2-Hydroxy-2′-deoxyadenosine 5′-Triphosphate Enhances A·T → C·G Mutations Caused by 8-Hydroxy-2′-deoxyguanosine 5′-Triphosphate by Suppressing Its Degradation upon Replication in a HeLa Extract†

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ABSTRACT: The coexistence effects of multiple kinds of oxidized deoxyribonucleotides were examined using an SV40 origin-dependent in vitro replication system with a HeLa extract. Oxidized dGTP and dATP, 8-hydroxy-2′-deoxyguanosine 5′-triphosphate (8-OH-dGTP) and 2-hydroxy-2′-deoxyadenosine 5′-triphosphate (2-OH-dATP), were used in this study. The mutation frequency synergistically increased when the two oxidized deoxyribonucleotides were together in the reaction. 2-OH-dATP enhanced the mutagenicity of 8-OH-dGTP, since the induced mutations were A·T → C·G transversions. The contribution of the highly error-prone DNA polymerase η was unlikely, since similar results were observed with an XP-V cell extract. The possible involvement of 2-hydroxyadenine in the complementary (template) strand was excluded on the basis of experiments using plasmids containing 2-hydroxyadenine as templates in the reactions with 8-OH-dGTP. 2-OH-dATP suppressed hydrolysis of 8-OH-dGTP, suggesting that the inhibition of the MTH1 protein played the major role in the enhancement. These results highlight the importance of specific hydrolysis of 8-OH-dGTP for the suppression of its induced mutation.

Reactive oxygen species (ROS)[1] are generated by aerobic metabolism and environmental exposure to ionizing radiation and chemicals. Cellular DNA and its precursor nucleotides are at high risk of being oxidized by ROS, and structurally diverse forms of oxidative damage are induced. The oxidation of DNA and DNA precursors seems to result in spontaneous mutagenesis or cell death and, consequently, in various age-related diseases such as cancer and neurodegeneration (*1*, *2*). Among the various types of oxidative damage, 8-hydroxyguanine (8-OH-Gua) and 2-hydroxyadenine (2-OH-Ade), oxidized forms of guanine and adenine, have been shown to pair with incorrect bases during DNA replication (*3*). Thus, the formation of 8-OH-Gua and 2-OH-Ade is considered to be one of the spontaneous causes of mutagenesis.

To our knowledge, most of the studies on mutagenicity have been performed using DNA containing a single oxidized base and using a single oxidized deoxyribonucleotide (ref *4* and references cited therein). However, it is likely that multiple oxidative lesions are present in DNA and in the nucleotide pool. Clustered DNA damage has been focused on recently (*5*−*7*). The mutagenicity of a DNA lesion is influenced by other lesions present in neighboring areas in various manners. At the same time, multiple kinds of oxidized deoxyribonucleotides are expected to be formed by ROS in the nucleotide pool. One damaged nucleotide might alter the biological effects of another nucleotide. Thus, examining the effects of the coexistence of multiple kinds of oxidized deoxyribonucleotides might be important to evaluate their mutagenic potentials.

8-OH-dGTP and 2-OH-dATP are damaged deoxyribonucleotides formed by in vitro oxidation reactions (*8*−*10*). Nunoshiba et al. reported that the mutations found in an *Escherichia coli* strain lacking superoxide dismutases and a repressor for iron-uptake systems were A·T → C·G and G·C → T·A transversions and concluded that these mutations would be caused by 8-OH-dGTP and 2-OH-dATP, respectively, on the basis of various experiments (*11*). Their conclusion suggests that the two deoxyribonucleotides are important as mutagenic, oxidized deoxyribonucleotides in cells. In agreement with this interpretation, 8-OH-dGTP and 2-OH-dATP are mutagenic when directly introduced into *E. coli* cells (*12*). Moreover, *E. coli*, yeast, and human cells contain enzymatic hydrolysis activities specific for 2-OH-dATP as well as 8-OH-dGTP (*13*−*17*),

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[1] Abbreviations: 8-OH-dGTP, 8-hydroxy-2′-deoxyguanosine 5′-triphosphate; 2-OH-dATP, 2-hydroxy-2′-deoxyadenosine 5′-triphosphate; ROS, reactive oxygen species; 8-OH-Gua, 8-hydroxyguanine; 2-OH-Ade, 2-hydroxyadenine; 2-OH-dADP, 2-hydroxy-2′-deoxyadenosine 5′-diphosphate; pol, polymerase.

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indicating that both are potentially harmful deoxyribonucleotides present in cells.

The SV40 origin-dependent in vitro replication system is a good model for replication in living cells. All of the factors required for bidirectional replication of double-stranded DNA, except for the SV40 large T antigen, are provided by the host cell extract (*18*, *19*). HeLa extracts are frequently used as a source of replication enzymes. The extract contains many DNA polymerases (pols). Replicative DNA pols, such as DNA pols α and δ, and specialized DNA pols, including DNA pol η (the XPV protein) (*20*), are present in the extract. Previously, 8-OH-dGTP and 2-OH-dATP were shown to be mutagenic in this in vitro replication system (*21*, *22*). Thus, the effects of the coexistence of 8-OH-dGTP and 2-OH-dATP in this replication system would be quite interesting.

