Mutations Induced by 2-Hydroxyadenine on a Shuttle Vector during Leading and Lagging Strand Syntheses in Mammalian Cells†

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ABSTRACT: An oxidatively damaged base, 2-hydroxyadenine (2-OH-Ade), was incorporated into a predetermined site of one of the strands {(+)- or (−)-strand} of the double-stranded shuttle vector, pSVK3, and the modified DNAs were transfected into simian COS-7 cells. The nucleotide sequences in which the modified base was incorporated were 5′-GTCGA*C and 5′-CTTA*AG (A* represents 2-OH-Ade). The former is the recognition site for the restriction enzyme *Sal*I, and the latter is that for *Afl*II. The DNAs replicated in the cells were recovered and were transfected again into *Escherichia coli*. The DNAs recovered from the COS-7 cells transfected with a plasmid containing 2-OH-Ade at either site of the (+)-strand (a template strand for lagging strand synthesis) formed colonies about 50%−70% as frequently as the unmodified DNA. This indicated that the base weakly blocked DNA replication during lagging strand synthesis. On the other hand, the base in the (−)-strand did not appear to affect the efficiency of leading strand synthesis in COS-7 cells. The mutation frequencies of 2-OH-Ade in COS-7 cells were 0.6%−0.1%, depending on the sequence and the strand location. Although the mutation spectra of 2-OH-Ade also differed with sequences and strands, the base elicited substitution and deletion mutations in mammalian cells, as in *E. coli*. These results indicate that 2-OH-Ade is mutagenic in eukaryotic cells as well as in prokaryotic cells.

Oxidative DNA damage produced by reactive oxygen species (ROS)[1] is an important source of mutations (Ames, 1983). The ROS are generated during the normal cellular respiration and oxygen metabolism. Thus, oxidative DNA lesions contribute to spontaneous mutations. In addition, some mutagens/carcinogens induce the formation of oxidative damage. Therefore, oxidative DNA lesions appear to be involved in many mutagenesis and carcinogenesis pathways that occur in organisms. However, only a few types of oxidative DNA lesions have been studied in detail. One well-investigated lesion is 8-hydroxyguanine (8-OH-Gua) (Kasai & Nishimura, 1984), and its abilities to induce mispairing and mutations have been shown (Kamiya et al., 1992a,b, 1995a,b; Cheng et al., 1992; Shibutani et al., 1991; Wood et al., 1990; Moriya, 1993). The mutagenic capacity of another lesion, 5-hydroxycytosine, has also been reported (Purmal et al., 1994; Feig et al., 1994). However, it is necessary to know the mutational properties of other forms of oxidative damage in order to reveal the overall effects on the mutations induced by ROS.

Treatment of DNA and its related compounds with a Fenton-type ROS-generating system (Fe^{2+}-EDTA) induces the formation of 2-hydroxyadenine (2-OH-Ade, 1,2-dihydro-2-oxoadenine, or isoguanine) from adenine. Its yield is very similar ($2-{}^{1}/_{6}$-fold) to that of 8-OH-Gua in monomers, but its formation in DNA is less efficient (1/40) than 8-OH-Gua (Kamiya & Kasai, 1995; Murata-Kamiya et al., 1997). It was reported that 2-OH-Ade is formed in DNA by the treatment of cultured human cells with H_2O_2 and that its amount is one-fifth that of 8-OH-Gua (Jaruga & Dizdaroglu, 1996). DNA polymerases induce the formation of "incorrect" base pairs involving the modified base, which is either in the DNA or present as the nucleotide, in *in vitro* DNA synthesis (Kamiya & Kasai, 1995, 1996; Kamiya et al., 1995c). Moreover, the base is as mutagenic as 8-OH-Gua in *Escherichia coli* (Kamiya & Kasai, 1997). Therefore 2-OH-Ade may be another type of major mutagenic DNA lesion produced by ROS, and further investigation of its mutational properties and, in particular, its mutagenicity in mammalian cells is important.

