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Comparison of Incorporation and Extension of Nucleotides *in vitro* opposite 8-Hydroxyguanine (7,8-Dihydro-8-oxoguanine) in Hot Spots of the c-Ha-*ras* Gene

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DNA templates with 8-hydroxyguanine (7,8-dihydro-8-oxoguanine, oh⁸Gua) at a site corresponding to the first or second position of codon 12 of the c-Ha-*ras* gene were prepared, and the nucleotides inserted opposite the modified base were compared. The Klenow fragment (KF) of *Escherichia coli* DNA polymerase I inserted C opposite oh⁸Gua at both positions. *Taq* DNA polymerase incorporated C and A opposite oh⁸Gua, and the ratio of C to A was higher at the first position than at the second position. DNA polymerase α (pol α) inserted A and C at the first position, and A at the second position of codon 12, indicating that the ratio of C to A was higher at the first position. Moreover, we studied the extensions of bases paired with oh⁸Gua by DNA polymerases with or without 3′-5′ exonuclease activity. G and T opposite oh⁸Gua were removed, and subsequently C was inserted by KF. We found that an oh⁸Gua:A pair was recognized by the exonuclease activity of the enzyme and that A was partially subsituted by C. On the other hand, pol α extended only C and A opposite oh⁸Gua. No difference was observed with oh⁸Gua at the two positions. These results indicate that the ratio of nucleotides incorporated opposite oh⁸Gua depends on the sequence context, while there is no particular difference in the extension of base pairs involving oh⁸Gua by DNA polymerases.

Key words: 8-Hydroxyguanine — c-Ha-*ras* gene — DNA polymerase — *In vitro* DNA synthesis — Extension

Oxygen free radicals induce a variety of DNA lesions. Among them, oh^{8}Gua[6] (also known as 7,8-dihydro-8-oxoguanine) formation has been studied most extensively. The oxygen radicals are produced during cellular respiration and cell injury, and the modified guanine is generated by exposure to environmental oxygen radical-forming agents.[1–5] Formation of oh^{8}Gua in DNA appears to be involved in mutagenesis, and consequently in carcinogenesis.[6]

We have reported that a synthetic c-Ha-*ras* proto-oncogene containing oh^{8}Gua at the second position of codon 12 (the 35th position) induces G→T and G→A mutations at the modified site, and random mutations of an adjacent G on the 5′-side of oh^{8}Gua in NIH3T3 cells.[7,8] On the other hand, we observed a different mutation spectrum (exclusively G→T transversions) when oh^{8}Gua was incorporated into the first position of codon 12 (the 34th position) of the c-Ha-*ras* gene.[8] One of the aims of the present study was to compare the mutagenic potential and the mutation spectra of oh^{8}Gua *in vitro* and in living cells in parallel to elucidate the molecular mechanism of mutations elicited by the DNA damage induced by oxygen radicals. The second aim was to compare the incorporation of nucleotides opposite oh^{8}Gua residues at different positions, because different sequence contexts may affect the incorporation of nucleotides opposite a DNA lesion. Indeed, the ratio of dTMP to dCMP incorporated opposite me^{6}Gua is affected by the 3′-neighboring base of the me^{6}Gua.[9] Also, it was reported that the type of nucleotide inserted opposite 5-hydroxycytosine or 5-hydroxyuracil is dependent on the sequence context.[10] The third aim was to compare the extensions of various base pairs involving oh^{8}Gua at different positions by DNA polymerases with or without proof-reading activity. The fourth aim was to study whether the tendencies of incorporation and extension are related to the thermodynamic stabilities, which we recently reported,[11] of duplexes with a base pair involving oh^{8}Gua. The final aim was to compare the tendencies

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[6] The abbreviations used are: oh^{8}Gua, 8-hydroxyguanine; 34-oh^{8}Gua, 8-hydroxyguanine at the first position of codon 12; 35-oh^{8}Gua, 8-hydroxyguanine at the second position of codon 12; KF, Klenow fragment; me^{6}Gua, O^6-methylguanine; PAGE, polyacrylamide gel electrophoresis; pol α, DNA polymerase α; *Taq* pol, *Taq* DNA polymerase; dNTP, 2′-deoxynucleoside 5′-triphosphate; PCR, polymerase chain reaction.

of nucleotide incorporation opposite oh^{8}Gua and those opposite another modified guanine, me^{6}Gua.

