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Author(s)	Satou, Kazuya; Kawai, Kazuaki; Kasai, Hiroshi et al.
Citation	Free Radical Biology and Medicine, 42(10), 1552-1560 https://doi.org/10.1016/j.freeradbiomed.2007.02.024
Issue Date	2007-05-15
Doc URL	https://hdl.handle.net/2115/20538
Type	journal article
File Information	FRBM.pdf



Mutagenic effects of 8-hydroxy-dGTP in live mammalian cells

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Running title: Mutations by 8-hydroxy-dGTP

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Abstract

The mutagenicity of an oxidized form of dGTP, 8-hydroxy-2'-deoxyguanosine 5'-triphosphate (8-OH-dGTP), was examined using COS-7 cells. 8-OH-dGTP and *supF* shuttle plasmid DNA were co-introduced by means of cationic liposomes, and the DNAs replicated in the cells were recovered and then transfected into *Escherichia coli*. 8-OH-dGTP induced A:T→C:G substitution mutations in the COS-7 cells. This result agrees with previous observations indicating that DNA polymerases misincorporate 8-OH-dGTP opposite A *in vitro*, and that the oxidized deoxyribonucleotide induces A:T→C:G transversions in *E. coli*. These results constitute the first direct evidence to show that 8-OH-dGTP actually induces mutations in living mammalian cells.

Keywords: reactive oxygen species; 8-hydroxy-dGTP; mutation; mammalian cells

Introduction

ROS are generated endogenously by normal oxygen metabolism, and are also produced by many environmental mutagens and carcinogens. For these reasons, ROS and the resulting DNA oxidation represent a potentially important source of mutations and one of the causative factors of carcinogenesis, neurodegeneration, and aging [1-3]. 8-OH-Gua (also known as 7,8-dihydro-8-oxoguanine) is one of the major oxidized bases [4,5]. Its highly mutagenic potential has been shown by site-directed mutagenesis experiments using *Escherichia coli* and mammalian cells [6-14]. 8-OH-Gua has been used as a marker of DNA oxidation, due to its sensitive detection by an HPLC-electrochemical detector system as well as its mutagenic properties [4].

In addition to the direct oxidation of DNA, DNA precursors (deoxyribonucleotides) are also affected by oxidation. 8-OH-dGTP (also known as 7,8-dihydro-8-oxo-2'-deoxyguanosine 5'-triphosphate) is the major oxidation product of dGTP in *in vitro* oxidation reactions [15]. Several lines of evidence indicate that the oxidation of dGTP in the nucleotide pool is another important endogenous source of mutagenesis. DNA polymerases incorporate 8-OH-dGTP opposite C and A *in vitro* [7,16-23]. In terms of the accumulation of 8-OH-Gua in DNA, the contribution of 8-OH-dGTP from the nucleotide pool and the direct oxidation of G bases in DNA has been reported to be almost equal [24].

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 the former was caused by 8-OH-dGTP, based on a variety of experimental data [25]. The *E. coli* MutT protein catalyzes the hydrolysis of 8-OH-dGTP [16], and A:T→C:G transversions are induced with high frequency in *mutT* mutant strains [24,26,27], indicating the importance of 8-OH-dGTP as a source of mutations. Moreover, the presence of mammalian homologues of MutT (MTH1 proteins) supports this speculation [28]. Indeed, a greater number of tumors were formed in the lungs, livers, and stomachs of MTH1-deficient mice than wild-type mice [29]. In addition, 8-OH-dGTP incorporated directly into bacterial cells induced chromosomal gene mutations, providing evidence that the damaged DNA precursor acts as a mutagen in *E. coli* [27,30].

These results raised the question of whether 8-OH-dGTP is mutagenic and induces A:T→C:G transversions in mammalian cells. In a previous study, the mutation spectrum of 8-OH-dGTP was examined using an SV40 origin-dependent *in vitro* replication system [22]. However, no assay system using live mammalian cells has been established. We developed a new assay system, and now show that 8-OH-dGTP clearly induces A:T→C:G transversion mutations in live simian COS-7 cells.

Materials and Methods

Materials

dGTP (FPLC-grade) was purchased from GE Healthcare Bio-Sciences (Piscataway, New Jersey, USA). 8-OH-dGTP was obtained from TriLink BioTechnologies (San Diego, California, USA). Purified oligonucleotides were from Hokkaido System Science (Sapporo, Japan) and Sigma Genosys Japan (Ishikari, Japan). pSVKAM189/Zeo(+) was constructed by insertion of the *Zeo^r* cassette (BglII-SalI) of pCMV/Zeo (Invitrogen, Carlsbad, California, USA) into pSVKAM189 [23]. The *E. coli* strain KS40 (*lacZ(am)*, *CA7070*, *lacY1*, *hsdR*, *hsdM*, $\Delta(\textit{araABC-leu})7679$, *galU*, *galK*, *rpsL*, *thi*, *gyrA*)/pOF105 [31] was provided by Dr. Tatsuo Nunoshiba, of Tohoku University, and was used as an indicator strain of the *supF* mutants.

