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The mutations induced by oxidatively damaged nucleotides, 5-formyl-dUTP and 5-hydroxy-dCTP, in *Escherichia coli*

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ABSTRACT

The mutational properties of 5-formyl-2′-deoxyuridine 5′-triphosphate (5-CHO-dUTP) and 5-hydroxy-2′-deoxycytidine 5′-triphosphate (5-OH-dCTP), the major oxidatively damaged pyrimidine nucleotides derived from dTTP and dCTP, respectively, were analyzed by an *in vivo* assay. 5-CHO-dUTP and 5-OH-dCTP were directly incorporated into *Escherichia coli*, and their mutagenicities were evaluated by the chromosomal *lacI* forward mutation assay. The mutation frequencies increased, depending on the dose of these damaged nucleotides, indicating that these nucleotides were incorporated into *E.coli* and acted as mutagens *in vivo*. The mutagenicities of 5-CHO-dUTP and 5-OH-dCTP were comparable to that of 8-hydroxy-2′-deoxyguanosine 5′-triphosphate, a major form of dGTP oxidative damage. 5-CHO-dUTP induced G·C to A·T, A·T to G·C and G·C to T·A mutations, and 5-OH-dCTP elicited G·C to A·T, A·T to C·G and G·C to T·A mutations.

INTRODUCTION

Reactive oxygen species (ROS) appear to induce genetic mutation by damaging DNA and DNA precursors. This damage is possibly related to aging and various diseases, including cancer (1), since it is produced endogenously in cells. Furthermore, oxidative lesions may be involved in the process of these diseases, since ROS are produced by exogenous exposure to environmental chemicals and ionizing radiation.

5-Formyl-2′-deoxyuridine and 5-hydroxy-2′-deoxycytidine are the major products when dT and dC, respectively, are treated with Fenton-type reagents (2,3). The formation of these oxidized products was also observed when DNA was γ-irradiated *in vitro* (4,5), and when cultured human cells were exposed to hydrogen peroxide (6). Thus, 5-formyl-2′-deoxyuridine 5′-triphosphate (5-CHO-dUTP) and 5-hydroxy-2′-deoxycytidine 5′-triphosphate (5-OH-dCTP) appear to be the major oxidatively damaged pyrimidine nucleotides derived from dTTP and dCTP, respectively. The incorporation of these damaged nucleotides into DNA by DNA polymerases *in vitro* has been reported (7,8). DNA polymerases insert these damaged nucleotides opposite Gua and Ade, suggesting that they induce A·T to G·C and G·C to A·T transitions. However, the mutational properties of these nucleotides in an *in vivo* system have never been reported.

Recently, we established a new evaluation method for mutagenesis by a damaged nucleotide *in vivo* (9). This method enables us to evaluate the mutagenic potency of a nucleotide, based on both the rate of misincorporation by a DNA polymerase and the efficiency of elimination by a MutT-type enzyme. Thus, it is interesting to know how mutagenic 5-CHO-dUTP and 5-OH-dCTP are *in vivo* by this method. It has been reported that 5-formyluracil (5-CHO-Ura) in DNA is one of the substrates for the AlkA DNA glycosylase (10), and that 5-hydroxycytosine (5-OH-Cyt) is excised from DNA by endonucleases III and VIII and the fapy DNA glycosylase (11,12). These DNA repair enzyme activities are also important determinant factors in our new evaluation method. In this assay, mutations induced by a nucleotide analog are detectable with less of the bias that originates in the specificity by the DNA sequence context and the types of DNA polymerases, because a 1.2 kb chromosomal region that is replicated in wild type *Escherichia coli* cells is analyzed.