We now report that 2-OH-dATP enhanced the mutagenicity of 8-OH-dGTP and A·T → C·G transversion mutations were increased during in vitro replication with a HeLa extract. The major reason for this enhancement was inhibition of degradation of 8-OH-dGTP by MTH1(-like proteins). These results highlight the importance of specific hydrolysis of 8-OH-dGTP for the suppression of its induced mutation.

MATERIALS AND METHODS

Materials. The FPLC-grade nucleoside triphosphates used in the replication reactions were from GE Healthcare Bio-Sciences (Piscataway, NJ). 2-OH-dATP, 2-OH-dADP, and 8-OH-dGTP were prepared from dATP, dADP, and dGTP, respectively, and were purified by HPLC as described (*23*, *24*). The purified nucleotides yielded the same ultraviolet spectra as those of the corresponding deoxyribonucleosides in the literature (*25*, *26*), and each eluted as a single peak in both reverse-phase and anion-exchange HPLC (data not shown). Their purities were estimated to be more than 99%. The SV40 large T antigen and the HeLa extract were purchased from CHIMERx (Milwaukee, WI). Oligodeoxyribonucleotides containing 2-OH-Ade and Ade for vector construction were purified as described (*27*). Other oligodeoxyribonucleotides were from Hokkaido System Science (Sapporo, Japan) and Sigma Genosys Japan (Ishikari, Japan) in purified forms. The pSVKAM189/M13R plasmid was previously constructed (*28*). The *E. coli* strain KS40 [*lacZ*(am), *CA7070*, *lacY*1, *hsdR*, *hsdM*, Δ(*araABC-leu*)7679, *galU*, *galK*, *rpsL*, *thi*, *gyrA*]/pOF105 (*29*) was provided by Dr. Tatsuo Nunoshiba, of Tohoku University, and was used as an indicator strain of the *supF* mutants.

Preparation of the XP-V Extract. XP-V cell extracts were prepared as described previously (*20*). Briefly, XP2SA-SV cells, grown in 10 roller bottles at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, were harvested by gentle scraping and low-speed centrifugation. After a wash with phosphate-buffered saline, the cells were suspended in four-packed cell volumes of hypotonic buffer [20 mM Hepes−KOH (pH 7.4), 1.5 mM $MgCl_2$, 5 mM KCl, 2 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride, 0.2 μg/mL aprotinin, 0.2 μg/mL leupeptin, 0.1 μg/mL antipain, and 50 μM EGTA] and were disrupted in an all-glass Dounce homogenizer. The suspension was adjusted to contain 0.2 M NaCl, and an extract was obtained by centrifugation for 1 h at 100000*g*. Then, the supernatant was brought to 70% saturation with ammonium sulfate and was centrifuged for 30 min at 20000*g*. The precipitates were dissolved by dialysis overnight against extract buffer [20 mM Hepes−KOH (pH 7.4), 0.1 mM EDTA, 50 mM NaCl, 10% glycerol, 2 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride] in an ice−water bath. After the removal of insoluble materials by centrifugation, the dialysate was stored at −80 °C as a cell-free extract.

Construction of Vectors. The following oligodeoxyribonucleotides were synthesized and chemically phosphorylated on the support: A, 5′-dGGCAGATTTAGAGTCTGCTCCC; AOH-94, 5′-dGGCAGATTTAGA*GTCTGCTCCC; AOH-96, 5′-dGGCAGATTTA*GAGTCTGCTCCC; where A* represents 2-OH-Ade. The single-stranded form of pSVKAM189/M13R was obtained as described (*30*). Double-stranded (ds) DNAs containing Ade or 2-OH-Ade at a predetermined site were constructed as described (*28*). After protein removal by passage through a Micropure EZ device (Millipore, Billerica, MA), the DNA was purified by ethanol precipitation. The ds DNA containing 2-OH-Ade was then treated with Dam methylase (New England Biolabs, Beverly, MA; 40 units), at 37 °C for at least 6 h, to methylate the N^6-position of adenine in the 5′-GATC-3′ sequences (to restore the bacterial methylation pattern) in the DNA. The DNA was purified by passage through a Micropure EZ device and by ethanol precipitation.

In Vitro DNA Replication. The plasmid with the *supF* gene and the SV40 origin (pSVKam189 and the constructed plasmids containing 2-OH-Ade) was replicated with the HeLa extract by an established method, as described (*31*, *32*). The ds plasmid (40 ng) was replicated by the HeLa extract (∼250 μg) in a buffer (total 25 μL) containing 30 mM Hepes (pH 7.5), 7 mM $MgCl_2$, 0.5 mM dithiothreitol, 4 mM ATP, 100 μM each of dATP, dGTP, dCTP, and dTTP, 50 μM each of GTP, CTP, and UTP, 40 mM phosphocreatine, 0.625 unit of creatine phosphokinase, and 1 μg of large T antigen, with or without oxidatively damaged nucleotide(s). The mixture was incubated at 37 °C for 4 h, and the reaction was terminated by the addition of EDTA to a final concentration of 15 mM. The proteins were removed by extractions with phenol−chloroform (twice) and chloroform, and the DNA was precipitated with ethanol in the presence of 10 μg of tRNA. The recovered DNA was treated with *Dpn*I to digest the unreplicated plasmids. After the removal of proteins by passage through a Micropure EZ device, the DNA was purified by ethanol precipitation. Replication reactions were carried out similarly, with 125 μg of the XP-V cell extract.