To study the frequency and the spectrum of mutations induced by 2-OH-Ade in mammalian cells, we incorporated the oxidized base into unique, predetermined sites in double-stranded (ds) vectors. The bases were incorporated into two different sequence contexts, in either the leading or the lagging template strand, and the mutational properties were investigated with simian COS-7 cells. We observed that (i) the 2-OH-Ade residue induced a weak DNA replication block during lagging strand synthesis, while the base elicited a slight, if any, replication block during leading strand synthesis. We found that (ii) the 2-OH-Ade residue was mutagenic in COS-7 cells. Moreover, we revealed that (iii) the mutation spectra elicited by 2-OH-Ade were affected by the sequence contexts and the strands within which the base was located, as in bacteria.

MATERIALS AND METHODS

Materials. pSVK3 was purchased from Pharmacia Biotech Inc. COS-7 cells were from the RIKEN Cell Bank

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[1] Abbreviations: ROS, reactive oxygen species; 2-OH-Ade, 2-hydroxyadenine; 2-OH-dATP, 2-hydroxy-2′-deoxyadenosine 5′-triphosphate; 8-OH-Gua, 8-hydroxyguanine; MF, mutation frequency; ds, double-stranded.

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(Tsukuba, Japan). *E. coli* strain DH5α cells {F^-, $\phi 80d$ *lacZ*ΔM15 Δ (*lacZ*YA-*argF*) U169, *endA*1, *recA*1, *hsdS*17 ($r\kappa^- m\kappa^+$), *deoR*, *thi*-1, *supE*44, λ^-, *gyrA*96, *relA*1} for $CaCl_2$ transformation were prepared according to the method described by Sambrook et al. (1989).

Construction of Vectors. The oligonucleotides indicated below were synthesized and purified as described (Kamiya & Kasai, 1997).

A-17, 5′ dGGTCGACTTAAGGTACC 3′

2-OH-Sal, 5′ dGGTCGA*CTTAAGGTACC 3′

2-OH-Afl, 5′ dGGTCGACTTA*AGGTACC3′

splint-1,
5′ dTCGAGGTACCTTAAGTCGACCGTAC 3′

splint-2,
5′ dAATTGGTACCTTAAGTCGACCGGCC 3′

where A* represents 2-OH-Ade.

The ds vectors were constructed as described (Kamiya & Kasai, 1997).

DNA Transfection into COS-7 Cells and Recovery of Plasmid. COS-7 cells (5×10^5 cells) were plated into a 6-cm dish and were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum at 37 °C under a 5% CO_2 atmosphere for 24 h. The constructed ds vectors (10 ng) were transfected into the cultured COS-7 cells by using Lipofectamine (Life Technologies Inc.) according to the supplier's recommendations. After 48 h, the plasmid amplified in the cells was recovered by the method of Stary and Sarasin (1992). The recovered DNA was treated with *Dpn*I to digest unreplicated plasmids. After the removal of proteins by passage through Probind (Millipore Co.) the DNA was purified by ethanol precipitation.

Calculation of Cytotoxicity of 2-OH-Ade in COS-7 Cells. *E. coli* DH5α cells were transfected with the recovered plasmid by the calcium chloride method (Sambrook et al., 1989). To measure the cytotoxicity, a small portion of the recovered DNA was used. The numbers of bacterial colonies were used for the calculation of the cytotoxicities in COS-7 cells.

Mutant Screening and Sequencing. The plasmid recovered from the COS-7 cells was digested with either *Sal*I or *Afl*II under the conditions recommended by the suppliers. The digested DNA was transfected into *E. coli* strain DH5α. Undigested DNA in the same buffer solution was also transfected, and the ratio of colonies obtained with digested DNA to colonies obtained with undigested DNA was calculated. This value (defined as *A*) was used for calculation of mutation frequency (MF) as described below.

In all, 350−2000 colonies were isolated from the *E. coli* colonies obtained with the digested DNAs (the "mutant" pool) and the plasmid was recovered from each colony by the alkaline lysis method (Sambrook et al., 1989). Each plasmid DNA was screened by dot blot hybridization using either A-17 or splint-1 and -2 as a probe (Kamiya & Kasai, 1997). The plasmids that were judged as mutants by the hybridization were treated with the targeted restriction enzyme, and no cleavage was confirmed.