In this study, we analyzed the mutagenicities and the mutation spectra of 5-CHO-dUTP and 5-OH-dCTP by this chromosomal *lacI* forward mutation assay in wild type *E.coli*. 5-CHO-dUTP and 5-OH-dCTP were as mutagenic as 8-hydroxy-2′-deoxyguanosine 5′-triphosphate (8-OH-dGTP), a major form of dGTP oxidative damage. Moreover, we found that 5-CHO-dUTP characteristically induced G·C to A·T, A·T to G·C and G·C to T·A mutations, and that 5-OH-dCTP elicited G·C to A·T, A·T to C·G and G·C to T·A mutations. Possible mechanisms of these mutations, by incorporation of the oxidized nucleotides and by base-pair specific repair systems, are discussed.

MATERIALS AND METHODS

Materials

All chemicals for the *E.coli* media were as described (13–15). *Escherichia coli* strain W3110 (wild type, F^-) was used for the chromosomal *lacI* forward mutation assay. The dTTP and dCTP used for the preparation of the oxidized products were from Sigma. Nucleotides for control treatments were from Amersham Pharmacia Biotech. Digoxygenin-labeled oligonucleotides and other unmodified oligonucleotides were from Nissinbo (Tokyo, Japan) and from Hokkaido System Science (Sapporo, Japan), respectively, in purified forms.

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Preparation of damaged nucleotides

5-CHO-dUTP and 5-OH-dCTP were prepared by vigorous shaking of solutions of dTTP and dCTP (10 mg), respectively, with Fe(II)-EDTA (40 mM $FeSO_4$ and 40 mM EDTA) in 1.5 ml of 50 mM sodium phosphate buffer (pH 7.4) under air, at room temperature, for 30 min. 5-CHO-dUTP was separated by reverse-phase HPLC using two Mightysil RP-18 5 µm columns (4.6 × 250 mm, Kanto Chemical) connected in series, with isocratic elution by buffer A (12.5 mM citric acid, 25 mM sodium acetate, 10 mM acetic acid and 30 mM sodium hydroxide). The fraction containing 5-CHO-dUTP, identified by on-line measurement of the UV spectra, was further separated by the same two columns using buffer A containing 0.7% formic acid and 10 mM sodium bisulfite as an eluent. The 5-OH-dCTP was isolated by reverse-phase HPLC using two Mightysil RP-18 5 µm columns connected in series, with isocratic elution by buffer A and monitoring of the UV spectra. The 5-OH-dCTP fraction was further purified under the same HPLC conditions. Both damaged nucleotides were finally desalted by HPLC using an Ultrasphere ODS 5 µ column (4.6 × 250 mm, Beckman). Detection was performed with a Hewlett Packard 1040M HPLC Detection System.

These oxidized nucleotides were eluted as a single peak in both reverse-phase and anion-exchange HPLC (data not shown). They were eluted in a similar position to other nucleoside triphosphates in anion-exchange HPLC. The dephosphorylated samples were eluted at the same time as the authentic nucleosides in reverse-phase HPLC (data not shown).

γ-^{33}P-labeled 5-CHO-dUTP and 5-OH-dCTP were prepared by an unpublished method. This procedure will be reported elsewhere (H.Kamiya and H.Kasai, manuscript in preparation).

Introduction of nucleotides into *E.coli* W3110 cells and selection of *lacI*$^-$ mutants

Introduction of damaged or undamaged nucleotides into W3110 cells and selection of *lacI*$^-$ mutants were carried out as described by Miller (16). A white colony (the *lacI*$^+$ genotype) of W3110 was taken from an X-gal minimal plate and was inoculated into LB medium. The *E.coli* culture was incubated at 37°C for ~2 h (OD_{600} = 1.0), and competent cells were prepared by treatment with 0.1 M calcium chloride (14,17). Nucleotide solution (5 µl) was added to 195 µl of the *E.coli* suspension and the mixture was placed on ice for 30 min (standard assay). Alternatively, 0.5 µl of nucleotide was added to 19.5 µl of the *E.coli* suspension (small scale assay). After heat shock treatment (42°C for 2 min and then 0°C for 2 min), 800 µl (standard assay) or 350 µl (small scale assay) of LB medium was added, and the cells were incubated at 37°C for 45 min. The culture was transferred onto a P-gal plate to determine the number of *lacI* plus *lacO* mutants, and onto an LB plate after appropriate dilution to determine the cell titer. These values were used to calculate the mutation frequency of *lacI*$^-$ plus *lacO*^{c}. About 30 mutants were picked randomly from a P-gal plate and were transferred onto an X-gal plate to discriminate between *lacI*$^-$ and *lacO*^{c} mutants. The ratio of *lacI*$^-$ to *lacO*^{c} was scored and used for the calculation of the mutation frequency in the *lacI* gene. The isolated *lacI*$^-$ mutants were transferred onto an LB plate and were incubated at 37°C overnight.