Mutagenesis experiments

COS-7 cells (5×10^4 cells) were plated into 24-well dishes and were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, at 37°C under a 5% CO₂ atmosphere for 24 hr. The pSVKam189/Zeo(+) plasmid (38 fmol, 100 ng) and 8-OH-dGTP (100 nmol, final concentration of 1 mM) were mixed with Lipofectamine (Invitrogen)

and introduced into the cultured COS-7 cells according to the supplier's recommendations. After 48 hr, the plasmid amplified in the cells was recovered by the method of Stary and Sarasin [32]. The recovered DNA was treated with DpnI to digest the unreplicated plasmids. After removing the protein by passage through a Micropure EZ device (Millipore, Billerica, Massachusetts, USA), the DNA was isolated by ethanol precipitation.

The DNAs recovered from the COS-7 cells 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, California, USA). 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 [31].

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 (Applied Biosystems, Norwalk, Connecticut, USA).

Analysis of 8-OH-Gua in DNA

COS-7 cells (5×10^5 cells) were plated into a 60-mm dish and were cultured in Dulbecco's modified Eagle medium supplemented with 10%

fetal calf serum, at 37°C under a 5% CO₂ atmosphere for 24 hr. The pSVKam189/Zeo(+) plasmid (380 fmol, 1 μg) and 8-OH-dGTP (1.0 μmol, final concentration of 1 mM) were mixed with Lipofectamine and introduced into the cultured COS-7 cells. After 16 hr, the treated cells were washed with the medium and then with phosphate-buffered saline. After trypsin treatment, the cells were washed twice with phosphate-buffered saline. The cellular DNA was isolated by the sodium iodide method, using a DNA Extraction WB Kit (Wako Pure Chemical Industries, Osaka, Japan). Desferal (deferoxamine mesylate, 1 mM) was added to the solution used for cell lysis. The total DNA isolated from the COS-7 cells was treated with nuclease P1 and calf intestine alkaline phosphatase. The amount of 8-OH-Gua was determined by HPLC, essentially as described previously [34]. Namely, the nucleoside solution was filtered through an Ultrafree-Probind filter (Millipore, Bedford, Massachusetts, USA). The filtrate was then injected onto an HPLC column (Capcellpak C18 MG, 4.6 X 250 mm, 5 μm, Shiseido Fine Chemicals, Tokyo, Japan) equipped with UV (UV-8020, Tosoh, Tokyo, Japan) and electrochemical (ECD-300, Eicom, Kyoto, Japan) detectors, with isocratic elution with a solution containing 8% methanol, 10 mM NaH₂PO₄, and 50 mg/l EDTA•2Na, at a flow rate of 1 ml/min. The effluent was monitored by UV absorption at 260 nm for guanosine and by the electrochemical response (applied voltage, 550 mV) for 8-hydroxy-2'-deoxyguanosine (also known as

7,8-dihydro-8-oxo-2'-deoxyguanosine). The 8-OH-Gua level in the DNA sample was calculated from HPLC runs of standard samples of 8-OH-dG and 2'-deoxyguanosine.

Results

Experimental design

To determine the mutation spectrum of 8-OH-dGTP in live mammalian cells, we planned to introduce it together with a shuttle plasmid containing the SV40 *ori*. Plasmid DNAs with the SV40 *ori* could be replicated in cells transformed with the SV40 large T antigen, as in COS-7 cells [35]. This SV40 *ori*-plasmid and the COS-7 system has previously been used to analyze the mutagenicities of various DNA lesions, including 8-OH-Gua [11,13,14,36,37]. In addition, the *supF* gene was chosen as the mutagenesis target. However, the replication of episomal DNAs in mammalian cells is error-prone [38,39]. Indeed, plasmid DNAs recovered from the transfected COS-7 cells frequently contained a considerable fraction of mutants (the maximum mutant frequency was $\sim 1 \times 10^{-2}$) that lacked some portion of the *supF* gene (data not shown). Thus, it is necessary to minimize this replication error to detect mutations induced by 8-OH-dGTP. To accomplish this, we examined various experimental

