculated

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Table 1 Distribution of three forms of AFM images in various reaction products

	Control	U100 DNA	Control + Pol B1	U100 DNA + Pol B1	Control + Pol B1 + dNTPs + primer	U100 DNA + Pol B1 + dNTPs + primer
ssDNA	87.5	91.3	80.8	81.6	34.1	61.2
	(300)	(95)	(84)	(129)	(30)	(123)
Other Forms*	12.5	8.7	14.4	18.4	40.9	34.3
	(43)	(9)	(15)	(29)	(36)	(69)
dsDNA	0	0	4.8	0	25.0	4.5
	(0)	(0)	(5)	(0)	(22)	(9)

Data are percentages of each form, i.e. ssDNA, dsDNA and other forms, relative to the total number of forms. The percentage and number (in parentheses) of each form are presented. For "Control (or U100 DNA) + Pol B1 + dNTPs +Primer" experiments, the reaction conditions are the same as those described in Experimental procedures. For "Control (or U100 DNA) +Pol B 1" experiments, R2 and F1 primers and dNTPs were omitted from the reaction mixture. For "Control or U100 DNA" experiments, PolB1, R2 and F1 primers and dNTPs were omitted from the mixture. *Other forms include the intermediate where Pol B1 appeared to bind to DNA. In these experiments, the ratio of Pol B1 (1 pmol) and ssDNA (control or U100 DNA, 2 pmol) in the reaction mixture (40 μL) was 1 : 2 (see Experimental procedures). The ratio of the enzyme (20 pmol) and ssDNA (0.1 pmol) in the reaction mixture (20 μL) was 200 : 1 for *in vitro* DNA synthesis where extensive digestion of U100 DNA was observed (Fig. 2).

length of 1.4 kb DNA (0.32 nm × 1400 = 448 nm). The reaction mixtures containing Pol B1, ssDNA (Control or U100 DNA), primers and dNTPs were subjected to AFM analysis before and after incubation for 15 min at 55 °C (Fig. 4B). There were many Form I (ssDNA) and Form III (Pol B1) molecules in the mixtures before the incubation. After the incubation, however, Form II (dsDNA) became apparent in the reaction mixtures containing control DNA. In contrast, Form II (dsDNA) was rare in the mixtures containing U100 DNA, and other forms such as those where Pol B1 appeared to bind to DNA were noted (Form IV, Fig. 4A,C). The sizes of the bound molecules (50–70 nm) were similar to those of monomeric forms of Pol B1 (Fig. 4A,D).

To analyze the dsDNA formation more quantitatively, individual molecules on the mica were counted, and the percentage of each form among total molecules was calculated (Table 1). It is evident that Form II (dsDNA) was generated, concomitant with the reduction of the number of Form I (ssDNA) in the reaction mixture containing control DNA, Pol B1, primers and dNTPs. The number of Form II (dsDNA) increased to a quarter of the total number of molecules (22/88 total molecules) while that of Form I (ssDNA) decreased to one third of it (30/88 total molecules). In contrast, Form II (dsDNA) accounted for only 5% of the total number of molecules (9/201 total molecules) and Form I (ssDNA) represented more than 60% (123/201 total molecules) in reaction mixtures containing U100 DNA, Pol B1, primers and dNTPs. The differences in the distribution of each form between two reaction products were statistically significant ($P < 0.0001$ by χ^2 test). These results confirm the inhibitory effects of template uracil on DNA synthesis by Pol B1. Form IV (DNA plus Pol B1) accounted for about 41% (36/88 total molecules) and 34% (69/201 total molecules), respectively, of total numbers of molecules in the two reaction products. These values are significantly larger than the percentages of Form IV observed in the samples of Control (13% = 43/343), U100 DNA (9% = 9/104), Control DNA plus Pol B1 (14% = 15/104) and U100 DNA plus Pol B1 (18% = 29/158), suggesting that DNA synthesis accelerates the formation of Form IV.