Mutagenesis Experiments. The DNAs obtained after the in vitro replication reactions were transfected into *E. coli* KS40/pOF105 cells by electroporation, using a Gene Pulser II transfection apparatus with a pulse controller II (Bio-Rad, Hercules, CA). The mutant frequency was calculated according to the number of colonies on a Luria−Bertani agar plate containing nalidixic acid (50 μg/mL), streptomycin (100 μg/mL), ampicillin (150 μg/mL), and chloramphenicol (30 μg/mL) and the number of colonies on an agar plate containing ampicillin and chloramphenicol, as described (*29*).

The nucleotide sequences of the *supF* gene were analyzed by sequencing as described previously (*33*), using an ABI PRISM big dye terminator cycle sequencing kit and an ABI model 377 DNA sequencer (Applera, Norwalk, CT).

Table 1: Mutant Frequencies after Replication in HeLa Extracts

oxidized nucleotides added	no. of replications	no. of colonies		mutant frequency ($\times 10^{-5}$)
		total	mutant	
none	6	33216500	1280	3.9
200 μM 2-OH-dADP	3	25559400	701	2.7
200 μM 2-OH-dATP	4	21416311	1329	6.2
200 μM 8-OH-dGTP	3	10625500	3300	31.1
200 μM 8-OH-dGTP + 200 μM 2-OH-dADP	3	7057254	6839	96.9
200 μM 8-OH-dGTP + 200 μM 2-OH-dATP	3	6850730	9319	136.0
100 μM 8-OH-dGTP	3	5671800	205	3.6
100 μM 8-OH-dGTP + 100 μM 2-OH-dATP	3	2117200	353	16.7
400 μM 8-OH-dGTP	3	307000	914	297.7
400 μM 8-OH-dGTP + 400 μM 2-OH-dATP	3	715400	7230	1013.5

Table 2: Spectra of Mutations Induced by Oxidized Nucleotides in HeLa Extracts

	none		8-OH-dGTP		8-OH-dGTP + 2-OH-dATP	
	cases found (%)	MF ($\times 10^{-5}$)[a]	cases found (%)	MF ($\times 10^{-5}$)[a]	cases found (%)	MF ($\times 10^{-5}$)[a]
single-base substitutions						
transitions						
G·C to A·T	17 (16)	0.6	4 (4)	1.3	0 (0)	0.0
A·T to G·C	6 (6)	0.2	0 (0)	0.0	1 (1)	1.3
transversions						
G·C to C·G	5 (5)	0.2	0 (0)	0.0	0 (0)	0.0
G·C to T·A	9 (8)	0.3	1 (1)	0.3	0 (0)	0.0
A·T to C·G	4 (4)	0.1	78 (80)	25.0	95 (94)	127.9
A·T to T·A	1 (1)	0.0	2 (2)	0.6	0 (0)	0.0
one-base deletion						
ΔA·T	10 (9)	0.4	2 (2)	0.6	2 (2)	2.7
ΔG·C	34 (32)	1.2	4 (4)	1.3	2 (2)	2.7
tandem base substitution						
GG to AA	17 (16)	0.6	1 (1)	0.3	0 (0)	0.0
others	4 (4)	0.1	5 (5)	1.6	1 (1)	1.3
total	107 (100)	3.9	97 (100)	31.1	101 (100)	136.0

[a] MF means mutation frequency.

Quantitation of 8-OH-dGTP. The amount of 8-OH-dGTP in a reaction mixture was determined by anion-exchange HPLC (*17*). Two hundred micromolar 8-OH-dGTP, 200 μM 8-OH-dGTP plus 200 μM 2-OH-dADP, and 200 μM 8-OH-dGTP plus 200 μM 2-OH-dATP were incubated with the HeLa extract at 37 °C, as described above, except that the plasmid and the large T antigen were not included in the mixture. Reactions were terminated by the addition of ice-cold EDTA to a final concentration of 15 mM. All samples were fractionated by a TSK-gel DEAE-2SW column (Tosoh, Tokyo, Japan), with isocratic elution by 75 mM phosphate buffer (pH 7.0) and 20% acetonitrile at a flow rate of 0.8 mL/min. The amounts of the nucleoside triphosphates and their hydrolyzed products were quantitated by measuring the area of UV absorbance (at 300 nm).

RESULTS

Increase of Mutant Frequency by the Addition of 8-OH-dGTP plus 2-OH-dATP. The mutagenic potentials of oxidized deoxyribonucleotides, 8-OH-dGTP and 2-OH-dATP, have been examined by in vitro replication reactions using a HeLa extract as a good model for replication in living cells (*21*, *22*). In this study, the two oxidized deoxyribonucleotides were added in the same replication reaction mixture to examine the effects of their coexistence. The *supF* gene was chosen as the mutagenesis target, and the plasmid DNA containing the gene, pSVKAM189 (*30*), was added as the template to the replication reaction mixtures. The plasmid DNA was replicated with the HeLa extract, purified, and then transfected into the indicator strain, KS40/pOF105 (*29*).