In a previous report we studied nucleotide incorporation opposite 35-oh^{8}Gua (oh^{8}Gua at the second position of codon 12 of the c-Ha-*ras* gene) by *Taq* pol and pol α.[12] In this paper we describe (1) the nucleotide incorporated opposite 35-oh^{8}Gua by KF and (2) the nucleotide(s) inserted opposite 34-oh^{8}Gua (oh^{8}Gua at the first position of the c-Ha-*ras* gene) by KF, *Taq* pol, and pol α, and a comparison of the results for 34- and 35-oh^{8}Gua. In addition, we compare (3) the nucleotides incorporated opposite me^{6}Gua at the 34th and 35th positions. We also describe (4) the extensions of various bases paired with oh^{8}Gua by DNA polymerases with or without proof-reading activity (KF or pol α).

MATERIALS AND METHODS

Enzymes Mouse DNA polymerase α-primase complex was purified from FM3A cells as described.[13] *Aat*II, *Bcl*I, and *Msc*I were purchased from Toyobo (Osaka). *Taq* pol was obtained from Wako Pure Chemical (Osaka). λ exonuclease was from BRL (Gaithersburg, MD). Other enzymes were obtained from Takara (Shiga).

Oligonucleotide synthesis The oligonucleotide with oh^{8}Gua, HRU2 34OH (Fig. 1), was synthesized by the phosphoramidite method in an Applied Biosystems model 394 DNA/RNA synthesizer using N^2-acetyl-8-methoxy-5′-*O*-monomethoxytrityl-2′-deoxyguanosine-3′-*O*-[(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite] as described previously.[7] HRU2 35OH (Fig. 1) was previously synthesized.[7] The oligonucleotides were purified by reversed-phase and anion-exchange high-performance liquid chromatographies as described previously.[14]

Preparation of DNA templates Sixty-five-mer DNA templates with oh^{8}Gua or me^{6}Gua (Fig. 1a) were prepared by enzymatic joining of HRU1, HRU2, and HRU3 as described.[12] Thirty-one-mer templates with oh^{8}Gua (Fig. 1a) were constructed in a similar way by ligation of HRU2 and HRU3. The ligation products were purified by denaturing PAGE using 20% gels containing 8 *M* urea.

***In vitro* DNA synthesis** Ten pmol of primed DNA template (65mer and Seq2 in Fig. 1) was treated with a DNA polymerase as specified. The reaction conditions of *Taq* pol and pol α were essentially the same as described previously.[12] Reactions catalyzed by KF were carried out at 37°C for 20 min using 2 pmol of the primed template and 0.2 units of the enzyme in a buffer solution containing 50 m*M* Tris-HCl (pH 7.4), 5 m*M* $MgCl_2$, 2 m*M* 2-mercaptoethanol, and 50 μM of each dNTP in a total volume of 10 μl.

Preparation of single-stranded DNA for sequence analysis DNA elongated by KF or pol α-primase complex was digested with *Alu*I as described.[12] This treatment generates a 5′-end with a phosphate group in the strand containing oh^{8}Gua or me^{6}Gua. After purification by PAGE, the cleaved products were treated with λ exonuclease, which digests DNA from the 5′-phosphorylated end to yield single-stranded DNA without a modified base, as described.[12]

Analysis of incorporated nucleotides Nucleotide(s) incorporated opposite oh^{8}Gua and me^{6}Gua were analyzed