Primer-1 (5′-dCCTCTGAGCTATTCCAGA 3′) and primer-2 (5′ dAATTTCTGCCATTCATCCG 3′) for the sequencing reactions were purchased from Hokkaido System Science Co. (Sapporo, Japan) in purified forms. The nucleotide sequences of the mutants were analyzed by plasmid sequencing with either primer-1 or -2 and the ABI PRISM Dye Terminator Cycle Sequencing Kit (Perkin-Elmer) in an Applied Biosystems model 373S DNA sequencer (Perkin-Elmer).

Calculation of Mutation Frequency. By the screening and the subsequent sequencing experiments, the plasmids in the "mutant" pool that were "resistant" to the restriction enzyme were divided into four categories: (1) plasmids with a mutation in the target site, (2) plasmids with mutation(s) in the enzyme site, (3) undigested, nonmutant plasmids, and (4) plasmids containing a deletion derived from error-prone replication in mammalian cells (Calos *et al*., 1983; Razzaque *et al*., 1983; Moriya, 1993). Almost all of the "resistant" DNAs belonged to the fourth group. The plasmids in the first three groups and the digested fraction were judged as effective. The MF, (mutant colonies)/(effective colonies), was calculated as follows:

$$\mathrm{MF} = (AB)/(1 - AC)$$

where $A =$ (colonies obtained with digested DNA)/(colonies obtained with undigested DNA); $B =$ (targeted mutant colonies)/(colonies screened from the mutant pool); $C =$ (colonies with a random deletion)/(colonies screened from the "mutant" pool); and $1 - AC =$ the ratio of effective colonies.

RESULTS

Vectors. DNA polymerases incorporate incorrect nucleotides, in addition to dTMP, opposite 2-OH-Ade *in vitro* in a sequence-dependent manner (Kamiya et al., 1995c; Kamiya & Kasai, 1996). Mammalian DNA polymerases α and β misinsert dCMP most frequently opposite 2-OH-Ade, and 2-OH-Ade in a 5′-TA*A context (A* represents 2-OH-Ade) induces misincorporation of dAMP (Kamiya et al., 1995c; Kamiya & Kasai, 1996). On the basis of these findings, we previously incorporated 2-OH-Ade into 5′-CTTA*AG (an *Afl*II site) and 5′-GTCGA*C (a *Sal*I site) sequences to investigate the mutational properties of the oxidized adenine in the 5′-TA*A context or in other sequences in *E. coli* (Kamiya & Kasai, 1997). It was found that 2-OH-Ade induces targeted substitution and deletion mutations in bacteria. Furthermore, the MF and the mutation spectrum are dependent on both the sequence contexts and the strand location (leading versus lagging strand synthesis). We used the same approach and the same vector (pSVK3, Figure 1) in this study. The shuttle plasmid pSVK3 has the bidirectional SV40 origin, which allows replication in COS-7 cells, in addition to the *E. coli* Co1E1 origin. The (+)-strand of pSVK3 (we define the (+)-strand as the same strand of single-stranded pSVK3 that is produced by the infection of helper phage) is replicated during lagging strand synthesis around the region where the linker DNA with 2-OH-Ade was inserted (Figure 1). Accordingly, the (−)-strand of pSVK3 around the inserted site is replicated by the leading strand apparatus. This is the same pattern as in *E. coli*, in which the vector is replicated under the control of the unidirectional Co1E1 origin (Figure 1). Thus, the mutational properties of 2-OH-Ade in the same sequence contexts located on lagging and leading template strands can be compared in the bacterial and mammalian systems.