Analysis of mutations

An *E.coli* colony containing the mutated *lacI* gene was picked from the LB plate, suspended in 20 µl of TE buffer (10 mM Tris–HCl, pH 7.5, and 1 mM EDTA), and boiled at 100°C for 5 min. The resultant bacterial lysate was centrifuged at 12 000 *g* for 10 min. The supernatant, containing the chromosomal DNA, was transferred into a fresh tube and used for amplification of the DNA fragment containing the *lacI* gene by the polymerase chain reaction (13,14).

The presence of an addition or deletion of the 5′-TGGC-3′ sequence, which is a mutation detected frequently in the *lacI* gene, was judged by dot blot hybridization, as described by Inoue *et al.* (9). The nucleotide sequences of the *lacI* gene fragments were analyzed by sequencing of the polymerase chain reaction products, using an Applied Biosystems PRISM Dye Primer Cycle Sequencing Kit (Perkin-Elmer) and an Applied Biosystems model 373S DNA sequencer (Perkin-Elmer), essentially as described (13,14), except that the first amplified product (1337 bp) was sequenced directly with the -21M13 dye primer.

RESULTS

In vivo mutagenicity of 5-CHO-dUTP and 5-OH-dCTP

We recently reported the mutational properties of 8-OH-dGTP and 2-hydroxy-2′-deoxyadenosine 5′-triphosphate (2-OH-dATP) *in vivo*, using the chromosomal *lacI* forward mutation assay (9). In this study, the *in vivo* mutagenicities of oxidatively damaged pyrimidine deoxynucleotides, 5-CHO-dUTP and 5-OH-dCTP, were investigated by our new method. We first evaluated the frequencies of mutations induced by these damaged nucleotides (Table 1). Mutations were induced by the addition of various concentrations of 5-CHO-dUTP and 5-OH-dCTP. In contrast, the mutation frequency was not increased by treatment with 1250 µM dTTP or dCTP. These results suggest that these damaged nucleotides were incorporated into *E.coli* and acted as mutagens *in vivo*. In the case of 5-CHO-dUTP, a dose dependency of the mutation frequency was observed within 1250 µM. A steady increase in the mutation frequency by the addition of 5-OH-dCTP was observed within 250 µM, and the mutation frequency slightly increased from 250 to 1250 µM.

Next we measured the mutation frequencies induced by 250 µM of damaged nucleotides in a standard assay (Materials and Methods). The conditions of this assay were used when the mutagenicities of 8-OH-dGTP and 2-OH-dATP were evaluated (9). As shown in Table 1, the mutation frequencies in the *lacI* plus *lacO* sequences were increased 1.8- and 2.4-fold by the 5-CHO-dUTP and 5-OH-dCTP treatments, respectively, over the control value. The frequencies of the mutations induced by 250 µM 5-OH-dCTP in standard and small scale assays were different. This result is suggestive of a lower incorporation of 5-OH-dCTP into *E.coli* in small scale assay than in standard assay. This speculation appears to be correct because lower cytotoxicity was observed in small scale assay (Table 1).