Fig. 1. Nucleotide sequences of a) DNA templates and b) primers used in this study. G* represents oh^{8}Gua (65mer and 31 mer) or me^{6}Gua (65mer). The sequence corresponding to codon 12 of the c-Ha-*ras* gene is shown in bold type. The *Alu*I sequence is underlined. The 3′ N in Seq3 and Seq4 represents one of the four bases (A, G, C, or T).

by the polymerase chain reaction-restriction enzyme (PCR-RE) method, as described previously.[12] The products elongated by *Taq* pol or the single-stranded DNA obtained by treatment with λ exonuclease were amplified by PCR, in which a mutagenic primer and Seq2 were used. The amplified DNAs were digested with an appropriate restriction enzyme as described previously.[12]

The digested PCR products were analyzed by 20% PAGE together with controls that had not been digested with the restriction enzyme.

Extensions of bases paired with oh^{8}Gua by KF The template (65mer) with oh^{8}Gua was annealed with a primer that has one of the four bases at the 3′-end (Seq3 or Seq4, Fig. 1). Two pmol of the primed template was treated with KF as described above. The reaction mixtures were subsequently treated with *Alu*I. The DNA fragments were analyzed by native 20% PAGE. The bands corresponding to the desired products were excised and the DNAs were extracted. The nucleotide opposite oh^{8}Gua was analyzed as described above.

Extensions of bases paired with oh^{8}Gua in a 65mer template by pol α The DNA template (65mer) with oh^{8}Gua was annealed with Seq3 or Seq4 (Fig. 1) and 4 pmol of the annealed DNA was treated with pol α under the described conditions[12] in a total volume of 40 μl. The reaction mixtures were subsequently treated with *Alu*I. The DNA fragments were analyzed by native 20% PAGE.

Extensions of bases paired with oh^{8}Gua in a 31mer template by pol α A primer with one of the four bases at the 3′-end (Seq3 or Seq4, Fig. 1) was labeled at the 5′-end with [γ-^{33}P]ATP (Amersham) and T4 polynucleotide kinase, and unincorporated ATP was removed with NENSORB 20 (Du Pont).

The DNA template (31mer) with oh^{8}Gua was annealed with the ^{33}P-labeled primer. One and a half pmol of the annealed template-primer complexes was treated with one unit of pol α in a total volume of 20 μl at 37°C, as described.[12] Aliquots of 4 μl were taken at 10, 30, 60, and 120 min after incubation, mixed with 4 μl of termination solution (95% formamide-0.1% xylene cyanol-0.1% bromophenol blue), and analyzed by 8 *M* urea-15% PAGE. Autoradiograms were obtained with a Fujix BAS-2000 Bio Image Analyzer.

RESULTS

Comparison of nucleotides incorporated opposite oh^{8}Gua at the 34th and 35th positions First, we analyzed the nucleotide(s) inserted opposite 34- and 35-oh^{8}Gua by DNA polymerases. Fig. 2 shows the results of the PCR-RE analysis with the 65mer template with 34-oh^{8}Gua. The DNA amplified from the product elongated by KF was only digested with *Hap*II (Fig. 2b). This indicates that KF had inserted dCMP opposite oh^{8}Gua. On the other hand, the DNAs amplified from the products elongated by *Taq* pol and pol α were cut by *Hap*II and *Stu*I, indicating that both dCMP and dAMP had been incorporated (Fig. 2a and c). *Taq* pol incorporated dCMP preferentially, and pol α inserted dAMP more frequently than dCMP (compare Fig. 2a and c). We also analyzed the nucleotide incorporation opposite 35-oh^{8}Gua by KF (data not shown). Table I summarizes the results of analyses with data reported previously.[12] KF inserted only dCMP opposite 34- and 35-oh^{8}Gua. *Taq* pol incorporated dCMP and dAMP, and the ratio of dCMP to dAMP was higher in the case of 34-oh^{8}Gua. pol α