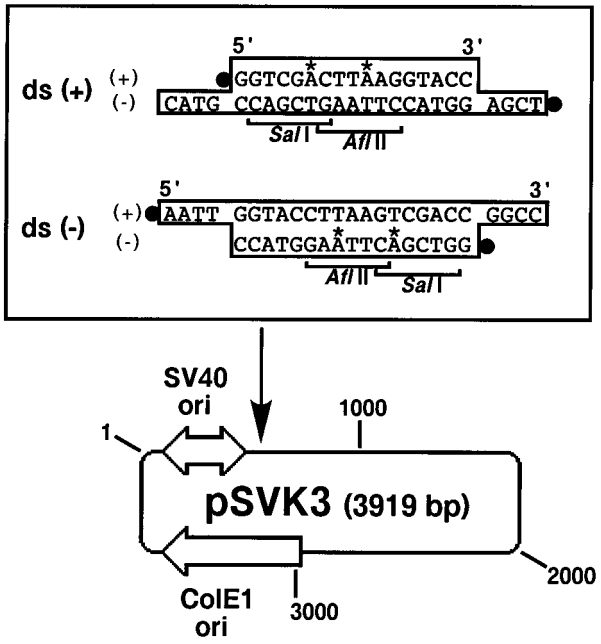

FIGURE 1: Insertion of double-stranded oligonucleotides with 2-OH-Ade into the shuttle vector pSVK3. The oligonucleotides contain ends that are compatible with the restriction enzyme-cleaved ends within the multiple cloning site. Closed and open circles represent 5′-phosphate and 5′-hydroxyl groups, respectively. The A* indicates the position where the 2-OH-Ade or the unmodified adenine was incorporated. The SV40 origin and the Co1E1 origin, which work in COS-7 cells and *E. coli*, respectively, are also shown.

We used the following nomenclature: (+)-*Sal*I and (+)-*Afl*II as the vectors containing 2-OH-Ade in the *Sal*I and *Afl*II sites, respectively, in the (+)-strand. The (+)-control vector has the same sequence with an unmodified adenine instead of 2-OH-Ade. The (−)-*Sal*I, (−)-*Afl*II, and (−)-control are named similarly, although the strand of interest is the (−)-strand (Figure 1).

Cytotoxicity of 2-OH-Ade in COS-7 Cells. We first evaluated the cytotoxicities of 2-OH-Ade in COS-7 cells to determine whether the base blocks DNA replication. First, we transfected the parental vector pSVK3 into COS-7 cells and the plasmid recovered from the cells was introduced into *E. coli* strain DH5α. We observed that the number of colonies and the amount of DNA transfected into COS-7 cells exhibited a linear correlation between 0 and 10 ng under our conditions (data not shown). This means that the number of relative bacterial colonies reflects the relative amount of plasmid DNA replicated in COS-7 cells. Thus, the number of *E. coli* colonies was a good indicator for the evaluation of the cytotoxicity of 2-OH-Ade residues in COS cells.

Modified and unmodified vectors (10 ng) were transfected into COS-7 cells and were allowed to replicate in the cells. The plasmid DNAs recovered from the cells were transfected into DH5α cells, and the colonies formed were counted. The numbers of *E. coli* colonies derived from (+)-*Sal*I and (+)-*Afl*II were 50%−70% of the (+)-control (Table 1). Since the 2-OH-Ade residues in the (+)-vectors were located on the template strand of lagging strand synthesis (Figure 1), these results mean that the oxidized base weakly blocked the lagging strand synthesis. On the other hand, the numbers of bacterial colonies derived from (−)-*Sal*I and (−)-*Afl*II were similar to those of the control vector (Table 1), showing that the replication block by 2-OH-Ade was negligible during leading strand synthesis. These data are consistent with the *in vitro* results that the existence of 2-OH-Ade only partially retards DNA synthesis (Kamiya et al., 1995c).

Table 1: Cytotoxicities of 2-OH-Ade Residues in COS-7 Cells[a]

vector	double strand (+), *lagging*	double strand (−), *leading*
control	100[b]	100[b]
GTCGA*C (*SalI*)[c]	54	103
CTTA*AG (*Afl*II)	69	91

[a] Percentage of colonies resulting from transformation of *E. coli* DH5α cells with the plasmid DNA recovered from COS-7 cells. [b] This value is defined as 100 for each experiment. The actual number of colonies ranged between 500 and 700. [c] A* represents 2-OH-Ade.

Table 2: Mutations Induced by 2-OH-Ade Residues in COS-7 Cells

vector	mutation frequency	targeted mutations[a] A→G	A→T	A→C	ΔA
double strand (+), *lagging*					
GTCGA*C (*SalI*)[b]	0.60	3 (13)	0 (0)	0 (0)	21 (88)
CTTA*AG (*Afl*II)	0.09	6 (46)	1 (8)	0 (0)	6 (46)
double strand (−), *leading*					
GTCGA*C (*Sal*I)	0.10	4 (29)	3 (21)	0 (0)	7 (50)
CTTA*AG (*Afl*II)	0.11	10 (67)	5 (33)	0 (0)	0 (0)

[a] Number of mutants observed. Percentage of each targeted mutation is represented in parentheses. [b] A* represents 2-OH-Ade.