When only the four unmodified dNTPs were present in the reaction mixture, the observed mutant frequency was 3.9×10^{-5} (Table 1). With the addition of 200 μM 8-OH-dGTP, the mutant frequency reached 31.1×10^{-5}. The mutant frequency also increased when 200 μM 2-OH-dATP was present during the DNA synthesis, although 2-OH-dATP was less mutagenic than 8-OH-dGTP in this assay system. Remarkably, the *supF* mutant frequency was quite high (~4-fold) when 200 μM 8-OH-dGTP was coexistent with 200 μM 2-OH-dATP (136.0×10^{-5}, Table 1). These results suggest that 8-OH-dGTP and 2-OH-dATP enhanced mutations in a synergistic manner. Similar enhancement was also observed when the two oxidized deoxyribonucleotides were included in the extract at 100 and 400 μM concentrations (Table 1). The mutant frequencies for 100 and 400 μM 8-OH-dGTP were increased from 3.6 to 16.7×10^{-5} and from 297.7 to 1013.5×10^{-5}, respectively, by addition of the same concentrations of 2-OH-dATP.

Mutation Spectrum of 8-OH-dGTP plus 2-OH-dATP. We then analyzed the sequences of the *supF* genes obtained in the control (without an oxidized deoxyribonucleotide), 8-OH-dGTP, and 8-OH-dGTP plus 2-OH-dATP experiments (Table 2). 8-OH-dGTP induced A·T → C·G transversions, as reported previously (*21*). Almost all of the substitution mutations found were also A·T → C·G transversions in the 8-OH-dGTP plus 2-OH-dATP experiment. Since 2-OH-dATP induces G·C → A·T and G·C → T·A mutations in

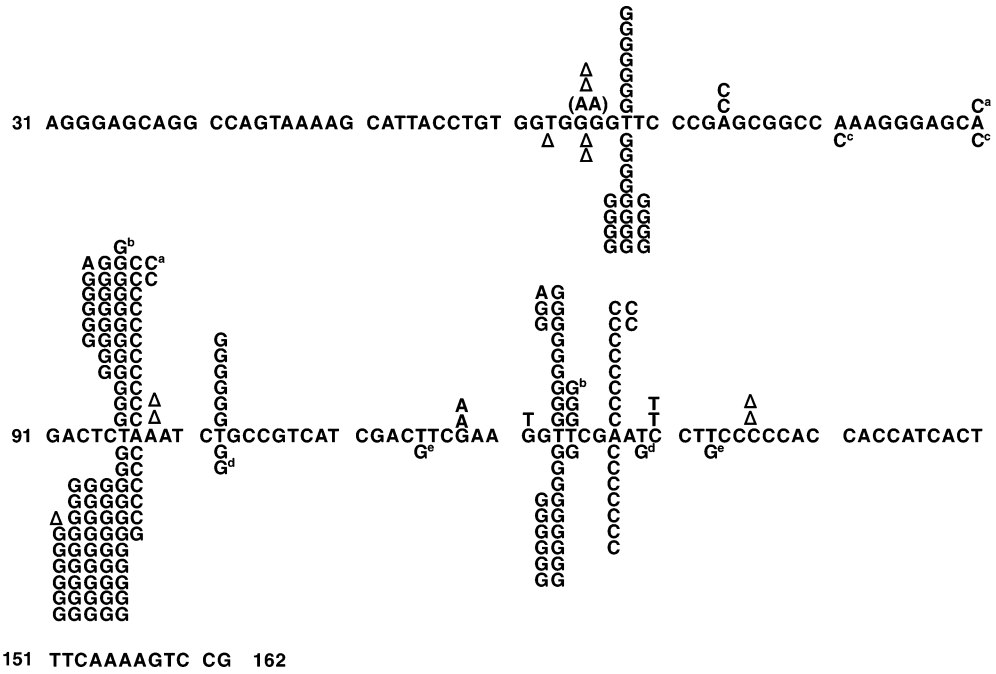

FIGURE 1: Overall distribution of the point mutations detected in the *supF* gene. The sequence of the upper strand of the plasmid is shown. The mutations obtained with 200 μM 8-OH-dGTP and those induced by 200 μM 8-OH-dGTP plus 200 μM 2-OH-dATP are shown above and below the sequence, respectively. The symbol Δ represents a deletion. A tandem two-base substitution is shown in parentheses. The symbol Δ above and below the midpoint of positions 65−66 represents the deletion of either of the G residues located at positions 64−67. The symbol Δ above the midpoint of positions 98−99 represents the deletion of either of the A residues located at positions 97−99. The symbol Δ above position 136 represents the deletion of either of the C residues located at positions 134−138. One *supF* mutant colony (a) obtained with the 8-OH-dGTP experiment contained two A → C mutations at positions 90 and 97. One *supF* mutant colony (b) obtained with the 8-OH-dGTP experiment contained two T → G mutations at positions 96 and 124. One *supF* mutant colony (c) obtained with the 8-OH-dGTP plus 2-OH-dATP experiment contained two A → C mutations at positions 81 and 90. One *supF* mutant colony (d) obtained with the 8-OH-dGTP plus 2-OH-dATP experiment contained two T → G mutations at positions 102 and 129. One *supF* mutant colony (e) obtained with the 8-OH-dGTP plus 2-OH-dATP experiment contained two T → G mutations at positions 115 and 133. The anticodon corresponds to positions 95−97.