Discussion

Uracil is one of the most ubiquitously occurring mutagenic damages in DNA (Lindahl 1993). It is generated by deamination of cytosine in DNA as well as by incorporation of dUTP into DNA by DNA polymerases (Vassylyev & Morikawa 1996). Since the deamination of cytosine results in G:U base pairs, and the uracil in template DNA directs DNA polymerases to incorporate adenine opposite it, the hydrolytic reaction can convert original G:C base pairs to A:T base pairs in a half of the progeny. To exclude uracil from DNA, organisms possess various uracil-DNA glycosylases, which specifically excise uracil in DNA (Pearl 2000), and dUTPases, which hydrolyze dUTP to dUMP plus PPi to maintain low levels of dUTP in the cellular nucleotide pool (Hogrefe

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et al. 2002). In addition, hyperthermophilic archaeal B family DNA polymerases such as Pol B1 or Pfu possess unique mechanisms that recognize uracil in template DNA and inhibit the progress of DNA synthesis upstream from the lesion (Greagg *et al.* 1999). Given the genotoxicity of uracil in DNA, the recognition mechanism by the polymerases appears to contribute to the maintenance of genome integrity of the organisms living in the habitats at high temperatures.

To better understand the binding mechanisms, we examined the binding modes of Pol B1 to uracil in DNA by gel mobility shift assays and atomic force microscopy. We used the same ssDNA substrates, i.e. control and U100 DNA, whose molecular sizes are 1.4 kb, throughout the analyses. These DNAs were chosen because the linear form of dsDNA (Form II) can be clearly visible with AFM and is distinct from Form III (Pol B1). In addition, the ssDNA is efficiently converted to dsDNA by Pol B1 within 10 min at 55 °C (Fig. 2). The efficient conversion from ssDNA to dsDNA suggests that the ssDNA does not form substantial intramolecular base interactions at 55 °C, thereby allowing the progress of DNA polymerase on the template strand. Although Pol B1 itself could specifically bind to ssDNA containing uracil, the molar ratio between ssDNA containing uracil (U100 DNA) and Pol B1 required for the band shift was 1 : 200 in the absence of DNA synthesis (Fig. 1). The presence of uracil in the ssDNA strongly inhibited DNA syntheses by Pol B1 and Pfu exo$^-$ but not by Taq DNA polymerases (Fig. 2). The inhibitory effect was also clearly observed when the formation of dsDNA (Form II) in reaction products containing U100 DNA in the presence of Pol B1, primers and dNTPs was analyzed by atomic force microscopy (Table 1). The percentage of dsDNA was significantly lower in the reaction mixture containing U100 DNA (5%) than in that containing control DNA (25%).

Since the presence of uracil in DNA strongly inhibits the DNA synthesis by DNA pol B1, we postulated DNA pol B1 might bind to uracil in DNA in the progress of DNA synthesis more efficiently compared to during random diffusion in solution. Interestingly, the binding efficiency of Pol B1 to uracil was greatly enhanced by initiating DNA synthesis (Fig. 3). The molar ratio between U100 DNA and Pol B1 was 1 : 10 when the reaction mixtures contained primers and dNTPs necessary for DNA synthesis. Thus, we suggested that Pol B1 binds more efficiently to uracil when it proceeds along with template DNA for DNA synthesis rather than during random collision with DNA in solution (Fig. 5). This conclusion is consistent with the result that the percentage of Form IV (DNA plus DNA pol B1) in the mixture containing DNA pol B1 and U100 DNA was significantly higher in the presence of DNA synthesis (34%) than in the absence (18%) (Table 1). As described in the Introduction, the expression level of Pol B1 is 1500 molecules per cell, which is noticeably higher than the ones reported for *E. coli* DNA polymerases (Gruz *et al.* 2003). Because of the preferential binding of Pol B1 to uracil during DNA synthesis, we suggest that the majority of Pol B1 molecules are engaged in DNA replication and recognize uracil during DNA synthesis rather than directly binding to uracil in the non-replicating chromosome as lesion-specific binding proteins. Since Pol B1 is less sensitive in sensing uracil in the template strand than Pfu exo$^-$ (Gruz *et al.* 2003), which binds tightly to uracil in template/primer DNA (Shuttleworth *et al.* 2004), we suggest that Pol B1 might continue DNA synthesis beyond template uracil to some extent and randomly halt replication before template uracil along with template DNA (Fig. 5). In fact, we observed Pol B1 bound to uracil-containing DNA at various positions in the template DNA. It should be noted that Pol B1 possesses 3′ to 5′ exonuclease activity (Pisani & Rossi 1994). Thus, the primer strand may be digested at least in part when Pol B1 encounters uracil in template DNA during DNA synthesis *in vivo*.