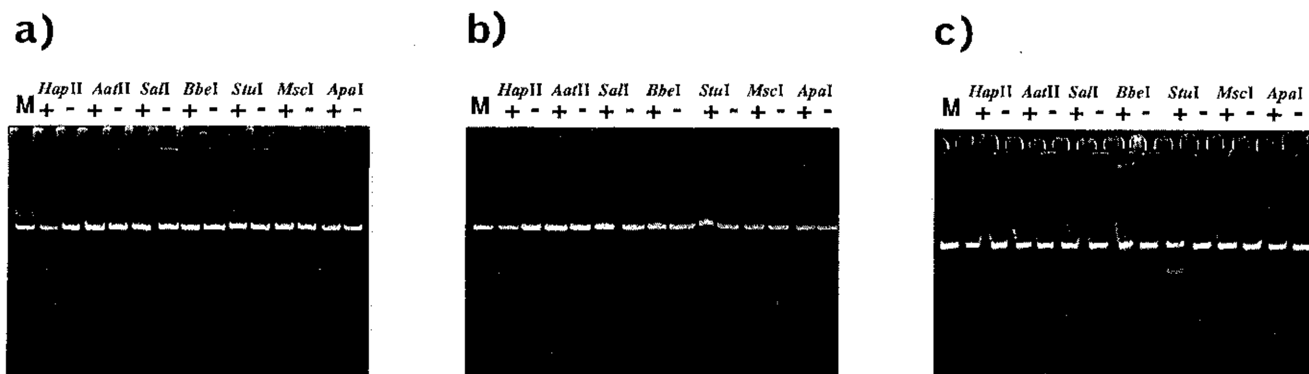

Fig. 2. Analysis of the nucleotide incorporated opposite 34-oh^{8}Gua by (a) *Taq* pol, (b) KF, and (c) pol α. The PCR-RE method was described previously.[12] Incorporation of dAMP, dTMP, or dGMP opposite 34-oh^{8}Gua is indicated by cleavage of the PCR products by *Stu*I, *Msc*I, or *Apa*I, respectively (shown as *Stu*I +, for example). To confirm the cleavage, the non-digested controls are also shown (as *Stu*I −, for example). Incorporation of dAMP, dTMP, or dGMP opposite the 3′-flanking site of oh^{8}Gua (the 35th position) are indicated by cleavage of the PCR products by *Aat*II, *Sal*I, or *Bbe*I, respectively. *Hap*II cleavage was carried out for detection of dCMP incorporation at both sites. M, an uncleaved PCR product.

Table I. Nucleotide Incorporated opposite oh^{8}Gua and me^{6}Gua

DNA lesion/position		DNA polymerase		
		KF	*Taq* pol	pol α
oh^{8}Gua	34	C	C>A$^{a)}$	A>C
	35	C	C∼A$^{b)}$	A$^{b)}$
me^{6}Gua	34	ND$^{c)}$	T>C	T∼C
	35	ND$^{c)}$	T≫C$^{b)}$	T≫C$^{b)}$

a) Ratios of nucleotides incorporated were estimated from relative band intensities of cleaved products in ethidium bromide-stained gels. X∼Y, X>Y, and X≫Y represent ratios of X to (X+Y) that are about 40–60%, 60–95%, and larger than 95%, respectively.
b) Data from reference 12.
c) Not determined.

Fig. 3. Analysis of the nucleotide incorporated opposite me^{6}Gua by pol α. Incorporation of dCMP, dAMP, dTMP, or dGMP opposite me^{6}Gua is indicated by cleavage of the PCR products by *Hap*II, *Stu*I, *Msc*I, or *Apa*I, respectively. M indicates marker DNA, pBR322-*Hae*III digests.