Table 3: Mutant Frequencies after Replication in XP-V Extracts

oxidized nucleotides added	no. of replications	no. of colonies		mutant frequency ($\times 10^{-5}$)
		total	mutant	
none	6	1688200	246	14.6
200 μM 2-OH-dADP	1	35480	8	22.6
200 μM 2-OH-dATP	2	42960	10	23.3
200 μM 8-OH-dGTP	5	1051040	369	35.1
200 μM 8-OH-dGTP + 200 μM 2-OH-dADP	3	939400	1036	110.1
200 μM 8-OH-dGTP + 200 μM 2-OH-dATP	5	722650	1570	217.3

this in vitro replication system (*22*), the observed A·T → C·G transversions in the 8-OH-dGTP plus 2-OH-dATP experiment suggest that 2-OH-dATP enhanced the mutagenicity of 8-OH-dGTP.

Mutational hot spots were observed at positions 68, 96, 97, 123, and 127 in the 8-OH-dGTP experiment (Figure 1). The major hot spot was present at position 96. On the other hand, the distribution of the A·T → C·G transversion mutations was slightly changed by the addition of 2-OH-dATP. These positions were also identified as mutational hot spots, and position 96 was also the major hot spot in the 8-OH-dGTP plus 2-OH-dATP experiment. However, only five colonies were found to have the A·T → C·G transversion at position 97 for 8-OH-dGTP plus 2-OH-dATP (Figure 1). Instead, position 68 seemed to be the second major hot spot.

DNA Pol η Is Not Involved in the Synergistic Effect. We next carried out in vitro replication reactions using an extract of XP-V cells lacking a Y-family DNA pol, pol η, since DNA pol η has been shown to incorporate 8-OH-dGTP and 2-OH-dATP in highly erroneous manners (*34*). As shown in Tables 3 and 4, the effects of 8-OH-dGTP and 8-OH-dGTP plus 2-OH-dATP on the mutant frequencies and the mutation spectra during in vitro replication with the XP-V extract were similar to those with the HeLa extract. Thus, it was unlikely that DNA pol η played a major role in the mutagenic effects of these oxidized deoxyribonucleotides.

Possibility of Enhanced 8-OH-dGTP Incorporation by 2-OH-Ade. We assumed that the effect of 2-OH-dATP on the mutagenicity of 8-OH-dGTP upon replication in the HeLa extract might originate from 2-OH-Ade·8-OH-Gua base pair formation. The base pair could be formed when the *supF* gene is replicated twice in the HeLa extract. Judging from the mutation spectrum of 8-OH-dGTP plus 2-OH-dATP, this putative base pair was expected to be formed at A·T sites in the wild-type *supF* gene. Thus, this base pair might be formed by the incorporation of 2-OH-dATP opposite T, and the subsequent 8-OH-dGTP insertion opposite the incorporated 2-OH-Ade base, during the next round of replication.

Table 4: Spectra of Mutations Induced by Oxidized Nucleotides in XP-V Extracts

	none		8-OH-dGTP		8-OH-dGTP + 2-OH-dATP	
	cases found (%)	MF ($\times 10^{-5}$)[a]	cases found (%)	MF ($\times 10^{-5}$)[a]	cases found (%)	MF ($\times 10^{-5}$)[a]
single-base substitutions						
transitions						
G·C to A·T	39 (19)	2.8	6 (8)	2.8	7 (6)	12.6
A·T to G·C	4 (2)	0.3	0 (0)	0.0	1 (1)	1.8
transversions						
G·C to C·G	14 (7)	1.0	1 (1)	0.5	3 (3)	5.4
G·C to T·A	36 (18)	2.6	4 (5)	1.9	2 (2)	3.6
A·T to C·G	6 (3)	0.4	35 (47)	16.6	103 (85)	185.0
A·T to T·A	9 (4)	0.6	2 (3)	0.9	0 (0)	0.0
one-base deletion						
ΔA·T	24 (12)	1.7	8 (11)	3.8	4 (3)	7.2
ΔG·C	56 (28)	4.0	11 (15)	5.2	1 (1)	1.8
tandem base substitution						
GG to AA	4 (2)	0.3	2 (3)	0.9	0 (0)	0.0
others	12 (6)	0.9	4 (5)	1.9	0 (0)	0.0
total	204 (100)	14.6	74 (100)	35.1	121 (100)	217.3

[a] MF means mutation frequency.