Each oxidized nucleotide induces specific types of single base substitutions

We isolated 60–82 *lacI*$^-$ mutant colonies that were obtained in the 250 µM (standard assay) and 1250 µM (small scale assay) 5-CHO-dUTP experiments and in the 250 µM 5-OH-dCTP (standard assay) experiments. Mutations in the *lacI*$^-$ gene were

Table 1. Mutagenicity and cytotoxicity of oxidatively damaged nucleotides[a]

Nucleotide added[b]		Mutation frequency[c] x10^{-5}	Viable cells[d] %
Small scale[e]			
None		1.06 (1.0)	100[h]
dTTP	1250 μM	0.96 (0.9)	94
dCTP	1250 μM	1.04 (1.0)	91
5-CHO-dUTP			
	125 μM	2.03 (1.9)[g]	48
	250 μM	2.50 (2.4)[g]	32
	1250 μM	3.70 (3.5)[g]	27
5-OH-dCTP			
	125 μM	1.18 (1.1)	99
	250 μM	1.73 (1.6)[g]	77
	1250 μM	1.89 (1.7)[g]	70
Standard scale[f]			
None		1.20 (1.0)	100[h]
5-CHO-dUTP	250 μM	2.23 (1.8)[g]	38
5-OH-dCTP	250 μM	2.97 (2.4)[g]	47

[a]The values represent the average of three independent experiments.
[b]Final concentrations in the *E.coli* suspension are shown.
[c]Mutations in the *lacI* and *lacO* loci are shown. Relative mutation frequencies are shown in parentheses.
[d]*Escherichia coli* colonies on LB plates are shown.
[e]Final volumes of *E.coli* suspensions were 20 μl.
[f]Final volumes of *E.coli* suspensions were 200 μl.
[g]$P < 0.05$ for difference from the no nucleotide added experiment in each condition.
[h]These values were defined as 100% for each experiment.

analyzed by dot blot hybridization and sequencing. We also analyzed 115 isolated *lacI*$^-$ mutants obtained in the treatment without any nucleotide (Table 2). We calculated the *lacI* mutation frequencies from the ratios of *lacI*$^-$ to *lacO*^{c} mutants on X-gal minimal plates, and obtained the frequency of each type of mutation by multiplying the percentages.

The frequencies of single base substitutions in the *lacI* gene increased 1.9-fold (21.3 to 39.5 × 10^{-7}) and 2.8-fold (21.3 to 59.2 × 10^{-7}) by the 250 μM 5-CHO-dUTP and 5-OH-dCTP treatments, respectively, over the control value. With 1250 μM 5-CHO-dUTP, the frequency was 4.1-fold higher (87.3 × 10^{-7}).

We subtracted the frequencies of each type of single base substitution for the control experiment from those induced by the nucleotide treatments. Several types of mutations were characteristically elicited (Table 3). By the 250 μM 5-CHO-dUTP treatment, transition and transversion mutations were induced at frequencies of 12.8 × 10^{-7} and 5.4 × 10^{-7}, respectively. Thus, 5-CHO-dUTP induced about twice as many transitions as transversions. By the further classification of substitution types, G·C to A·T and A·T to G·C transitions and G·C to T·A transversions were induced by ~6 × 10^{-7} each. At a 5-CHO-dUTP concentration of 1250 μM, a similar tendency was also observed, except for an increase of G·C to A·T transitions (28.0 × 10^{-7}). An increase in A·T to C·G transversions was not detectable. To summarize, 5-CHO-dUTP induced G·C to A·T ≥ A·T to G·C ≈ G·C to T·A.

In contrast, transition and transversion mutations were increased by 12.5 × 10^{-7} and by 25.4 × 10^{-7}, respectively, by the 5-OH-dCTP treatment (Table 3). G·C to A·T transitions (11.4 × 10^{-7}), and G·C to T·A (7.6 × 10^{-7}) and A·T to C·G (14.4 × 10^{-7}) transversions were induced. An increase in A·T to G·C transitions was less

Table 2. Classified mutation frequency induced by oxidatively damaged pyrimidine nucleotides[a]