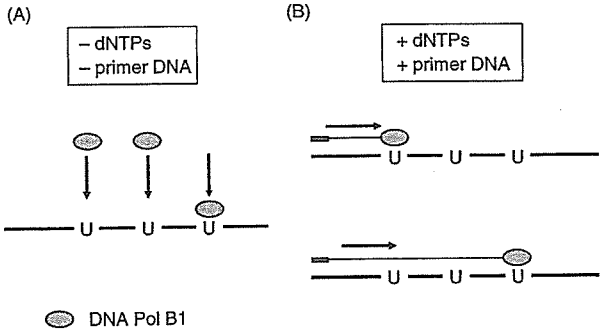

Figure 5 Schematic model of the mechanism of recognition of uracil in DNA by Pol B1. (A) Random diffusion-mediated recognition. Pol B1 randomly diffuses in solution and binds to uracil in DNA. (B) DNA replication-mediated recognition. Pol B1 slides on template DNA for elongation of primer and binds to uracil in DNA.

Since archaeal DNA polymerases bind to uracil-containing ssDNA, it is possible that one polymerase molecule actively extending the primer is blocked by a second polymerase bound to uracil in the template strand (Greagg *et al.* 1999). This "blocking" model seems plausible in particular for the long-range primer extension, and contrasts with the "read-ahead" model where single molecules of DNA polymerases directly bind to uracil in the template DNA and stop replication. To distinguish the two possibilities, we used AFM and suggested that Pol B1 bound to uracil-containing DNA as a monomeric

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form (Fig. 4). This finding directly supports the "read-ahead" model as a mechanism by which archaeal DNA polymerases halt copying DNA before template uracil. Since Form IV (DNA plus DNA pol B1) was observed even in "Control" or "U 100 DNA" samples where no Pol B1 was added, some of Form IV observed were not necessarily true replication intermediates where DNA polymerases bound to DNA. However, Form IV was more frequently generated when DNA replication was initiated (Table 1). Thus, we suggest that a significant portion of Form IV observed in the samples of "Control + Pol B1 + dNTPs + Primer" and "U100 DNA + Pol B1 + dNTPs +Primer" include the true replication intermediates. The sizes of the molecules bound to control DNA and U100 DNA in the presence of dNTPs and primers were indistinguishable. Hence, we suggested that monomeric forms of Pol B1 bind to uracil in template DNA. However, more extensive quantification of the size of many of the blobs at the end of the dsDNA in different conditions is needed for the final conclusion.

Both Pol B1 and Pfu exo$^-$ bind not only to template uracil but also to template hypoxanthine, a deamination product of adenine (Gruz *et al.* 2003). Pfu exo$^-$ stalls three to four bps before hypoxanthine as well as uracil in the template strand, and Pol B1 displays a similar stalling behavior. Hypoxanthine pairs with cytosine during DNA replication and induces A:T to G:C transitions if not repaired. Thus, this stalling behavior seems to suppress the incorporation of cytosine opposite template hypoxanthine, thereby reducing the mutagenic potential of hypoxanthine. Stalling Pol B1 at template uracil or hypoxanthine may generate ss gap DNA region downstream of the lesions. Recombination that seals the gap with DNA sequence of sister chromatid or translesion DNA synthesis with Y-family DNA polymerase may contribute to the gap filling (Fig. 6). In fact, *Sulfolobus solfataricus* possesses a homolog of Rad51, i.e. RadA, a recombination protein, and a homolog of *E. coli* DNA pol IV, i.e. *Sso* DNA pol Y1 or Dpo4, a Y-family DNA polymerase(Seitz *et al.* 1998; Gruz *et al.* 2001; Ohmori *et al.* 2001; She *et al.* 2001). In addition, it has uracil DNA glycosylase to excise uracil in DNA (She *et al.* 2001). Thus, it is important to investigate how these proteins are involved in the subsequent steps leading to repair of deaminated bases in archaeal DNA.