conditions and found that the conditions described in this paper gave the lowest nonspecific background mutant frequency (data not shown). In addition, the transfected COS-7 cells were recovered from a well and divided into four sub-fractions and the mutant frequencies were measured for each sub-fraction. The frequencies were different from one sub-fraction to another, and a sub-fraction occasionally showed a mutant frequency $>1 \times 10^{-4}$, while others did not. For example, sub-fractions with 18.2, 4.0, 1.4, and 0.3×10^{-5} mutant frequencies were obtained from transfected COS-7 cells in a single transfection experiment. Nearly 90% of the *supF* mutants had ≥ 3 base deletions in a sub-fraction with a 18.2×10^{-5} mutant frequency (data not shown). These results suggested that a very small fraction of the transfected COS-7 cells contained the nonspecific mutant DNAs. To avoid this effect, the sub-fraction that showed the highest mutant frequency was not used for further analysis. Namely, among the four sub-fractions, the three with the lower mutant frequencies, obtained in a single 8-OH-dGTP introduction experiment, were further analyzed.

Accumulation of 8-OH-Gua by 8-OH-dGTP treatment

Before the introduction of 8-OH-dGTP, we tested our procedures with 5-Br-dUTP to determine whether the exogenously added deoxyribonucleotide is actually incorporated into the treated cells. COS-7 cells were treated with 5-Br-dUTP, as described under Materials and

Methods. When a fluorescently labeled anti-Br-dU antibody was added to cells fixed with paraformaldehyde, the nuclei were stained with the antibody (data not shown). This result indicates that the deoxyribonucleotide was in fact delivered to the inside of the cells and then incorporated into the DNA.

We then measured the 8-OH-Gua content in the DNA after the 8-OH-dGTP treatment. Due to the amount of DNA required, the introduction of 8-OH-dGTP was carried out on a 10-fold larger scale than that of the mutagenesis experiment, keeping the same concentration of 8-OH-dGTP. 8-OH-Gua was detected by the HPLC-electrochemical detector method, which has a high sensitivity [4]. Upon the dGTP treatment (control), the 8-OH-Gua level in the cells was $1.73/10^6$ Gua, at 16 hr after the treatment (Fig. 1). This 8-OH-Gua level can be attributed to DNA oxidation and the incorporation of 8-OH-dGTP into DNA, and also to oxidation that could occur during the experimental procedure. On the other hand, we found that the 8-OH-Gua level of the cells treated with 8-OH-dGTP increased to $2.91/10^6$ Gua at the same time point. Thus, the 8-OH-Gua level of $1.18/10^6$ Gua over the background was induced by the 8-OH-dGTP treatment, suggesting that the added damaged nucleotide actually entered the live mammalian cells and was incorporated by DNA pol(s).

Induction of A:T→C:G transversions by 8-OH-dGTP in COS-7 cells

We examined the mutagenic potential of 8-OH-dGTP in living COS-7 cells. Plasmid DNA containing the *supF* gene, pSVKAM189/Zeo(+), was introduced together with 8-OH-dGTP. The replicated DNA was recovered from the transfected COS-7 cells and then transfected again into the indicator strain, KS40/pOF105 [31].

When the unmodified dGTP was co-introduced with the *supF* plasmid, the observed *supF* mutant frequency was 3.24×10^{-5} (Table 1). The total mutant frequency was similar when 8-OH-dGTP was introduced (3.44×10^{-5} , Table 2). However, a sequence analysis of the *supF* mutants revealed that A:T→C:G transversions were specifically induced by the addition of 8-OH-dGTP (Tables 1 and 2). The induction of an A:T→C:G transversion mutation indicates that the introduced 8-OH-dGTP caused mutations in COS-7 cells, since the A:T→C:G mutation is the ‘fingerprint’ of 8-OH-dGTP observed in *E. coli* [30].

Mutation spectrum of 8-OH-dGTP

We analyzed the sequences of the *supF* genes in 205 and 207 colonies, obtained in control (dGTP) and 8-OH-dGTP experiments, respectively. Since the total mutant frequencies and the numbers of the mutants analyzed were different in each transfection experiment, we

multiplied the total mutant frequency by the proportion of each type of mutation, and expressed them as mutation frequencies (Tables 3 and 4).

The most frequent mutation in the control experiment was the deletion of ≥ 3 bases (2.25×10^{-5}). The frequency of this type of mutation was reduced in the 8-OH-dGTP experiment (0.85×10^{-5}). The frequency of single-base substitution mutations in the 8-OH-dGTP experiment (1.47×10^{-5}) was much higher than that in the control experiment (0.30×10^{-5}). Among the substitutions, 8-OH-dGTP induced A:T \rightarrow C:G transversions specifically (0.95×10^{-5}). Nearly 60% of the single-base substitution mutations were A:T \rightarrow C:G mutations when 8-OH-dGTP was introduced. In contrast, no A:T \rightarrow C:G mutation was found in the control experiment. This result is in agreement with the previously reported A:T \rightarrow C:G induction by 8-OH-dGTP in *E. coli* [30].