	Nucleotide added							
			5-CHO-dUTP				5-OH-dCTP	
	None		250 μM[b]		1250 μM[c]		250 μM[b]	
Single base substitution	21.3	(31)	39.5	(21)	87.3	(17)	59.2	(25)
Transition	4.1	(6)	16.9	(9)	46.2	(9)	16.6	(7)
G·C -> A·T	2.8	(4)	9.4	(5)	30.8	(6)	14.2	(6)
A·T -> G·C	1.4	(2)	7.5	(4)	15.4	(3)	2.4	(1)
Transversion	17.2	(25)	22.6	(12)	41.1	(8)	42.6	(18)
G·C -> T·A	9.0	(13)	15.0	(8)	20.5	(4)	16.6	(7)
A·T -> C·G	6.9	(10)	7.5	(4)	5.1	(1)	21.3	(9)
A·T -> T·A	1.4	(2)	<1.9	(0)	5.1	(1)	4.7	(2)
G·C -> C·G	<0.7	(0)	<1.9	(0)	10.3	(2)	<2.4	(0)
Multi base mutation	57.9	(84)	102	(54)	220	(43)	135	(57)
+TGGC[d]	42.7	(62)	75.2	(40)	149	(29)	116	(49)
-TGGC[d]	11.7	(17)	22.6	(12)	46.2	(9)	16.6	(7)
Insertion/Deletion[e]	3.4	(5)	3.8	(2)	25.7	(5)	2.4	(1)
Total	79.2	(115)	141	(75)	308	(60)	194	(82)

[a]Frequencies of each type of mutation (×10^{-7}) in the *lacI* gene are shown. Cases found are shown in parentheses.
[b]Standard scale assay.
[c]Small scale assay.
[d]All of the mutations occurred at positions 621–632 from the *lacI* mRNA transcription start.
[e]+/–TGGC mutations are not included.

Table 3. Frequencies of single base substitutions ($\times 10^{-7}$)[a]

	5-CHO-dUTP		5-OH-dCTP
	250 μM	1250 μM	250 μM
Single base substitution	18.2	66.0	37.9
Transition	12.8	42.1	12.5
G·C -> A·T	6.6	28.0	11.4
A·T -> G·C	6.1	14.0	1.0
Transversion[b]	5.4	23.9	25.4
G·C -> T·A	6.0	11.5	7.6
A·T -> C·G	0.6	-1.8	14.4

[a]Frequencies of each type of single base substitution for the control experiment are subtracted from those induced by the nucleotide treatments (Table 2).
[b]These values include the frequencies of A·T to T·A and G·C to C·G transversions.

Table 4. The single base substitutions detected in the *lacI* gene[a]

Mutation type	Control	5-CHO-dUTP	5-OH-dCTP
G•C -> A•T	638 733 884 (X2)	469 (X2) 718 731 884	154 251 484 500 884 (X2)
A•T -> G•C	944 965	44 419 830 922	776
G•C -> T•A	45 260 305 568 594 677 695 703 737 752 817 845 (X2)	278 305 433 484 589 735 853 1043	248 353 589 693 817 (X2) 978
A•T -> C•G	91 446 (X2) 692 803 818 (X3) 896 904	370 446 904 1063	446 596 818 (X2) 904 936 976 1063 (X2)
A•T -> T•A	602 806		562 644

[a]The positions of single base substitutions found at *lacI* locus (–50 to 1111) are shown. Numbering starts at the mRNA transcription start. In the case that the same type of mutations occurred at the same position, the number of mutations are shown in parentheses.

detectable. To summarize, 5-OH-dCTP induced mutation in the order A·T to C·G ≥ G·C to A·T ≥ G·C to T·A.

The distribution of the single base substitutions observed in the *lacI* gene is shown in Table 4. We found no hotspot for the single base substitutions.

The addition or deletion of the 5′-TGGC-3′ sequence was also induced by the treatment with 5-CHO-dUTP (1.8-fold) and 5-OH-dCTP (2.4-fold) (Table 2). Interestingly, these increased rates of the TGGC mutations are similar to the overall mutation rates in the *lacI* gene.