2-OH-Ade Is Mutagenic in Mammalian Cells. We introduced 2-OH-Ade residues into unique restriction enzyme sites, and thus we could select mutants as bacterial cells with a plasmid resistant to the restriction enzyme. However, almost all of the *E. coli* colonies with the restriction enzyme-resistant plasmids contained random deletions that occurred in the mammalian cells (Calos et al., 1983; Razzaque et al., 1983; Moriya, 1993). We used dot blot hybridization to select against this type of event (Kamiya & Kasai, 1997). By this technique, we obtained plasmids mutated within the inserted linker region. The existence of the mutation was confirmed by digestion of the plasmid with the targeted restriction enzyme.

Table 2 shows the MFs of the (+)- and (−)-vectors with 2-OH-Ade. The 2-OH-Ade residues in the *Sal*I site in the (+)-DNA induced mutations with an efficiency of 0.60%. The maximum MF was expected to be 50% because the complementary strand is also replicated. Thus, 1.2% of the 2-OH-Ade residues were estimated to induce misincorporation of nucleotides during lagging strand synthesis. On the other hand, the MF of the 2-OH-Ade residues in the *Afl*II site was 0.09% during lagging strand synthesis (Table 2). These values were comparable to the MF of 8-OH-Gua in a ds vector in *E. coli* (Cheng et al., 1992; Wagner et al., 1997) and with that of the modified guanine on a chromosome in NIH3T3 cells (Kamiya et al., 1992a, 1995b).

The 2-OH-Ade bases in both sites of the ds (−)-vectors induced mutations with an efficiency of about 0.1% (Table 2). The fact that the MFs of 2-OH-Ade in the same sequence in different strands were not identical indicates that the ratios of misincorporation were affected by the leading or lagging strand synthesis during replication.

Mutation Spectra of 2-OH-Ade in COS-7 Cells. We analyzed the sequences of the mutants obtained with the (+)-*Sal*I DNA. The mutation detected most frequently was a −1 deletion (ΔA, 21 colonies, Table 2). Three colonies contained A→G transitions, which were consistent with the

results (incorporation of dCMP opposite 2-OH-Ade) obtained in our previous *in vitro* experiments (Kamiya & Kasai, 1996). Moreover, the observed deletion mutations (ΔA) appeared to occur due to the incorporation of dCMP by the loop-out mechanism [Figure 3 in Kamiya and Kasai (1997)] (Kunkel, 1990). Therefore, the results agreed with our previous findings in an *in vitro* system.

The ΔA mutation was also detected in six colonies that were derived from the (+)-*Afl*II vector (Table 2). This type of mutation in the *Afl*II site is postulated to be due to the incorporation of dAMP opposite the oxidized adenine (Kamiya & Kasai, 1997). The same number of colonies contained targeted A→G transitions, indicating that the incorporations of dCMP and dAMP opposite 2-OH-Ade in the 5′-TA*A sequence occurred with similar efficiencies during lagging strand synthesis in COS-7 cells.

In the case of the (−)-*Sal*I vector, −1 deletions (ΔA), A→G transitions, and A→T transversions were observed (seven, four, and three cases, respectively) (Table 2). As described above, the observed deletion mutations appeared to be due to the expected incorporation of dCMP. Thus, eleven of the fourteen mutations would be derived from the incorporation of dCMP. On the other hand, ten and five cases were A→G transitions and A→T transversions, respectively, in the *Afl*II site (Table 2). No deletion mutants were detected. Thus, the ratios of dCMP to dAMP incorporated opposite 2-OH-Ade in the *Sal*I and *Afl*II sites were expected to be 11:3 and 2:1, respectively, during leading strand synthesis.