In addition, several mutants with G:C \rightarrow T:A transversions were observed for the 8-OH-dGTP treated group (0.27×10^{-5} vs 0.06×10^{-5} in the control group). However, it is not clear whether 8-OH-dGTP induced the G:C \rightarrow T:A transversions, when the tandem mutations observed in the control experiment are taken into consideration (see Discussion).

The distributions of mutations in the *supF* gene found in this study are shown in Tables 5 and 6. Positions 95-97 correspond to those of the anticodon in the encoded tRNA, and many mutations were found at the

95th and 96th positions in the case of the 8-OH-dGTP-treated group. An A:T→C:G transversion hotspot was found at position 96. G:C→T:A and G:C→C:G transversion hotspots were found at position 95.

Discussion

8-OH-dGTP induces A:T→C:G transversions with a high frequency in a chromosomal gene in *E. coli* [30]. This *in vivo* result is supported by *in vitro* experiments, which revealed that *E. coli* DNA pols misincorporate 8-OH-dGTP opposite A [16-18,40]. Likewise, mammalian DNA pols misinsert this oxidized dGTP opposite A [19-22,41]. These results suggest that 8-OH-dGTP also induces A:T→C:G transversions in living mammalian cells. Indeed, Table 4 clearly indicates that A:T→C:G mutations were induced by 8-OH-dGTP in COS-7 cells. These findings suggest that 8-OH-dGTP was incorporated opposite A, and dCTP was then inserted opposite the incorporated 8-OH-Gua residue during the next round of replication.

In contrast, when 8-OH-dGTP is incorporated opposite C, and dATP is then inserted opposite the incorporated 8-OH-Gua residue during the next round of replication, G:C→T:A transversion mutations are induced by 8-OH-dGTP. In *E. coli*, exogenous 8-OH-dGTP mainly causes A:T→C:G transversions. In the case of the *lacI* gene, 61 colonies contained

an A:T→C:G mutation, while only two contained a G:C→T:A mutation [30]. A:T→C:G transversions were found in 55 colonies of *rpoB* mutants, as the result of 8-OH-dGTP treatment, and none were found to have a G:C→T:A transversion [42]. In this study, the frequency of G:C→T:A transversion mutations was higher in the 8-OH-dGTP-treated group than in the control group (0.27×10^{-5} vs 0.06×10^{-5} , Tables 3 and 4). These results suggest that G:C→T:A mutations are induced by the oxidized dGTP. However, this interpretation should be reviewed carefully, when tandem mutations are considered. In the control experiment (2-1), CC→TA mutations were observed. If this tandem mutation is characterized as independent G:C→A:T and G:C→T:A mutations, then this mutation frequency (0.24×10^{-5}) should be reflected in that for the G:C→T:A mutation. Moreover, if the GG→TT tandem mutations observed in the control experiment (2-2) are considered as above, then this mutation frequency (1.08×10^{-5}) after multiplying by two should be added to that for the G:C→T:A mutation. Namely, the mutation frequency for the G:C→T:A mutation in the control experiment was calculated as follows:

$$(0.06 + (0.24/9) + ((1.08 \times 2)/9)) \times 10^{-5} = 0.33 \times 10^{-5}.$$

Likewise, the mutation frequency for the G:C→T:A mutation in the 8-OH-dGTP experiment was re-evaluated. If the CC→AT mutations observed in the 8-OH-dGTP experiment (2-2) are regarded as independent

G:C→T:A and G:C→A:T mutations, then this mutation frequency (0.51×10^{-5}) was considered. Moreover, if the GG→TT tandem mutations observed in the 8-OH-dGTP experiment (4-3) are considered as above, then this mutation frequency (0.06×10^{-5}) after multiplying by two should be added to that for the G:C→T:A mutation. Thus, the frequency for the G:C→T:A mutation was calculated as follows:

$$(0.27 + (0.51/12) + ((0.06 \times 2)/12)) \times 10^{-5} = 0.32 \times 10^{-5}.$$

Therefore, it is uncertain whether the G:C→T:A transversions were actually induced by 8-OH-dGTP in COS-7 cells.