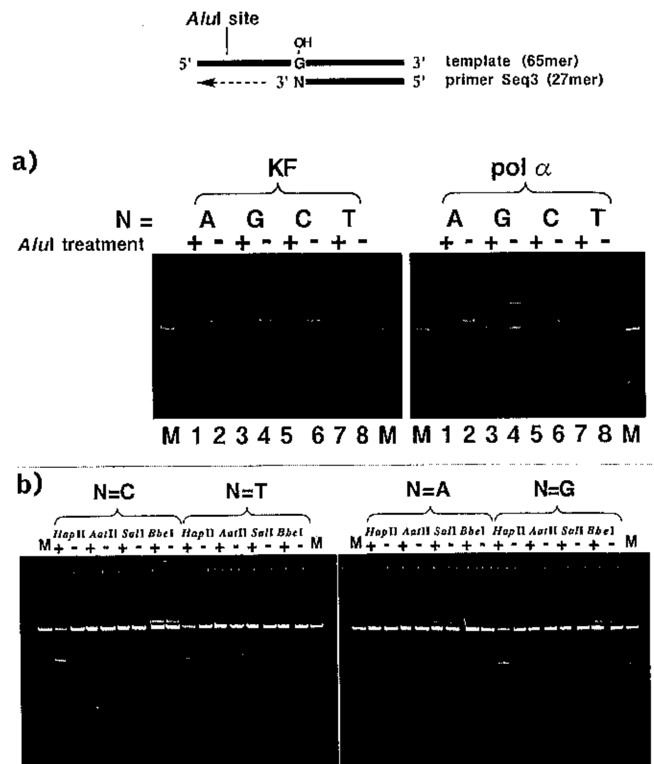

Fig. 4. Extensions of bases paired with 35-oh^{8}Gua by KF and pol α. a) The DNA template (65mer) with 35-oh^{8}Gua was annealed with a primer, Seq3, which has one of the four bases (N=A, G, C, or T), and the complexes were treated with either KF or pol α. The *Alu*I site was formed when the 3′-base was extended, and a 49 bp-fragment was produced by treatment with the enzyme. Lanes 1 and 2, N=A; lanes 3 and 4, N=G; lanes 5 and 6, N=C; lanes 7 and 8, N=T. Lanes 1, 3, 5, and 7, treated with *Alu*I; lanes 2, 4, 6, and 8, non-digested controls. M indicates marker DNA (49 bp). b) Analysis of the products extended by KF. Full-length products obtained by treatment with KF were analyzed. Incorporation of dCMP, dAMP, dTMP, or dGMP opposite oh^{8}Gua is indicated by cleavage of the PCR products by *Hap*II, *Aat*II, *Sal*I, or *Bbe*I, respectively. M, an uncleaved PCR product.

inserted A and C opposite 34-oh^{8}Gua, and A opposite 35-oh^{8}Gua. These results suggest that the ratios of dCMP to dAMP incorporated by the DNA polymerases (*Taq* pol and pol α) were higher opposite 34-oh^{8}Gua than opposite 35-oh^{8}Gua.

Comparison of nucleotides incorporated opposite me^{6}Gua at the 34th and 35th positions To determine whether the tendency to insert dCMP more frequently opposite the 34th position than opposite the 35th position was specific for oh^{8}Gua, the nucleotides incorporated opposite another modified guanine, me^{6}Gua, were compared. Fig. 3 shows that the PCR products obtained from the elongated product obtained with me^{6}Gua at the 34th position by pol α were cut by *Hap*II and *Stu*I. This indicates that dCMP and dTMP were incorporated at similar levels opposite me^{6}Gua at the 34th position by pol α. Preferential incorporation of dTMP over dCMP was observed when *Taq* pol was used in DNA synthesis (Table I). On the other hand, we previously observed that dTMP was incorporated almost exclusively opposite me^{6}Gua at the 35th position by the two DNA polymerases.[12] Therefore, a similar tendency was observed in the cases of oh^{8}Gua and me^{6}Gua; i.e., less dCMP is inserted opposite modified guanine residues at the 35th position by DNA polymerases when the 34th and 35th positions are compared.