DISCUSSION

We have used a site-specific mutagenesis approach to investigate the mutational properties of 2-OH-Ade in mammalian cells. The vector used in this study contains the SV40 origin (Figure 1), and the (+)- and (−)-strands near the region where the 2-OH-Ade residues were inserted appeared to be replicated during lagging and leading strand syntheses, respectively. In previous experiments this plasmid was used as the parental vector (Kamiya & Kasai, 1997), and it contains the unidirectional Co1E1 origin (Figure 1). Thus, the results obtained with bacteria and COS-7 cells can be compared easily.

During lagging strand synthesis in COS-7 cells, the 2-OH-Ade residue in the *Sal*I site elicited more mutations than that in the *Afl*II site (Table 2 and Figure 2). This fact is consistent with the observations in our previous study using *E. coli* as a host (Kamiya & Kasai, 1997). The frequency of the mutations by the base in the *Sal*I site was higher during lagging strand synthesis than during leading strand replication in both COS-7 and bacterial cells. However, the frequency by the base in the *Sal*I site was similar to that in the *Afl*II site during leading strand synthesis was different from the observations in *E. coli*. As a result, the 2-OH-Ade residue in the *Sal*I site induced the targeted mutations most frequently during lagging strand synthesis in both COS-7 and *E. coli* cells (Figure 2). The 2-OH-Ade residue in the *Afl*II (5′-TA*A) sequence was a rather exceptional sequence, because the incorporation of dAMP opposite 2-OH-Ade is predominant in *in vitro* DNA synthesis (Kamiya et al., 1995c; Kamiya & Kasai, 1996). Therefore, the formation of 2-OH-Ade opposite T in DNA will induce targeted mutations at a frequency of 0.1%−0.6%. The maximum MF was expected

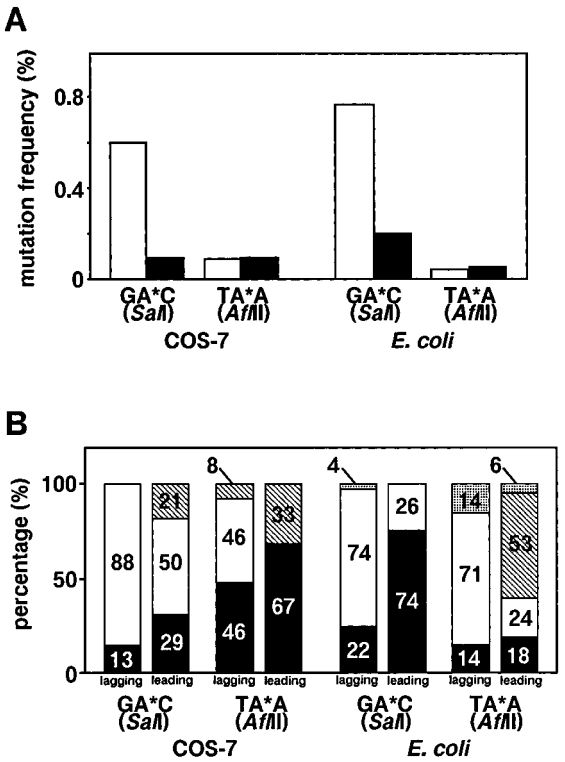

FIGURE 2: Comparison of mutations induced by 2-OH-Ade residues in COS-7 cells and *E. coli*. (A) Mutation frequencies in COS-7 cells and *E. coli* are shown. Open and closed bars represent the MFs during lagging and leading DNA syntheses, respectively. (B) Content of the induced mutations. Closed box, A→G; open box, ΔA; hatched box, A→T; dotted box, A→C. The COS-7 cell data are from Table 2. The *E. coli* data are from Kamiya and Kasai (1997).

to be 50%, and thus, 2-OH-Ade in DNA appears to induce targeted mutations within the range of 0.2%−1.2% in COS-7 cells.