5-OH-dCTP is more mutagenic than 5-CHO-dUTP

We measured the incorporation rates of 5-CHO-dUTP and 5-OH-dCTP in wild type *E.coli* to determine their mutagenicities per incorporation dose. Competent W3110 cells were treated with ^{33}P-labeled 5-CHO-dUTP or 5-OH-dCTP under standard assay conditions (250 μM). The *E.coli* pellet was washed with ice-cold LB medium to remove an unincorporated nucleotide. The radioactivity incorporated into the cells was counted after lysis. We observed that 0.26% (130 pmol/assay) and 0.16% (80 pmol/assay) of the added 5-CHO-dUTP and 5-OH-dCTP, respectively, were present in the cells. The frequencies of single base substitutions in the *lacI* gene increased 1.9- and 2.8-fold by the addition of 250 μM 5-CHO-dUTP and 5-OH-dCTP, respectively (Table 2). Thus, single base substitutions corresponding to 0.9-fold of the background were induced by 130 pmol of 5-CHO-dUTP. Similarly, 80 pmol of 5-OH-dCTP elicited single base substitutions corresponding to 1.8-fold of the background. Therefore, the mutagenicities of 5-CHO-dUTP and 5-OH-dCTP were calculated to be 6.9 (0.9-fold induction/0.13 nmol) and 23 (1.8-fold induction/0.08 nmol), respectively. Thus, the mutagenicity of 5-OH-dCTP was estimated to be 3-fold higher than that of 5-CHO-dUTP.

Cytotoxicity of the damaged pyrimidine nucleotides

Viable cells after various treatments were counted, as shown in Table 1. In the small scale assay for damaged pyrimidine nucleotides, the cell viability decreased, depending on the increase in the mutation frequency. The correlation of the cell viability and the mutation frequency coincided well with the results in the standard scale assay. Thus, the oxidatively damaged pyrimidine nucleotides showed high cytotoxicities as well as high mutagenicities. In contrast, the oxidatively damaged purine nucleotides, 8-OH-dGTP and 2-OH-dATP, show no cytotoxicities but high mutagenicities (9).

As described above, 130 and 80 pmol of 5-CHO-dUTP and 5-OH-dCTP, respectively, were incorporated into *E.coli* cells in the standard assay. Thus, 5-OH-dCTP was estimated to be more cytotoxic than 5-CHO-dUTP.

DISCUSSION

The direct incorporations of 5-CHO-dUTP and 5-OH-dCTP into *E.coli* induced single base substitutions (Table 2). The mutagenicities of 5-CHO-dUTP and 5-OH-dCTP were calculated to be 6.9 (-fold induction/nmol) and 23 (-fold induction/nmol), respectively. Recently, we reported that 670 pmol of 8-OH-dGTP, a major form of dGTP oxidative damage, is incorporated into the bacteria in the standard assay and induces single base substitutions at a frequency corresponding to 11-fold over the background (9). From these data, the mutagenicity of 8-OH-dGTP is calculated to

be 16 (11-fold induction/0.67 nmol). Thus, the mutagenicities of 5-CHO-dUTP and 5-OH-dCTP were 0.4 and 1.4 times higher, respectively, than that of 8-OH-dGTP. These results suggest the importance of 5-CHO-dUTP and 5-OH-dCTP as mutagens. We previously found that 2-OH-dATP is mutagenic three times higher than 8-OH-dGTP *in vivo* (9). Taken together, the mutagenicities of oxidatively damaged nucleotides appear to be in the order of 2-OH-dATP > 5-OH-dCTP > 8-OH-dGTP > 5-CHO-dUTP.

The incorporation of these damaged nucleotides into DNA by DNA polymerases *in vitro* has been reported (7,8,18–21). The data obtained in our present and previous studies (the order of the mutagenicities) reflect this incorporation by DNA polymerase III. In addition, our data reflect the removal of these damaged nucleotides from the nucleotide pool by MutT-like enzyme(s). The other factor that affects mutagenicities *in vivo* is the existence of repair systems. It is known that 5-CHO-Ura in DNA is a substrate for the AlkA protein (10) and that endonucleases III and VIII and fapy DNA glycosylase excise 5-OH-Cyt from DNA strands (11,12). At present, no *E.coli* repair enzymes have been found to act on 2-hydroxyadenosine (2-OH-Ade) (22). Thus, the lack of 2-OH-Ade repair may be a reason for the high mutagenicity of 2-OH-dATP.