Extensions of bases paired with oh^{8}Gua by KF It is important to know whether bases paired with oh^{8}Gua are

recognized by the proof-reading activity of a DNA polymerase. We used KF as the model DNA polymerase with 3′-5′ exonuclease activity. Template (65mer) and primer (Seq3 or Seq4) complexes with various bases opposite oh⁸Gua (Fig. 1) were treated with KF in the presence of the four dNTPs. Fig. 4a (left) shows the extensions of bases paired with 35-oh⁸Gua. When the annealed primer (Seq3) was extended by KF, an *Alu*I site was formed and therefore could be digested with the restriction enzyme (Fig. 1). All the base pairs appeared to be extended. The *Alu*I-digested products were isolated and the single-stranded DNAs obtained after λ exonuclease treatment were analyzed by the PCR-RE method. Cleavage by *Hap*II was observed when the bases opposite oh⁸Gua were C, G, and T (Fig. 4b). These results indicate that G and T opposite oh⁸Gua were removed by the 3′-5′ exonuclease activity of KF, and that dCMP was inserted instead. On the other hand, digestion was observed by *Hap*II as well as by *Aat*II in the case of the oh⁸Gua:A pair (Fig. 4b), indicating that the A paired with oh⁸Gua was partially removed by the proof-reading activity, and that dCMP was incorporated at the site of the eliminated A. Similar results were obtained in the case of base pairs with 34-oh⁸Gua (data not shown). Shibutani *et al.* reported that an A paired with oh⁸Gua was not eliminated by either KF or DNA polymerase I from *Escherichia coli.*[15] Our present findings are in contrast to their results.

Extensions of bases paired with oh⁸Gua or guanine by pol α Next we determined which bases paired with oh⁸Gua were extended by pol α, which does not contain the proof-reading activity. Fig. 4a (right) shows PAGE analysis of the extensions of various bases paired with 35-oh⁸Gua. A and C paired with oh⁸Gua were extended, and thus the cleaved band was obtained upon treatment with *Alu*I. On the other hand, a very small amount of products corresponding to the extended band was observed, as well as other products with unexpected lengths, in the cases with G and T opposite oh⁸Gua (Fig. 4a). However, sequencing of the DNA of the "extended band" after subcloning revealed that the band contained a deleted sequence, probably as the result of looped-out structures, caused by misannealing due to the presence of the "mismatched" base pair (data not shown).

In order to obtain clearer results, we prepared a shorter template (31mer, Fig. 1) and the extension of the radio-labeled primer was analyzed by denaturing PAGE. Again, A and C paired with 35-oh⁸Gua were extended (Fig. 5b). On the other hand, extension of G or T opposite oh⁸Gua was not observed (Fig. 5b). In the control experiments in which the normal sequence (G instead of oh⁸Gua) was used, C, T, and A opposite G were extended (Fig. 5a). Similar results were observed when the base pairs involving oh⁸Gua were located at the 34th position, although A opposite G was not extended (data not shown). These results, together with the results of the KF analysis, indicate that only C and A opposite oh⁸Gua were extended by DNA polymerases, either with or without 3′-5′ exonuclease activity.