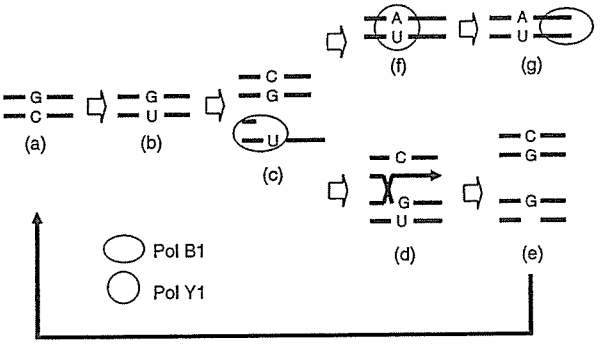

Figure 6 Possible mechanisms by which daughter strand gaps generated by stalling of Pol B1 at template uracil (or hypoxanthine) are sealed by homologous recombination and/or translesion bypass by Pol Y1. Cytosine in DNA (a) is deaminated by heat, thereby generating G:U mismatch (b). When the strand containing uracil is copied by Pol B1 before the uracil is removed by repair enzymes, Pol B1 may stop before uracil (c). ssDNA region downstream of the uracil can be sealed by homologous recombination with DNA sequence from sister chromatid (d). Uracil DNA glycosylase may excise the uracil (e) and provides a chance to regenerate normal G:C bp (a). However, if Pol B1 is switched to Pol Y1, the Y-family DNA polymerase may bypass uracil by incorporating adenine opposite template uracil (f). Then, another polymerase switch occurs and Pol B1 extends the primer strand containing adenine opposite uracil, thereby generating a mutagenic mispair in DNA (g).

Experimental procedures

Preparation of ssDNAs with or without uracil

TaKaRa Taq DNA polymerase, Pfu DNA polymerase exo$^-$, and FPLC-grade dNTPs were purchased from TaKaRa (Shiga, Japan), Stratagene (La Jolla, CA, USA) and Amersham Biosciences, respectively. Pol B1 was purified as previously described (Pisani & Rossi 1994). About 1.4 kb DNA was amplified by PCR using plasmid pUC118 DNA (3.2 kb), F1-biotin primer (5′ biotin-GGGAGAAAGGCGGACAGGTA-3′, 20 pmol), R2 primer (5′-GGCTGGCTTAACTATGCGGC-3′, 20 pmol), Taq DNA polymerase (1 unit) and four dNTPs (4 nmol each) in a total volume of 20 μL. Taq DNA polymerase almost equally incorporates dUTP and dTTP opposite template adenine (Lasken *et al.* 1996). Advantage was taken of this property and 1.4 kb DNA containing uracil was prepared by the same PCR conditions except for the presence of dUTP (4 nmol) instead of dTTP. The former and latter amplified DNAs were named "control DNA" and "U100 DNA," respectively. After the amplification, excess primers were digested with ssDNA specific exonuclease, i.e. ExoSAP-IT (Amersham Biosciences). The proteins were inactivated by heat and proteinase K treatments, and removed by phenol/chloroform/isoamylalcohol extraction. The DNAs were purified by ethanol precipitation, and re-suspended in TE buffer. After removal of low-molecular-weight contaminants by filtration with Microcon YM100 (Millipore, Bedford, MA, USA), ssDNAs were prepared by heating (100 °C × 5 min) followed by rapid cooling on ice. The resulting ssDNAs were used for the analysis with AFM. ssDNAs for gel shift assays were prepared with magnetic streptavidin bead according to the manufacturer's protocol (MAGNOTEX-SA, TaKaRa, Japan). Briefly, the filtrated DNAs having biotin were mixed with MAGNOTEX-SA, and ssDNAs without biotin

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were obtained in the supernatant after treatments with alkaline solution. The incorporation of uracil into DNA was confirmed by treating the DNAs with uracil DNA glycosylase (Gibco BRL, Gaithersburg, MD, USA), which converts uracil into abasic sites in DNA (Krokan *et al.* 2002). U100 DNA, but not control DNA, became a poor substrate for PCR amplification with Taq because of the generation of abasic sites.