Similar MFs were obtained in *in vitro* and prokaryotic systems (Kamiya et al., 1995c; Kamiya & Kasai, 1996, 1997). In the case of 8-OH-Gua, the observed MFs in prokaryotic and mammalian systems are much lower than that expected from the results of the *in vitro* system (Cheng et al., 1992; Wagner et al., 1997; Kamiya et al., 1995a,b; Shibutani et al., 1991) and the MFs induced by 8-OH-Gua in repair-deficient *E. coli* strains are very close to the *in vitro* results (Wagner et al., 1997). These results suggest that in cells, 2-OH-Ade showed similar MFs to those found *in vitro* because the oxidized adenine is not repaired efficiently in cells. In fact, we observed that crude extracts of *E. coli* and rat tissues poorly nicked a ds DNA fragment with 2-OH-Ade (Tsurudome et al., unpublished results). Moreover, Jaruga and Dizdaroglu observed that the removal of 2-OH-Ade formed in the DNA of H_2O_2-treated cells is very slow (Jaruga & Dizdaroglu, 1996). Thus, it is likely that the 2-OH-Ade residues in DNA showed similar MFs to that of 8-OH-Gua, since the repair of 2-OH-Ade is much less efficient than that of 8-OH-Gua.

In the 5′-GA*C sequence (*Sal*I site), 2-OH-Ade induced deletion (ΔA) and A→G substitution mutations, both of which appeared to be derived from the incorporation of dCMP opposite 2-OH-Ade. In *E. coli*, the ratios of the deletion to the substitution are 74:22 (∼3:1) during lagging strand synthesis and 26:74 (∼1:3) during leading strand synthesis (Figure 2). This "reversed" tendency may be

generated from the difference in the efficiency of the extension of the 2-OH-Ade:C pair (Kamiya & Kasai, 1997). In COS-7 cells, the ratios of the deletion to the substitution are 88:13 (~7:1) during lagging strand synthesis and 50:29 (~7:4) during leading strand synthesis (Figure 2). The ratio of the A→G transition increased in leading strand synthesis, as in *E. coli*. These results suggest that the 2-OH-Ade:C pairs were extended more easily by the leading strand replication proteins than the lagging strand apparatus in mammalian cells. The incorporation of dAMP (A→T transversion) by the leading strand apparatus was observed in COS-7 cells.

The 2-OH-Ade residue in the 5′-TA*A sequence (*Afl*II site) elicited deletions and A→T and A→G substitutions. Again, the deletion and the A→T transversion appeared to be derived from the formation of a 2-OH-Ade:A pair. In *E. coli*, the ratios of dAMP to dCMP incorporated (the ratios of the deletion plus the A→T transversion to A→G transition) are similar with both vectors (71:14 and 77:18, Figure 2). On the other hand, the ratios of dAMP to dCMP incorporated were 54:46 (~1:1) and 33:67 (1:2) during lagging and leading strand syntheses, respectively, in COS-7 cells. It is noteworthy that dCMP was incorporated predominantly opposite 2-OH-Ade in the 5′-TA*A sequence in COS-7 cells. This is in contrast to the observation that DNA polymerases α and β do not insert dCMP opposite the modified adenine in the 5′-TA*A sequence *in vitro* (Kamiya et al., 1995c; Kamiya & Kasai, 1996). The ratios of the A→T mutation to the deletion generated during leading strand synthesis were higher than those detected during lagging strand replication in both COS-7 and *E. coli* cells (Figure 2). These findings also suggest that 2-OH-Ade:A "mispairs" were extended more easily by the leading replication proteins than the lagging strand apparatus (Kamiya & Kasai, 1997).

The MFs during leading and lagging strand syntheses in bacteria and by human DNA polymerases have been compared by some investigators. It appeared that the misincorporation rates opposite some modified and unmodified bases are higher during lagging strand synthesis than during leading strand synthesis in bacteria (Veaute & Fuchs, 1993; Iwaki et al., 1996; Kamiya & Kasai, 1997), although similar fidelities were also observed (Wagner et al., 1997). The different (Thomas et al., 1993; Roberts et al., 1994; Calcagnile et al., 1996) and similar (Roberts et al., 1991; Izuta et al., 1995) fidelities of leading and lagging strand syntheses by human DNA polymerases have been reported by the use of *in vitro* SV40 origin-dependent replication systems. In this study, we showed that the fidelities of the leading and lagging DNA replication apparatuses were different in COS-7 cells. The MF of 2-OH-Ade in the *Sal*I site during lagging strand synthesis was higher than that during leading strand replication (Table 2), and the mutation spectra of the bases in both strands were different (Table 2). Therefore, the fidelities of leading and lagging strand syntheses appear to be different in cultured mammalian cells, as in *E. coli* and in the *in vitro* replication system. Basic-Zaninovic et al. analyzed mutations induced by *N*-methyl-*N*-nitrosourea in a reporter gene in cultured human cells (1992). They reported that the distribution and the MF at each position were not identical, although the overall error rates opposite methylated DNA lesion(s) are not affected by leading and lagging strand syntheses and half of the mutated positions are in common. Thus, our results appear to be consistent with their results regarding the point that the misincorporation rates opposite 2-OH-Ade or the methylated lesion(s) (probably O^6-methylguanine) of the leading and lagging strand apparatuses are not completely identical. The variations may be due in part to the differences in the of DNA polymerases and the other accessory proteins involved in DNA replication (Wang, 1991).