The mammalian MTH1 protein functions as the hydrolyzing enzyme for 8-OH-dGTP [43]. In the spleens of MTH1-deficient mice, the A:T→C:G mutation frequency is increased slightly, and no induction of G:C→T:A transversions was observed [44]. The enhanced occurrence of A:T→C:G mutations may reflect the accumulation of 8-OH-dGTP in the spleen cells of the knock-out mice. Single-base deletions are another type of mutation, which is found more frequently in the spleens of MTH1-deficient mice than the wild-type mice. However, only one colony contained this type of mutation in the 8-OH-dGTP experiment (Table 6). Thus, the induction of a single-base deletion by 8-OH-dGTP was a rare occurrence in COS-7 cells. The MTH1 protein catalyzes the hydrolysis of 2-OH-dATP in addition to 8-OH-dGTP [43]. Since single-base deletions were induced by 2-OH-dATP in *in vitro* replication reactions using a HeLa

extract [45], the accumulation of 2-OH-dATP in the spleen cells of the knock-out mice may partly explain the occurrence of this type of mutation.

8-OH-Gua in DNA is mutagenic in mammalian cells [10-14]. In the cases of shuttle vectors, the mutation frequency of 8-OH-Gua opposite C is <1% and that of 8-OH-Gua opposite A is 64% in COS-7 cells [46]. This implies that once it is incorporated, 8-OH-dGTP opposite A is much more mutagenic than 8-OH-dGTP opposite C. The ratio of 8-OH-dGTP incorporations opposite C and A is unknown. If we hypothesize that the oxidized dGTP is incorporated opposite C and A at similar frequencies, then the A:T→C:G mutation would be more frequently observed than the G:C→T:A mutation. Further studies will be necessary to interpret the data obtained in this study.

Many reports have described mammalian DNA repair enzymes that act on base pairs involving 8-OH-Gua. The OGG1 (MMH) protein excises the 8-OH-Gua in DNA, and its activity is dependent on the opposite bases [47-53]. In addition to the proportion of 8-OH-dGTP incorporation opposite C and A, the removal efficiencies of 8-OH-Gua residues would be a determinant of the mutation spectrum of oxidized dGTP. Since duplexes containing an 8-OH-Gua:C are preferred substrates, the 8-OH-Gua residues formed by the incorporation of 8-OH-dGTP opposite C would be eliminated more rapidly than those derived from the incorporation of 8-OH-dGTP opposite A. Mammalian MYH (MUTYH) is an adenine

glycosylase that removes the base opposite 8-OH-Gua [54-57]. If the MYH protein acts on A:8-OH-Gua pairs efficiently, then it might accelerate the occurrence of A:T→C:G, mutations, due to the loss of the original genetic information (the adenine base).

Kim *et al.* previously reported that, although exogenous 8-OH-dG was not incorporated into DNA, it enhanced the accumulation of 8-OH-Gua in DNA [58]. They suggested that 8-OH-dG enhanced the incorporation of endogenous 8-OH-dGTP by DNA pol β. The 8-OH-dG formed by the enzymatic hydrolysis of 8-OH-dGTP may induce mutations by this mechanism. Although 8-OH-dG, when added at the same concentration as that of 8-OH-dGTP, did not induce A:T→C:G transversions (data not shown), 8-OH-dG produced in the cytosol from 8-OH-dGTP may enhance the incorporation of endogenous and exogenous 8-OH-dGTP into DNA by DNA pol β. However, under our experimental conditions, no A:T→C:G transversion mutations were found after the recovery from the transfected COS-7 cells, in the case of a *supF* plasmid DNA lacking the SV40 replication *ori* (data not shown). This result indicates that the A:T→C:G transversion mutations were induced in a replication-dependent manner, excluding the possibility that the enhanced incorporation of 8-OH-dGTP by DNA pol β is the major reason for the A:T→C:G mutations observed in this study.

In conclusion, 8-OH-dGTP was mutagenic and induced A:T→C:G transversions in live mammalian cells. These results agree with previous findings that mammalian DNA pols insert 8-OH-dGTP opposite A in DNA [19-22,41]. Defense mechanisms against this damaged deoxyribonucleotide produced by ROS in mammalian cells are of great interest. Experiments along this line using live cells are currently in progress.

Acknowledgements

We are grateful to Dr. T. Nunoshiba for the *E. coli* strain KS40/pOF105. This work was supported in part by Grants-in-Aid from the Japan Society for the Promotion of Science, and by a fellowship and grant from Research Fellowships of the Japan Society for the Promotion of Science to K. S.

Abbreviations: ROS, reactive oxygen species; 8-OH-dGTP, 8-hydroxy-2'-deoxyguanosine 5'-triphosphate; 8-OH-Gua, 8-hydroxyguanine; 8-OH-dG, 8-hydroxy-2'-deoxyguanosine; 2-OH-dATP, 2-hydroxy-2'-deoxyadenosine 5'-triphosphate; pol, polymerase.