Table 5: A·T to C·G Mutations Induced by 8-OH-dGTP at Position 96 of the *supF* Gene[a]

	control		94-AOH		96-AOH	
	none	8-OH-dGTP	none	8-OH-dGTP	none	8-OH-dGTP
A·T to C·G at position 96	1 (2)	25 (136)	0 (0)	7 (34)	2 (4)	9 (56)
total A·T to C·G	12 (24)	69 (375)	9 (21)	50 (240)	11 (24)	44 (278)

[a] Cases found in sequencing experiments. Mutation frequencies ($\times 10^{-5}$) are shown in parentheses.

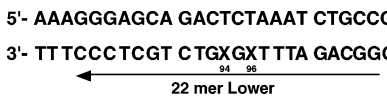

FIGURE 2: Double-stranded plasmid DNAs containing 2-OH-Ade in the *supF* gene. A portion of the nucleotide sequence of the *supF* gene (nts 81−110) is shown. The 22mer Lower corresponds to the oligodeoxyribonucleotides described in the Materials and Methods section. The X's indicate the positions where either the 2-OH-Ade or the unmodified Ade base was incorporated.

We examined this possibility by the replication of template plasmid DNA containing a 2-OH-Ade base. Since position 96 was a major hot spot, the 2-OH-Ade base was introduced into this position (Figure 2). If 8-OH-dGTP is incorporated into a template containing 2-OH-Ade more efficiently than into one containing A, then the A·T → C·G (2-OH-Ade → C) transversion would be induced more frequently.

Alternatively, the presence of 2-OH-Ade might promote a DNA pol switch, and the specialized DNA pol thus recruited might be involved in the incorporation of 8-OH-dGTP opposite A. The 2-OH-Ade base was introduced into position 94, upstream of the major hot spot (position 96), and this oxidized base might promote the A·T → C·G (A → C) transversion.

The plasmid DNAs containing 2-OH-Ade were constructed by extension of a primer with 2-OH-Ade, as described (*28*) (Figure 2). They were added as the template for in vitro replication reactions using the HeLa extract. As shown in Table 5, the presence of 2-OH-Ade did not cause an increase in A·T → C·G (A → C) transversions at either position 96 or at other positions. Thus, these results exclude the possibility that 2-OH-Ade in the template DNA caused the enhanced mutation frequency. In addition, the results suggest that 2-OH-Ade in the complementary strand did not suppress the removal of 8-OH-Gua in the extract (see Discussion).

Suppression of 8-OH-dGTP Degradation. The increased mutation induction by 8-OH-dGTP plus 2-OH-dATP might be due to a protective effect of 2-OH-dATP on 8-OH-dGTP degradation. 8-OH-dGTP might be hydrolyzed by a nonspecific nucleotidase/phosphatase(s) in the extract. Although we added 200 μM CTP into the replication reaction mixture containing 200 μM 8-OH-dGTP, the mutant frequency was not increased (data not shown). Since much higher amounts of nucleotides (in total) were included in the mixture (see the Materials and Methods section), this possibility is unlikely.

Both 8-OH-dGTP and 2-OH-dATP are substrates of the human MTH1 protein (*17*), and in the HeLa extract, this protein may specifically hydrolyze these oxidized deoxyribonucleotides during the incubation. Since 2-OH-dATP is hydrolyzed more efficiently than 8-OH-dGTP by the human MTH1 protein, the hydrolysis of 8-OH-dGTP by MTH1 in the extract might be inhibited by the addition of 2-OH-dATP. Indeed, the coexistence of 2-OH-dATP suppressed the hydrolysis of 8-OH-dGTP catalyzed by the purified MTH1 protein in vitro (*17*). Since 2-OH-dADP is a more potent inhibitor of MTH1 than 2-OH-dATP, the addition of 2-OH-dADP might increase the mutagenicity of 8-OH-dGTP, by protecting it from degradation by MTH1 (*17*). Previously, it was observed that the addition of 2-OH-dADP enhanced the mutagenicity of 2-OH-dATP during replication reactions with the extract, possibly due to inhibition of the MTH1 protein (and/or its functional homologue) (*22*). Thus, we added 200 μM 2-OH-dADP into the replication reaction mixture with 200 μM 8-OH-dGTP. As shown in Table 1, 2-OH-dADP actually enhanced the mutagenicity of 8-OH-dGTP [(31.1−96.9) $\times 10^{-5}$]. However, the enhancement seemed to be less efficient than that obtained by the addition of the same concentration of 2-OH-dATP, although this difference is