We previously treated the four unmodified deoxynucleosides with Fe(II)-EDTA-O_2, and found that the nucleosides containing 5-OH-Cyt, 5-CHO-Ura, 2-OH-Ade and 8-hydroxyguanine (8-OH-Gua) are formed at a similar level in this Fenton-type reaction (2). Thus, it appears that the four oxidatively damaged nucleotides are produced at a similar level by ROS in cells. It was already shown that 8-OH-dGTP is important in the accumulation of 8-OH-Gua in DNA and in mutagenic events (23). Therefore, our present and previous results show that the other three damaged nucleotides are involved in ROS-induced mutations.

5-CHO-dUTP induced the transition mutations, G·C to A·T and A·T to G·C (Table 3). It was shown that 5-CHO-dUTP is incorporated opposite Ade and Gua by DNA polymerases *in vitro* (7). Thus, our results indicate that 5-CHO-Ura·Gua and 5-CHO-Ura·Ade pairs were formed as intermediates for these mutations (Fig. 1). 5-CHO-dUTP also induced G·C to T·A transversions. This type of mutation may occur through the formation of a Cyt·5-CHO-UTP pair and the subsequent formation of a 5-CHO-Ura·dATP pair. On the other hand, 5-OH-dCTP induced A·T to C·G and G·C to T·A transversions (Table 3). These types of mutations appear to occur by the formation of 5-OH-Cyt·Gua and 5-OH-Cyt·Thy pairs (Fig. 1). 5-OH-dCTP also induced G·C to A·T transition mutations. The G·C to A·T mutation suggests that 5-OH-Cyt base-pairs with both Gua and Ade.

5-CHO-dUTP induced G·C to T·A mutations, but not A·T to C·G mutations. This asymmetrical pattern of mutations may be explained on the assumption of a difference in the incorporation efficiency. In the case that DNA polymerase III prefers the incorporation of 5-CHO-dUTP opposite Cyt to the incorporation of dCTP opposite 5-CHO-Ura, this mutational pattern will appear. Likewise, 5-OH-dCTP elicited G·C to A·T transitions, but not A·T to G·C mutations. This pattern is explained by the assumption that DNA polymerase III incorporates dATP opposite 5-OH-Cyt much more efficiently than 5-OH-dCTP opposite Ade. Deamination of 5-OH-Cyt forms 5-hydroxyuracil, and this secondary product might be involved in the putative preferential incorporation of dATP (24).

Alternatively, these asymmetrical mutation patterns may be explained by the action of DNA repair enzyme(s). 5-OH-Cyt has been identified as one of the substrates for *E.coli* endonuclease III, which excises various damaged pyrimidine bases from DNA (11). This enzyme catalyzes the removal of 5-OH-Cyt from 5-OH-Cyt·Ade with 70% of the efficiency of that from 5-OH-Cyt·Gua (25). However, significantly preferential formation of Gua·5-OH-dCTP over that of Ade·5-OH-dCTP has been estimated from *in vitro* results (8). Thus, the 5-OH-Cyt·Gua pair may be present much more than the 5-OH-Cyt·Ade pair before the second round of replication. The asymmetrical pattern of mutations may appear by this mechanism. 5-OH-dCTP induced A·T to C·G mutations. The excising activity for 5-OH-Cyt·Thy may be weak in *E.coli*. Likewise, an imbalance between the incorporation by DNA polymerase III and the removal by DNA repair enzymes might be involved in the asymmetrical mutation spectrum of 5-CHO-dUTP.