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but enhances the accumulation of 8-oxo-Gua in DNA through error-prone DNA synthesis. *Mutat. Res.* **596**:128-136; 2006.

Figure legend

Fig. 1 Increased 8-OH-Gua in DNA after 8-OH-dGTP treatment. Total DNA was isolated from COS-7 cells treated with 8-OH-dGTP. The 8-OH-Gua level was measured as described in the Materials and Methods section. Data are the means of three independent experiments. Error bars represent S.D. * $P < 0.05$ (Student's t -test)

Table 1. Mutation frequency for A:T to C:G transversions in the control experiment

Expt. ^a	Colonies		Mutant frequency (X 10 ⁻⁵)	Mutants analyzed		Mutation frequency for A:T to C:G (x10 ⁻⁵) ^b	
	Total (X 10 ²)	Mutant		Total	A:T to C:G		
Expt-1	1-1	12585	111	8.82	30	0	<0.29
	1-2	14816	73	4.93	60	0	<0.08
	1-3	14027	67	4.78	24	0	<0.20
Expt-2	2-1	11647	24	2.06	26	0	<0.08
	2-2	11555	25	2.16	18	0	<0.12
	2-3	4339	3	0.69	3	0	<0.23
Expt-3	3-1	12198	17	1.39	17	0	<0.08
	3-2	12100	48	3.97	23	0	<0.17
	3-3	12758	4	0.31	4	0	<0.08
Average			3.24 ± 2.69				

^a Three separate COS-7 transfection experiments (Expts-1 to -3) were carried out, and the recovered cells were divided into four sub-fractions for each transfection experiment. The three sub-fractions, except for one with the highest mutant frequency, are shown (see text).

^b The mutation frequency was calculated by multiplying the mutant frequency by the proportion of mutants containing A:T to C:G substitutions.

Table 2. Mutation frequency for A:T to C:G transversions in the 8-OH-dGTP experiment

Expt. ^a	Colonies		Mutant frequency (X 10 ⁻⁵)	Mutants analyzed		Mutation frequency for A:T to C:G (x10 ⁻⁵) ^b	
	Total (X 10 ²)	Mutant		Total	A:T to C:G		
Expt-1	1-1	6786	64	9.43	16	2	1.18
	1-2	9606	64	6.66	16	6	2.50
	1-3	8974	46	5.13	16	1	0.32
Expt-2	2-1	10304	27	2.62	16	5	0.82
	2-2	9642	39	4.04	32	15	1.89
	2-3	18686	36	1.93	16	2	0.24
Expt-3	3-1	7024	23	3.27	16	2	0.41
	3-2	13368	31	2.32	16	12	1.74
	3-3	8254	16	1.94	15	14	1.81
Expt-4	4-1	14846	16	1.08	16	1	0.07
	4-2	15382	29	1.89	16	3	0.35
	4-3	25382	24	0.95	16	2	0.12
Average			3.44 ± 2.52		0.95 ± 0.84		

^a Four separate COS-7 transfection experiments (Expts-1 to -4) were carried out, and the recovered cells were divided into four sub-fractions for each transfection experiment. The three sub-fractions, except for one with the highest mutant frequency, are shown (see text).

^b The mutation frequency was calculated by multiplying the mutant frequency by the proportion of mutants containing A:T to C:G substitutions.

Table 3. Frequencies of each type of mutation in the control experiment ^a

	Expt.									Average	
	1-1	1-2	1-3	2-1	2-2	2-3	3-1	3-2	3-3		
Single-base substitutions											
Transitions											
G:C to A:T	0.59 (2)	0 (0)	0 (0)	0 (0)	0.60 (5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.13
A:T to G:C	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0
Transversions											
G:C to C:G	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.90 (22) ^b	0 (0)	0 (0)	0 (0)	0.10
G:C to T:A	0.29 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0.23 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0.06
A:T to C:G	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0
A:T to T:A	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.08 (1)	0 (0)	0 (0)	0 (0)	0.01
1-2 bp addition	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0
≥3 bp addition	0 (0)	0.33 (4)	0 (0)	0 (0)	0.12 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.05
1-2 bp deletion	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.16 (2)	0.02
≥3 bp deletion	6.20 (21)	4.27 (52)	4.58 (23)	0.79 (10)	0 (0)	0 (0)	0.33 (4)	3.97 (23)	0.08 (1)	0.08 (1)	2.25
Others	0 (0)	0 (0)	0 (0)	0.24 (3) ^c	1.08 (9) ^d	0.23 (1) ^e	0.08 (1) ^f	0 (0)	0 (0)	0 (0)	0.18
No mutation	1.76 (6)	0.33 (4)	0.20 (1)	1.03 (13)	0.36 (3)	0.23 (1)	0 (0)	0 (0)	0.08 (1)	0.08 (1)	0.44
Total	8.82 (30)	4.93 (60)	4.78 (24)	2.06 (26)	2.16 (18)	0.69 (3)	1.39 (17)	3.97 (23)	0.31 (4)	0.31 (4)	3.24