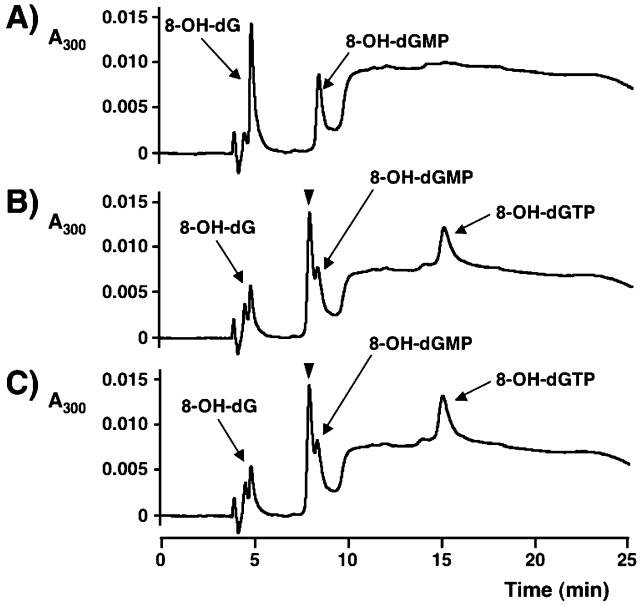

FIGURE 3: Hydrolysis of 8-OH-dGTP in the HeLa extract, monitored by anion-exchange HPLC. (A) 200 μM 8-OH-dGTP, (B) 200 μM 8-OH-dGTP plus 200 μM 2-OH-dADP, and (C) 200 μM 8-OH-dGTP plus 200 μM 2-OH-dATP were incubated with the HeLa extract at 37 °C for 30 min. The elution solution was 20% acetonitrile and 75 mM sodium phosphate buffer, pH 7.0 (isocratic). The triangles in panels B and C represent 2-hydroxy-2′-deoxyadenosine 5′-monophosphate. Abbreviations: 8-OH-dG, 8-hydroxy-2′-deoxyguanosine; 8-OH-dGMP, 8-hydroxy-2′-deoxyguanosine 5′-monophosphate.

statistically insignificant [mutant frequencies were 91.8 ($\pm$12.3) $\times$ 10^{-5} for 8-OH-dGTP plus 2-OH-dADP and 119.6 ($\pm$17.4) $\times$ 10^{-5} for 8-OH-dGTP plus 2-OH-dATP, when mutant frequencies were calculated using those obtained in each replication experiment and expressed as average ($\pm$standard deviation), $P = 0.087$].

We measured the concentration changes of 8-OH-dGTP in the presence and absence of 2-OH-dATP/2-OH-dADP during DNA replication reactions. The assay was performed by anion-exchange HPLC, as described previously (*17*). To reduce possible interruption of the detection by various nucleotides and proteins included in the reaction mixture, the wavelength was set at 300 nm rather than 293 nm that is λ_{max} of 8-OH-dGTP. When no 2-OH-dATP/2-OH-dADP was added, 8-OH-dGTP was almost completely hydrolyzed to the monophosphate and nucleoside derivatives for 30 min (Figure 3A). This hydrolysis might be catalyzed by the MTH1 protein. The 8-OH-dGTP degradation was suppressed by addition of 2-OH-dADP and 2-OH-dATP (Figure 3B,C). Nearly 50% of 8-OH-dGTP was present in these cases. Thus, 2-OH-dATP and 2-OH-dADP inhibited the hydrolysis of 8-OH-dGTP by MTH1(-like proteins). Unexpectedly, however, 2-OH-dATP but not 2-OH-dADP was detected when 2-OH-dADP was added to the reaction mixture at 2 min after the incubation (data not shown). This conversion from 2-OH-dADP to 2-OH-dATP was conducted by the extract and creatine phosphokinase in the reaction (data not shown). The half-life of 8-OH-dGTP was extended from ~15 to ~30 min by the addition of 2-OH-dADP or 2-OH-dATP (data not shown). Thus, both 2-OH-dATP and 2-OH-dADP suppressed the hydrolysis of 8-OH-dGTP, and this would be the major reason of the enhanced mutagenicity of the oxidized dGTP. However, the inhibition of the MTH1(-like) protein could not completely explain the effect of 2-OH-dATP since 2-OH-dATP seemed to enhance mutagenicity of 8-OH-dGTP more efficiently than 2-OH-dADP in the HeLa and XP-V extracts (Tables 1 and 3).

DISCUSSION

We found that 2-OH-dATP enhanced the mutagenicity of 8-OH-dGTP in in vitro replication (Table 1). We used 200 μM 8-OH-dGTP and 2-OH-dATP in the experiments but obtained similar results when the two oxidized deoxyribonucleotides were included in the extract at 100 μM concentrations as unmodified deoxyribonucleotides (Table 1). This effect could be interpreted as (i) higher incorporation of 8-OH-dGTP, (ii) inhibition of the removal of incorporated 8-OH-Gua residues, and/or (iii) protection of 8-OH-dGTP from (pyro)phosphatase(s).

The first hypothesis is that 2-OH-dATP enhances the incorporation of 8-OH-dGTP into the growing DNA chain. We assumed that the incorporation of 8-OH-dGTP would occur opposite 2-OH-Ade in the template DNA strand and examined this possibility with plasmid DNA containing 2-OH-Ade at position 96, the major hot spot of the induced A·T → C·G mutations. Moreover, plasmid DNA containing 2-OH-Ade at position 94, two bases upstream, was also prepared, under the assumption that an error-prone, specialized DNA pol would be recruited by the upstream 2-OH-Ade residue. As shown in Table 5, however, the results obtained with the plasmid DNAs containing a 2-OH-Ade base did not support these possibilities. Alternatively, 2-OH-dATP might act as a regulator of DNA pol(s). DNA synthesis in a *Xenopus* egg lysate is delayed by 8-OH-dGTP through the activation of protein kinases (*35*). 2-OH-dATP might reduce the fidelity of DNA pol(s) by direct and/or indirect mechanism(s) in extracts of human cells. Moreover, 2-OH-dATP might enhance the pol switch, and an error-prone, specialized DNA pol might be recruited.