Gel mobility shift assay

Interactions between Pol B1 and ssDNA with or without uracil were examined under various conditions, the details of which are described in the legends of Figs 1–4. Briefly, the reaction mixture (20 μL) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl and 1.5 mM $MgCl_2$ (Tris/KCl/$MgCl_2$). In addition, it contained F1 primer (5′-GGGAGAAAGGCGGACAGGTA-3′), four dNTPs, Pol B1 and ssDNA. When the interactions were analyzed without DNA synthesis, F1 primer and dNTPs were omitted from the mixture. The reaction was carried out at 55 °C and terminated by the addition of 1/10 volume of 40 mM ethylenediaminetetraacetic acid (EDTA) solution. The reaction products were separated by electrophoresis with 1% agarose gel, and transferred to nylon membrane (Hybond-N+, Amersham Biosciences) for Southern hybridization analysis. Probe DNA (0.7 kb) was prepared by PCR in a reaction mixture containing pUC118, F1 primer and R1 primer (5′-GGCCTCT-TCGCTATTACGCC-3′). R1 primer anneals a DNA sequence close to the multiple-cloning site of pUC118, while F1 and R2 primers anneal DNA sequences each 0.7 kb apart from the cloning site in opposite directions. The amplified DNA was fluorescently labeled and used for the hybridization with ECL Direct Nucleic Acid Labeling and Detection System (Amersham Biosciences). The hybridized DNA was quantified using a chemiluminescent detection system, i.e. ChemiDoc (Bio-Rad, Richmond, CA, USA).

AFM analysis

The reaction mixture (40 μL) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 20 pmol each of R2 and F1 primers, 8 nmol each of four dNTPs, 1 pmol Pol B1 and 2 pmol of ssDNA (control or U100 DNA). The reaction was carried out for 15 min at 55 °C, and the samples were directly deposited on to freshly cleaved and discharged mica, followed by a rinse with distilled water and blown dry with dry nitrogen. The plates were placed in a desiccator for 1 h before analysis. The samples were visualized with AFM (Model SPI 3800 N, Seiko Instruments Inc., Japan) in dynamic force mode (DFM) operation with a 20 μm scanner (Murakami *et al.* 2000, 2001). The cantilevers for the DFM-AFM (Micro Cantilever, Type SI-DF40, Seiko Instruments Inc., Japan) were used for the analysis. Images were collected at room temperature. Scan frequencies were typically 2.0 Hz, and all images contain 512 × 512 data points.

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Short communication

Detection of Genotoxic Nucleosides, 8-Hydroxydeoxyguanosine, 8-Hydroxyguanosine and Free Base 8-Hydroxyguanine, in Fish Food Products

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8-Hydroxydeoxyguanosine (8-OH-dG) is a DNA base modification induced by reactive oxygen species (ROS), and is analyzed in cellular DNA and urine as a marker of oxidative stress. We now report that 8-OH-dG, the ribonucleoside 8-OH-Guo and the free base 8-OH-Gua were detected in fish food products, such as salted dried sardines (Maruboshi) and dried small fishes (Iriko and Shirasu), which are often consumed in Japan. Water extracts of these fish products were analyzed by an HPLC-ECD method, using anion exchange- and reverse phase-columns. Various amounts of 8-OH-Gua (25 ng–22 μg/g), 8-OH-Guo (28–406 ng/g) and 8-OH-dG (5–60 ng/g) were detected in these foods. The amounts of 8-OH-dG and 8-OH-Guo increased upon heating or broiling of these fish products. This is the first report on the presence of genotoxic nucleoside analogues in food. These results provide the warnings that these nucleosides analogues might be food carcinogens, because they are incorporated into DNA by a salvage pathway and show genotoxicity.

Key words: Food mutagen, 8-hydroxyguanine, 8-hydroxydeoxyguanosine, 8-hydroxyguanosine

Introduction

8-Hydroxydeoxyguanosine (8-OH-dG) or 7,8-dihydro-8-oxodeoxyguanosine is a base modification produced in cellular DNA and the nucleotide pool by environmental agents and endogenous oxidative stress (1). The induction of mutations by 8-OH-dG formation in cellular DNA or by the incorporation of 8-OH-dGTP is well documented (2). 8-OH-dG is also widely used as a biomarker to assess cellular oxidative stress (3). In animal experiments many oxidative stress-inducing carcinogens increased the 8-OH-dG levels in the target organ DNA, suggesting a significant role of reactive oxygen species in carcinogenesis.