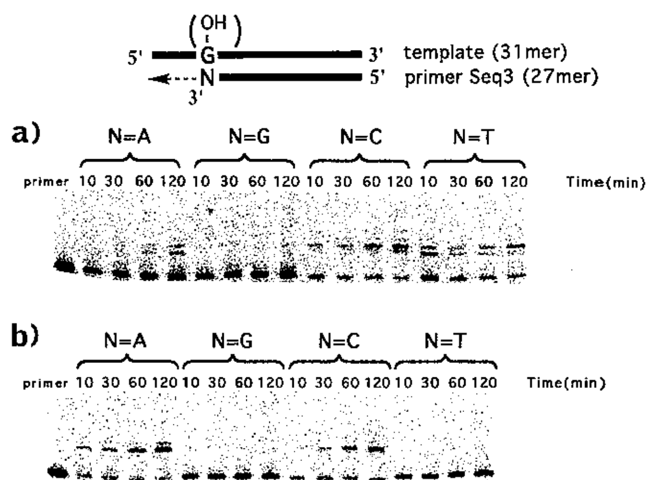

Fig. 5. Extensions of bases paired with (a) G or (b) oh⁸Gua at the 35th position by pol α. The DNA template (31mer) containing oh⁸Gua was annealed with the ^{33}P-labeled primer, which has one of the four bases at the 3′-end (Seq3 or Seq4, Fig. 1). One and a half pmol of the primed templates was treated with one unit of pol α in a total volume of 20 μl at 37°C, as described.[12] Aliquots of 4 μl were taken 10, 30, 60, and 120 min after incubation, mixed with 4 μl of termination solution (95% formamide-0.1% xylene cyanol-0.1% bromophenol blue), and analyzed by 8 *M* urea-15% PAGE. Autoradiograms were obtained with a Fujix BAS-2000 Bio Image Analyzer.

We also investigated whether extensions of bases mispaired with G at the 34th position were affected by the oh⁸Gua:C pairs at the 35th position, because we previously observed point mutations at the 34th position induced by 35-oh⁸Gua in NIH3T3 cells.[7,8] When the 31mer-template with 35-oh⁸Gua was annealed with Seq4 (Fig. 1) and treated with pol α, C and T opposite G were extended. No difference was observed as compared with mismatches at the 34th position without 35-oh⁸Gua (data not shown).

DISCUSSION

We have reported that oh⁸Gua at the 35th position of the c-Ha-*ras* gene induces a variety of point mutations at the position of oh⁸Gua and its 5′-adjacent position in NIH3T3 cells.[7,8] Moreover, we have shown that a G→T transversion takes place at the modified site almost exclusively when oh⁸Gua is incorporated into the 34th position.[8] In the present study, we first analyzed nucleo-

tides incorporated opposite 34- and 35-oh^{8}Gua in the fully elongated products to compare the mutation spectra of oh^{8}Gua in mammalian and *in vitro* systems, and to compare the nucleotide incorporated opposite the modified guanine located at different positions.

Different DNA polymerases inserted C and A in different proportions (Table I). At the 35th position, KF incorporated C, while pol α inserted only A. *Taq* pol inserted both C and A. At the 34th position, KF incorporated C, and pol α and *Taq* pol inserted both C and A. These results indicate that different DNA polymerases should be employed in mutation studies, because KF and pol α inserted different nucleotides opposite 35-oh^{8}Gua (Table I). The results also indicate the importance of using different sequences in mutation studies because pol α incorporated C and A opposite 34-oh^{8}Gua and A oppoite 35-oh^{8}Gua (Table I). It appeared that dCMP was incorporated less frequently opposite 35-oh^{8}Gua than opposite 34-oh^{8}Gua by the DNA polymerases.

The *in vitro* results described above suggest that the G→T transversion induced by 35-oh^{8}Gua may occur more frequently than the mutation by 34-oh^{8}Gua. However, the following facts should also be considered: 35-oh^{8}Gua induces a G→A transition and 5′-flanking mutations, as well as a G→T transversion, while 34-oh^{8}Gua elicits a G→T mutation almost exclusively, and oh^{8}Gua in DNA transfected into NIH3T3 cells seems to be repaired.[7,8] The discrepancy in the mutation spectrum of 35-oh^{8}Gua between the *in vitro* and semi-*in vivo* studies is possibly due to proteins involved in DNA replication. Such associated proteins, which usually contribute to the accuracy of DNA replication, probably affect the fidelity of reactions catalyzed by DNA polymerases. Replication protein-A, which is a candidate component of the DNA replication machinery, possibly reduces the incorporation of a noncomplementary nucleotide by pol α into a pol α pause site.[16] The protein may be involved in the G→A transitions and the 5′-flanking mutations detected in NIH3T3 cells.[7,8]