^a The mutation frequency was calculated by multiplying the mutant frequency by the proportion of mutants containing each type of mutation. The number of actual events is shown in parentheses.

^b Eleven colonies contained two C to G mutations at positions 95 and 125. Each mutation was counted as a single C to T mutation event.

^c CC to TA tandem mutations at positions 130 and 131 (three colonies).

^d GG to TT tandem mutations at positions 84 and 85 (nine colonies).

^e A CC to TT tandem mutation at positions 70 and 71 (one colony).

^f A GG to AA tandem mutation at positions 121 and 122 (one colony).

Table 4. Frequencies of each type of mutation in the 8-OH-dGTP experiment^a

	Expt.															Average
	1-1	1-2	1-3	2-1	2-2	2-3	3-1	3-2	3-3	4-1	4-2	4-3				
Single-base substitutions																
Transitions																
G:C to A:T	0 (0)	0.42 (1)	0 (0)	0.33 (2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.24 (2) ^b	0 (0)	0.08
A:T to G:C	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.20 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.02
Transversions																
G:C to C:G	0 (0)	0 (0)	0 (0)	0.49 (3)	0 (0)	0 (0)	1.02 (5)	0.15 (1)	0 (0)	0.07 (1)	0 (0)	0.06 (1)	0.15			
G:C to T:A	0 (0)	0 (0)	0 (0)	0.82 (5)	0.63 (5)	0.12 (1)	0.41 (2)	0.15 (1)	0 (0)	0.61 (9)	0.24 (2) ^b	0.24 (4)	0.27			
A:T to C:G	1.18 (2)	2.50 (6)	0.32 (1)	0.82 (5)	1.89 (15)	0.24 (2)	0.41 (2)	1.74 (12)	1.81 (14)	0.07 (1)	0.35 (3)	0.12 (2)	0.95			
A:T to T:A	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0			
1-2 bp addition	0 (0)	0 (0)	0 (0)	0.16 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.01			
≥3 bp addition	0 (0)	0 (0)	0.64 (2)	0 (0)	0 (0)	0.12 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.06			
1-2 bp deletion	0 (0)	0 (0)	0 (0)	0 (0)	0.13 (1)	0.24 (2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.06 (1)	0.04			
≥3 bp deletion	5.89 (10)	0.83 (2)	0 (0)	0 (0)	0.38 (3)	1.09 (9)	0.20 (1)	0.29 (2)	0 (0)	0.34 (5)	0.95 (8)	0.18 (3)	0.85			
Others	0 (0)	0 (0)	0 (0)	0 (0)	0.51 (4) ^c	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.12 (1) ^d	0.06 (1) ^e	0.06			
No mutation	2.36 (4)	2.91 (7)	4.17 (13)	0 (0)	0.51 (4)	0.12 (1)	1.02 (5)	0 (0)	0.13 (1)	0 (0)	0.24 (2)	0.24 (4)	0.98			
Total	9.43 (16)	6.66 (16)	5.13 (16)	2.62 (16)	4.04 (32)	1.93 (16)	3.27 (16)	2.32 (16)	1.94 (15)	1.08 (16)	1.89 (16)	0.95 (16)	3.44			

^a The mutation frequency was calculated by multiplying the mutant frequency by the proportion of mutants containing each type of mutation. The number of actual events is shown in parentheses.

^b One colony contained three mutations at positions 56 (C to T), 95 (C to A) and 104 (C to T). Each mutation was counted as a single mutation event.

^c CC to AT tandem mutations at positions 70 and 71 (four colonies).

^d A CC to A mutation at positions 71 and 72 (one colony).

^e A GG to TT tandem mutation at positions 121 and 122 (one colony).