Recently, it was reported that 8-OH-dG in cell culture medium is incorporated into the DNA of human cultured cells possibly by a salvage pathway (4). The induction of apoptosis due to the incorporation of 8-OH-dG into DNA and its accumulation in OGG1-deficient human cells has also been reported (5). These facts are compatible with our previous observation that 8-OH-dG and the ribonucleoside 8-hydroxyguanosine (8-OH-Guo) induce sister chromatid exchange (SCE) in human lymphocytes (6). In this communication, we report that these genotoxic modified nucleosides, 8-OH-dG, 8-OH-Guo and the free base 8-hydroxyguanine (8-OH-Gua), are present in considerably large amounts in processed fish foods, such as salted dried sardines (Maruboshi) and dried small fishes (Iriko), which are often consumed in Japan.

Materials and Methods

Preparation of food extracts: The fish food products, Maruboshi, Iriko and Shirasu, and the meat and ham were bought at a supermarket. The Maruboshi, Iriko and meat were heated in a frying pan until they attained a light brown color. These food products (100–500 mg) were cut into small pieces and extracted with 1 ml of water at 4°C for 15 h. The resulting supernatants were properly diluted with water and mixed with the same volume of a dilution solution, containing 4% acetonitrile with 130 mM sodium acetate (pH 4.5) and 0.6 mM H_2SO_4. The solutions were centrifuged at 13,000 rpm for 5 min, and the supernatants obtained were subjected to HPLC analysis.

HPLC analysis: A 20 μL aliquot of each supernatant was injected into the HPLC-ECD apparatus. The HPLC conditions and apparatus are basically the same as those employed for the urinary 8-OH-dG analysis (7). This method uses an anion exchange column at the first step (HPLC-1) followed by separation with a reverse phase column (HPLC-2), which allows the

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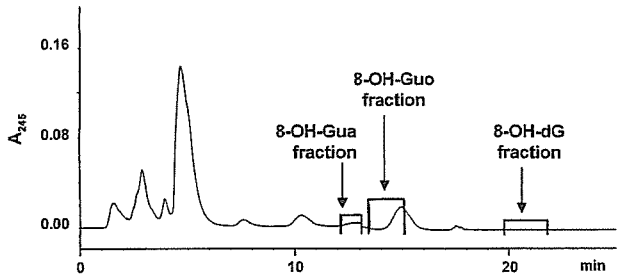

Fig. 1. HPLC-1 chromatogram of the Maruboshi sample. Column, manually packed anion exchange resin (MCI GEL CA08F, 7 μm, sulfate form); temperature, 65°C; elution speed, 50 μl/min; elution solvent, 2% acetonitrile in 0.3 mM sulfuric acid; detection, UV detector (Gilson UV/VIS-151), $A_{245\,nm}$.

detection of each 8-OH-Gua compound as a single peak by an EC detector. Prior to the analyses, the elution positions of 8-OH-dG, the ribonucleoside 8-OH-Guo and the free base 8-OH-Gua in HPLC-1 were determined by injecting standard compounds. After injecting the food extract into HPLC-1, the fraction containing 8-OH-dG, 8-OH-Guo or 8-OH-Gua was automatically injected into HPLC-2, by column switching technology. The detection limit for 8-OH-dG, 8-OH-Guo or 8-OH-Gua by this analytical method is about 5 pg.

Results

When food extracts were analyzed by the two step-HPLC system, using the anion exchange column (HPLC-1) and the reverse phase column (HPLC-2), all three 8-OH-Gua compounds were clearly detected as single peaks. As typical examples, HPLC-1 and -2 chromatograms of the Maruboshi sample are shown in Figs. 1 and 2, respectively. Various levels of 8-OH-Gua (25 ng–22 μg/g), 8-OH-Guo (28–406 ng/g) and 8-OH-dG (5–60 ng/g) were detected in these food extracts (Table 1). All of these foods contained higher levels of 8-OH-Guo than 8-OH-dG. Fresh Iriko contained an extremely high level of 8-OH-Gua (22 μg/g). Heating the fish foods (Maruboshi, Iriko, etc.) caused the amounts of 8-OH-dG and 8-OH-Guo to increase, while the amount of 8-OH-Gua decreased. On the other hand, cooked meat and a processed meat product, ham, showed lower levels of 8-OH-Gua compounds.