Shibutani *et al.* analyzed the nucleotides incorporated opposite oh^{8}Gua by KF and pol α in fully elongated products.[15] They reported that KF and pol α preferentially inserted C and A, respectively. Their results resemble ours, for the template with 35-oh^{8}Gua. The nucleotide sequence that they used was 5′···C oh^{8}Gua C···3′, while the sequences around 34- and 35-oh^{8}Gua used in this study were 5′···C oh^{8}Gua G···3′ and 5′···G oh^{8}Gua C···3′, respectively. The fact that similar results were obtained with 35-oh^{8}Gua and with oh^{8}Gua flanking cytosines, used by Shibutani *et al.*, may suggest an important role for the 3′-flanking base (C in these cases) of the modified base in the selection of the incoming dNTP by the DNA polymerases. It is possible that a base pair of the 3′-flanking base of oh^{8}Gua affects nucleotide selection through an interaction (e.g., stacking) with the incoming nucleobase. Alternatively, the secondary structure around codon 12 of the c-Ha-*ras* gene may be related to the base selection. Hoffmann *et al.* reported that the region around codon 12 was a pause site for pol α, and suggested the existence of secondary structure.[17] We used 65mer template DNAs, which can assume the putative secondary structure for *in vitro* DNA synthesis. The putative involvement of 3′-flanking bases in the base selection may be related to the nucleotide incorporation opposite me^{6}Gua at the 34th and 35th positions. Moreover, it is possible that the putative secondary structure may affect nucleotide incorporation opposite me^{6}Gua, located at the 34th and 35th positions.

We studied the extensions of various bases paired with oh^{8}Gua by KF, with 3′-5′ exonuclease activity, and by pol α, which lacks this activity (Figs. 4 and 5). KF removed G and T opposite oh^{8}Gua through its 3′-5′ exonuclease activity and inserted dCMP instead. The enzyme partially removed A paired with oh^{8}Gua, and dCMP was incorporated at the site of the eliminated A (Fig. 4). Shibutani *et al.* reported that A paired with oh^{8}Gua was not eliminated by either KF or DNA polymerase I from *E. coli.*[15] Our present findings are in contrast to their results. Again, the neighboring sequence may affect the recognition of the oh^{8}Gua:A pair by the exonuclease activity.

It is also important to examine the extension of the base pair involving oh^{8}Gua by pol α, a replicative DNA polymerase, because mutations other than the G→T transversion were detected in NIH3T3 cells[7,8] and because Sibutani *et al.* did not observe the extension of G and T paired with oh^{8}Gua by pol α. As shown in Fig. 5, C and A opposite oh^{8}Gua were extended readily, while T and G opposite oh^{8}Gua were not. It appeared that nucleotides that are not inserted *in vitro* are not extended *in vitro.*

In our thermodynamic study of oligonucleotide duplexes that have various bases opposite oh^{8}Gua, the duplexes with an oh^{8}Gua:C pair at either the 34th or 35th position are more stable than oligonucleotides with an oh^{8}Gua:A pair at the same site.[11] The differences in the stabilities (differences in the ΔG^O values) are 0.9 and 0.4 kcal/mol in the cases of the 34th and 35th positions, respectively. The fact that pol α inserted A exclusively opposite 35-oh^{8}Gua indicates a lack of direct correlation between the thermodynamic stability and the kind of dNMP incorporated by a DNA polymerase *in vitro.* However, the higher ratio of dCMP to dAMP incorporated opposite 34-oh^{8}Gua than opposite 35-oh^{8}Gua may reflect the larger difference in the stability of oh^{8}Gua:C and oh^{8}Gua:A pairs at the 34th position. In duplexes containing various bases at the 3′-end located opposite oh^{8}Gua, a remarkably small ΔG^O value (high stability)

was found with the oh^8Gua:T and oh^8Gua:G pairs at the 35th position.[11] However, we did not observe the insertion of dTMP and dGMP, or chain extensions of T and G residues opposite 35-oh^8Gua in this study. Thus, this finding suggests that there is no direct correlation between the thermodynamic stability and the kind of dNMP incorporated by a DNA polymerase *in vitro*.

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