The second hypothesis is the inhibition of DNA repair and/or proofreading. Recently, it was shown that the presence of a DNA lesion suppressed the removal of another DNA lesion (refs *5*−*7* and references cited therein). The incorporated 2-OH-Ade base might decrease the removal efficiency of 8-OH-Gua. This possibility was partially examined by the experiments with plasmid DNAs with 2-OH-Ade at positions 94 and 96. After 8-OH-dGTP is incorporated at position 96, a clustered DNA damage site is formed near position 96. However, as described above, the 2-OH-Ade bases in the plasmid DNAs did not increase the mutagenicity of 8-OH-dGTP, excluding the possibility of reduced DNA repair caused by 2-OH-Ade in the complementary strand (Table 5). Alternatively, 2-OH-Ade might suppress the 8-OH-Gua removal from the same DNA strand. OGG1 and OGG2 are base excision repair proteins involved in the removal of 8-OH-Gua in DNA although OGG1 has a very weak activity on 8-OH-Gua·A that seemed to cause the A·T → C·G mutations observed in this study (*36*−*43*). In addition, mismatch repair might act as defense against 8-OH-Gua derived from 8-OH-dGTP (*44*). Although the involvement of these repair proteins in 2-OH-Ade removal has not been reported, they might bind to 2-OH-Ade in DNA in the extract, thus suppressing the removal of 8-OH-Gua. We

cannot disregard this possibility at this time. Another possible mechanism for the removal of incorporated 8-OH-Gua is the proofreading activity of DNA pols. However, it is difficult to imagine that 2-OH-Ade in the growing DNA strand inhibits the proofreading activity-catalyzed elimination of 8-OH-Gua.

The A·T → C·G transversions could occur via A·8-OH-Gua pair formation. The MYH (MUTYH) protein removes the adenine base from the A·8-OH-Gua pair (*45*−*48*). If the removal occurred in the extract, then it would enhance the A·T → C·G transversions. The MUTYH protein also removes 2-OH-Ade from any base pair involving the oxidized base (*48*). Therefore, the presence of 2-OH-Ade bases in DNA could reduce the adenine removal from the A·8-OH-Gua pairs under certain conditions. Thus, the suppression of the MYH activity toward A·8-OH-Gua cannot explain the 2-OH-dATP effects observed in this study.

We examined the third hypothesis by quantitation of 8-OH-dGTP in the reaction mixture. The MTH1 protein, a specific pyrophosphatase for 8-OH-dGTP, seems to be the major hydrolysis enzyme for 8-OH-dGTP in the HeLa extract, since MTH1-null mouse embryo fibroblasts were reportedly unable to hydrolyze 8-OH-dGTP (*49*). Addition of 2-OH-dATP and 2-OH-dADP actually suppressed the hydrolysis of 8-OH-dGTP by MTH1(-like proteins), and the suppressive effects were similar for both deoxyribonucleotides (Figure 3). However, the enhancement of the mutations by 8-OH-dGTP seemed to be larger for 2-OH-dATP than 2-OH-dADP (Tables 1 and 3). Thus, the inhibition of the MTH1(-like) protein did not completely explain the effect of 2-OH-dATP. Further studies are necessary for a full understanding of the 2-OH-dATP effects observed in this study.

Another interesting point is which DNA pol was involved in the A·T → C·G transversions induced by 8-OH-dGTP (plus 2-OH-dATP). Previously, Shimizu et al. observed that human DNA pol η, one of the specialized DNA pols, incorporates 8-OH-dGTP and 2-OH-dATP in highly erroneous manners (*34*). We examined this possibility by the use of an extract prepared from XP-V cells lacking the functional XP-V protein (DNA pol η). As shown in Tables 3 and 4, we did not observe marked changes in the mutant frequencies and the mutation spectra of the reactions with the XP-V cell extract as compared to the replication with the HeLa extract. Thus, DNA pol η seemed to play a minor role, if any, in the synergistic effects of 8-OH-dGTP plus 2-OH-dATP (in addition to the incorporation of 8-OH-dGTP) in this in vitro replication system.

In conclusion, we found that 2-OH-dATP enhanced the mutagenicity of 8-OH-dGTP during in vitro replication using a HeLa extract. In the present study, the well-established, SV40 origin-dependent in vitro replication system, which is commonly used as a good model for replication in living cells, was employed. Multiple kinds of oxidized deoxyribonucleotides could be formed by the actions of ROS in the nucleotide pool. As a similar situation, 8-OH-dGTP and 2-OH-dATP could coexist in a single cell. Thus, the effects of the coexistence of the two oxidized deoxyribonucleotides in mammalian cells are of great interest. Experiments along this line are in progress.

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