We have studied the mutational properties of 2-OH-Ade in DNA in *in vitro*, prokaryotic, and mammalian systems. We carried out *in vitro* DNA synthesis experiments with 12 oligonucleotide templates containing 2-OH-Ade and found that the mammalian DNA polymerases α and β misinsert dCMP most frequently opposite the base, and that 2-OH-Ade in a 5′-TA*A sequence (of two oligonucleotide templates) induces misincorporation of dAMP (Kamiya et al., 1995c; Kamiya & Kasai, 1996). In the site-specific mutagenesis experiments with bacterial and mammalian cells, similar results were obtained. The 2-OH-Ade residues in the *Afl*II (5′-TA*A) and *Sal*I (5′-GA*C) sites induced a deletion plus an A→T transversion and a deletion plus an A→G transition, respectively (Figure 2). The deletions were assumed to be caused by the incorporations of dAMP and dCMP, respectively. Thus, these results were consistent with the *in vitro* data.

Incorporations of dCMP opposite the 2-OH-Ade residues in the *Afl*II site were observed in *E. coli* and particularly in COS-7 cells (Figure 2). Moreover, the incorporation of dAMP opposite the base in the *Sal*I site of the leading template strand was observed in COS-7 cells. These results suggest that the DNA polymerase III of *E. coli* and the simian DNA polymerase δ have different properties regarding the insertion of nucleotides opposite 2-OH-Ade. Alternatively, an accessory protein involved in replication may modify the fidelity of the DNA polymerases as reported (Shavitt & Livneh, 1989; O'day et al., 1992; Tadmor et al., 1992; Suzuki et al., 1994; Mozzherin et al., 1996). However, the actual reasons for these discrepancies remain to be resolved.

We assume that 2-OH-Ade in DNA (paired with T) will mainly elicit an A→G transition because (i) the incorporation of dCMP opposite the modified adenine is observed *in vitro* with most of the template oligonucleotides used (Kamiya & Kasai, 1996) and (ii) a deletion will not be produced by the loop-out mechanism when the 5′-flanking base of 2-OH-Ade is not G. The facts that the formation of 2-OH-Ade by ROS occurs in monomers (dATP or dA) much more efficiently than in DNA and that the incorporation of the 2-OH-dATP by DNA polymerases is as efficient as that of 8-hydroxy-2′-deoxyguanosine 5′-triphosphate (Kamiya & Kasai, 1995) suggest that the incorporation of 2-OH-dATP by DNA polymerases is a major pathway in the formation of 2-OH-Ade in DNA. Taken together, the observations that the oxidized nucleotide is incorporated opposite T and C residues in DNA, in a ratio of about 4.5:1 (Kamiya & Kasai, 1995) and that the nucleotide incorporated most frequently opposite 2-OH-Ade in DNA was dTMP in COS-7 cells, the formation of 2-OH-dATP by the ROS may induce a G·C→A·T transition, which was expected in the *E. coli* case (Kamiya & Kasai, 1997). This type of mutation was detected most frequently in the *E. coli supF* gene replicated in H_2O_2-treated simian cells (Moraes et al., 1990).

In this paper, we observed that 2-OH-Ade in DNA was as mutagenic as 8-OH-Gua in COS-7 cells and induced A·T→G·C transitions. Taken together with other data, the

formation of 2-OH-Ade in the nucleotide pool will induce G·C→A·T mutations. The formation of this oxidative base is an important mechanism in ROS-mediated mutagenesis.

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