Discussion

One of the mechanisms of mutation is the incorporation of modified bases, ribo- and deoxyribo-nucleosides into DNA. DNA base- and nucleoside-analogues, such as 2-amino-N^6-hydroxyadenine and N^4-aminocytidine, have extremely high mutagenic activities (8,9). However, these compounds are synthetic compounds and are not detected in either food or the environment. 8-OH-Gua, 8-OH-dG and 8-OH-Guo are an oxidatively damaged guanine base and its nucleosides. We have

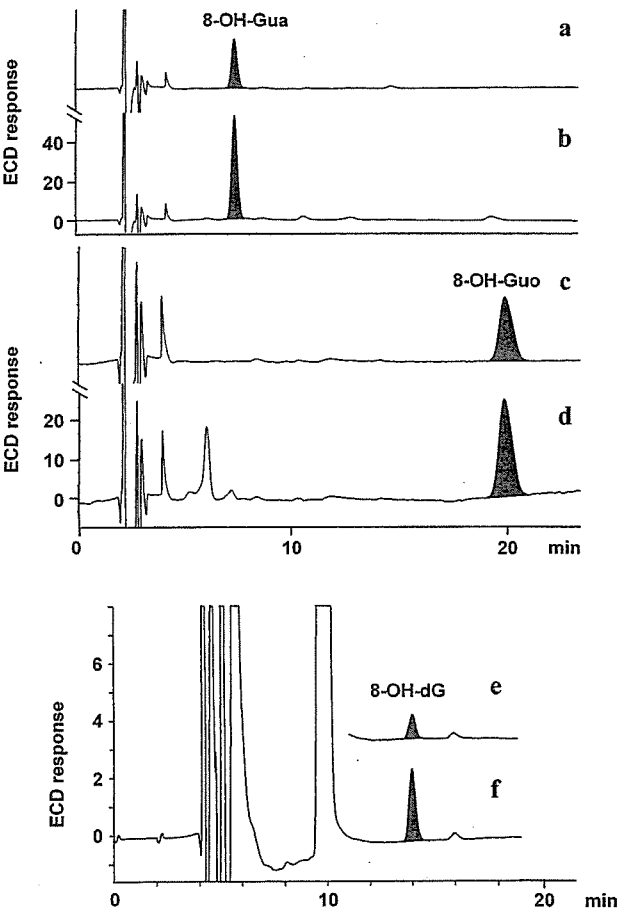

Fig. 2. HPLC-2 chromatograms to detect 8-OH-Gua, 8-OH-Guo and 8-OH-dG. a) standard 8-OH-Gua (220 pg); b) 8-OH-Gua fraction obtained by HPLC-1; c) standard 8-OH-Guo (500 pg); d) 8-OH-Guo fraction obtained by HPLC-1; e) standard 8-OH-dG (50 pg); f) 8-OH-dG fraction obtained by HPLC-1. Column, Shiseido Capcell Pak C18, (5 μm, 4.6×250 mm); elution solvent, 7% (for 8-OH-dG analysis) or 2% (for 8-OH-Gua and 8-OH-Guo analyses) methanol in 10 mM sodium phosphate buffer (pH 6.7); elution speed, 1 mL/min; detection, electrochemical detector (Eicom ECD-300, 550 mV).

Table 1. Contents of 8-OH-dG, 8-OH-Guo and 8-OH-Gua in various foods (ng/g)

Food	8-OH-dG	8-OH-Guo	8-OH-Gua
Maruboshi	35.2	190	114
Heated Maruboshi	60.5	315	413
Iriko	5.53	76.1	22000
Heated Iriko	33.9	406	2700
Old Iriko	21.3	141	820
Shirasu	5.54	28.3	101
Heated meat	ND	ND	24.9
Ham	ND	4.5	4.6

ND, not determined.