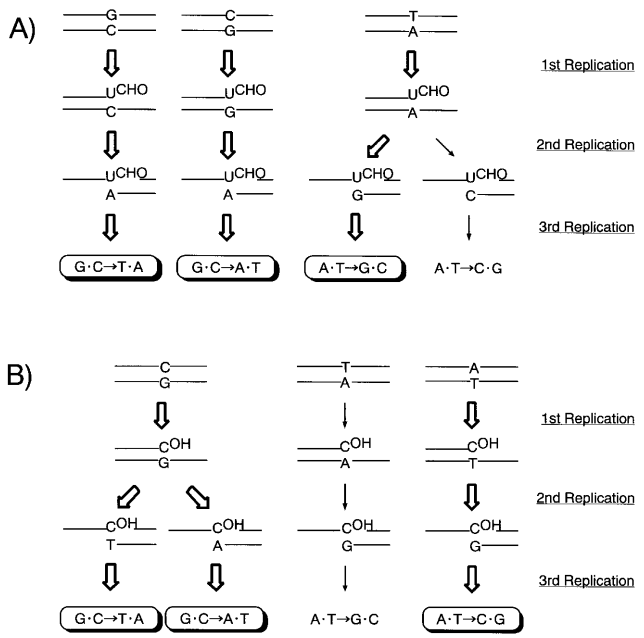

Figure 1. Proposed model for mutations induced by (**A**) 5-CHO-dUTP and (**B**) 5-OH-dCTP in *E.coli*.

In the case of 8-OH-dGTP, the MutY protein facilitates the A·T to C·G transversions induced by this damaged nucleotide, by the removal of Ade from 8-OH-Gua·Ade pairs (26,27). Cellular protein(s) may remove the base or nucleoside opposite 5-CHO-Ura, and this action may influence the mutation spectra.

The mutation spectra induced by these damaged pyrimidine nucleotides *in vivo* showed broad specificity. The present results are in contrast to our previous results with the damaged purine nucleotides, 8-OH-dGTP and 2-OH-dATP. The nucleotides specifically induce simple types of base substitutions, A·T to C·G for 8-OH-dGTP and G·C to T·A for 2-OH-dATP. Since pyrimidine bases are smaller than purines, it may be possible to form various base pairs with normal purine and pyrimidine bases via hydrogen bond(s), either directly or mediated by a water molecule.

These oxidatively damaged pyrimidine nucleotides induced not only single base substitutions, but also the loss or gain of TGGC repeats. It was in contrast to our previous findings that the damaged purine nucleotides induce mainly single base substitutions

(9). It was reported that ROS are involved in telomere shortening (28). Thus, 5-CHO-dUTP and/or 5-OH-dCTP may be involved in the process of aging.

5-CHO-dUTP and 5-OH-dCTP showed high lethalities in *E.coli*. In contrast, the cytotoxicities of the damaged purine nucleotides, 8-OH-dGTP and 2-OH-dATP, are not detected by the same procedure (9). It is possible that the lethalities of 5-CHO-dUTP and 5-OH-dCTP reflect the replication block ratio. Moreover, this possibility may be related to the induction of the TGGC mutations described above. 5-CHO-Ura and 5-OH-Cyt may cause slippage of the DNA replication machinery and misalignment through illegitimate recombination. Moreover, the incorporation of the damaged pyrimidine nucleotides may inhibit the normal metabolism of the nucleotides and/or nucleosides. These cytotoxic effects may occur in *E.coli*, which incorporates relatively large amounts of 5-CHO-dUTP and 5-OH-dCTP. If this is true, the actual mutation frequencies of these nucleotides are higher than those observed in this study. Several repair enzymes act on 5-OH-Cyt in DNA (11,12). These repair proteins would be involved in the defense against the cytotoxic and mutagenic effects of 5-OH-dCTP.

In this study, we showed that 5-CHO-dUTP and 5-OH-dCTP are mutagenic *in vivo*. MutT-type hydrolyzing activities for these nucleotides may be present in cells. The mutagenicities of the damaged nucleotides will be re-evaluated after the putative gene(s) for the sanitizing enzyme(s) are found.

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