Table 5. Distribution of mutations obtained in the control experiment

Base change	Position	No. of colonies	Expt.
Transitions			
G:C to A:T	122	1	1-1
	130	1	1-1
	130	5	2-2
Transversions			
G:C to C:G	95 & 125	11 ^a	3-1
G:C to T:A	85	1	2-3
	95	1	1-1
A:T to T:A	96	1	3-1
1-2 bp addition/deletion			
ΔC	134-138	2	3-3
Others			
GG to TT	84 & 85	9	2-2
GG to AA	121 & 122	1	3-1
CC to TT	70 & 71	1	2-3
CC to TA	130 & 131	3	2-1

^a These mutant colonies contained double G:C to C:G substitutions.

Table 6. Distribution of mutations obtained in the 8-OH-dGTP experiment

Base change	Position	No. of colonies	Expt.	
Transitions				
G:C to A:T	56 ^a	1	4-2	
	85	1	1-2	
	104 ^a	1	4-2	
	118	2	2-1	
A:T to G:C	68	1	3-1	
Transversions				
G:C to C:G	71	1	3-2	
	71	1	4-3	
	95	3	2-1	
	95	5	3-1	
	95	1	4-1	
G:C to T:A	75	2	3-1	
	85	1	2-2	
	95	5	2-1	
	95	1	3-1	
	95	1	4-2	
	95 ^a	1	4-2	
	121	4	2-2	
	121	9	4-1	
	130	3	4-3	
	131	1	4-3	
	A:T to C:G	68	1	1-2
		68	1	2-2
		68	1	3-1
		96	2	1-1
		96	5	1-2
96		1	1-3	
96		9	2-2	
96		1	3-1	
96		5	3-2	
96		1	4-1	
96		1	4-2	
97		5	3-2	
97		3	3-3	
97		1	4-3	
102		2	2-1	
102		1	4-3	
123		2	2-1	
123		1	2-2	
123		2	3-2	
123		8	3-3	
123	2	4-2		
124	2	2-2		
124	1	2-3		
124	1	3-3		
127	1	2-1		
127	2	2-2		
127	1	2-3		
127	2	3-3		
1-2 bp addition/deletion				
+CT	129-130	1	2-1	
ΔC	134-138	1	2-2	
ΔC	134-138	2	2-3	
ΔC	134-138	1	4-3	
Others				
GG to TT	121 & 122	1	4-3	
CC to AT	70 & 71	4	2-2	
CC to A	71 & 72	1	4-2	

^a One colony contained three base substitutions.

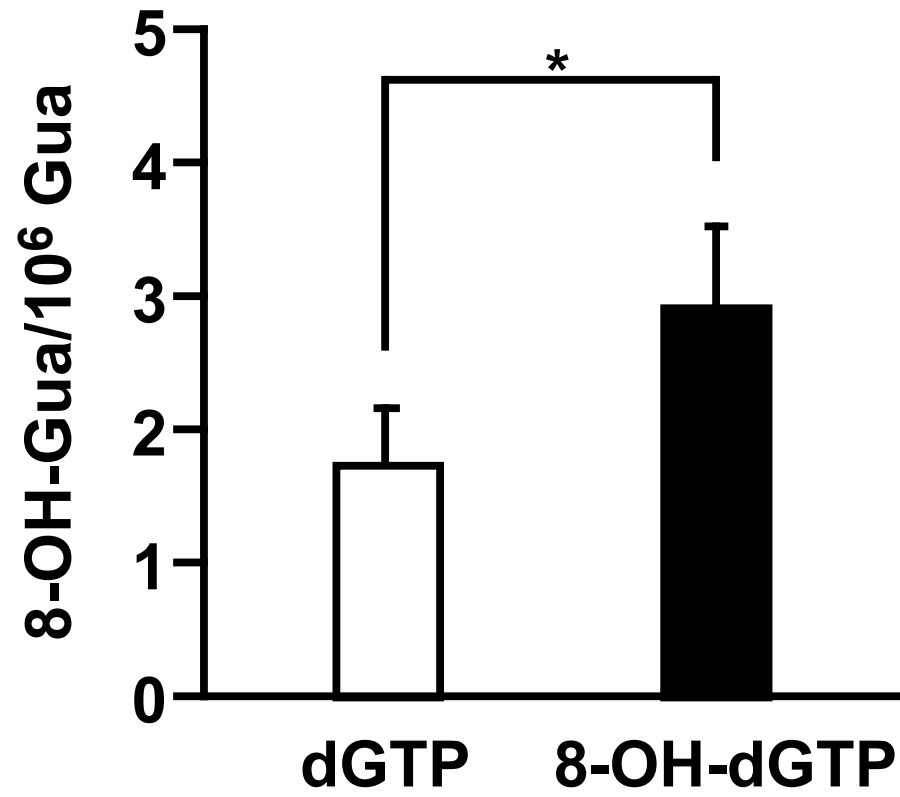


Fig. 1