8-Hydroxyadenine (7,8-dihydro-8-oxoadenine) induces misincorporation in *in vitro* DNA synthesis and mutations in NIH 3T3 cells

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ABSTRACT

An oligodeoxyribonucleotide containing 8-hydroxy-adenine (OH[8]Ade) was chemically synthesized and single- and double-stranded c-Ha-*ras* gene fragments with OH[8]Ade at the second position of codon 61 were prepared. The single-stranded *ras* gene fragment was used as a template for *in vitro* DNA synthesis with the Klenow fragment of *Escherichia coli* DNA polymerase I, *Taq* DNA polymerase, rat DNA polymerase β and mouse DNA polymerase α. The former two enzymes exclusively incorporated dTMP opposite OH[8]Ade. The DNA polymerases α and β misinserted dGMP, and dAMP and dGMP, respectively. The c-Ha-*ras* gene was constructed using the double-stranded *ras* gene fragment containing OH[8]Ade and was transfected into NIH 3T3 cells. The gene with OH[8]Ade induced focus formation, indicating that OH[8]Ade elicited point mutations in cells. When c-Ha-*ras* genes present in trans-formed cells were analyzed, an A→G transition and an A→C transversion were detected. These results indicate that OH[8]Ade induced misincorporation in *in vitro* DNA synthesis and mutations in mammalian cells.

INTRODUCTION

Oxygen radicals attack DNA, producing various DNA lesions. Among them, a modified guanine base, 8-hydroxyguanine (7,8-dihydro-8-oxoguanine, hereafter abbreviated to OH[8]Gua) (1) has been investigated extensively. DNA fragments with OH[8]Gua were used as templates for *in vitro* DNA synthesis and misincorporation of dAMP opposite OH[8]Gua by DNA polymerases was observed (2–4). In bacteria the modified base exclusively induces a G→T transversion (5,6). We introduced OH[8]Gua into the first and second positions of codon 12, as well as into the first position of codon 61, of a synthetic c-Ha-*ras* gene and found that

G→T mutations at the modified site were elicited in NIH 3T3 cells (7,8). Moreover, we observed that G→A transitions and mutations at the 5'-flanking positions were induced (7,8). The G→T transversion and other types of mutations induced by OH[8]Gua in mammalian cells were also examined using shuttle vectors, which are replicated as episomes (9,10). These reports indicate that OH[8]Gua causes mutations in cells.

An adenine analog of OH[8]Gua, 8-hydroxyadenine (7,8-dihydro-8-oxoadenine, hereafter abbreviated to OH[8]Ade) is produced by hydroxylation of adenine at the C8-position (Fig. 1). It was reported that OH[8]Ade was formed in DNA by γ-irradiation of DNA (11) and mice (12). Moreover, OH[8]Ade is present in the neoplastic liver of fish (13), in human cancerous tissues (14) and in the urine of ferrets and humans (15). These results suggest that OH[8]Ade in DNA may be involved in carcinogenesis. Therefore, it is important to study the misincorporation of deoxynucleotides during replication in mammalian cells and the mutations induced by OH[8]Ade in detail.

Shibutani *et al.* carried out *in vitro* DNA synthesis catalyzed by DNA polymerases using synthetic templates with OH[8]Ade and observed almost exclusive incorporation of dTMP opposite the base (16). Wood *et al.* reported that OH[8]Ade was <10% as mutagenic as OH[8]Gua in bacteria (17). However, different mutagenic potentials and mutation spectra using different (*in vitro*, prokaryotic and eukaryotic) systems have been reported for some DNA lesions. An abasic site and its analog induce a variety of mutations in mammalian cells (18–20), while they elicit the incorporation of dAMP opposite their positions *in vitro* (18,21–23) and mutations to T in bacteria (24,25). We previously demonstrated that *cis-syn* and *trans-syn* cyclobutane thymine dimers in the same nucleotide sequences showed different mutagenic potentials and mutation spectra in *in vitro* and mammalian systems (26). Moreover, nucleotide incorporation opposite ethenocytosine and propanoguanine is markedly different in bacterial and mammalian cells (27). Therefore, OH[8]Ade may

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a)   b)   c)
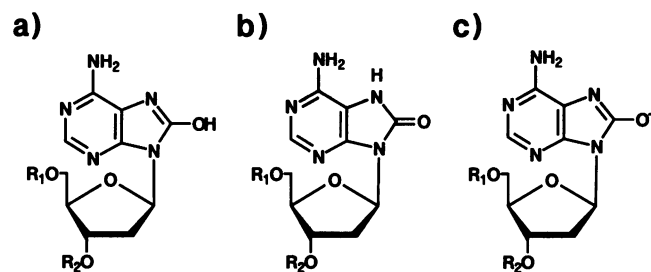
Figure 1. Structure of OH^{8}Ade. (**a**) 8-Enol (8-hydroxy) form, (**b**) 8-keto (8-oxo) form and (**c**) 8-enolate form (see Discussion).

induce mutations in mammalian cells, although the mutagenicity of the modified base is negligible in *Escherichia coli* (17).

To investigate whether OH^{8}Ade is mutagenic in mammalian cells, we transfected a synthetic c-Ha-*ras* gene with OH^{8}Ade at a hot spot, the second position of codon 61, into NIH 3T3 cells and analyzed *ras* genes present in transformed cells. Moreover, we studied nucleotide incorporation opposite OH^{8}Ade by the Klenow fragment of *E.coli* DNA polymerase I (KF), *Taq* DNA polymerase (*Taq* pol), rat DNA polymerase β (pol β) and mouse DNA polymerase α–primase complex (pol α) *in vitro*, for comparison. In this paper we describe how KF and *Taq* pol exclusively incorporate dTMP opposite OH^{8}Ade and that the DNA polymerases α and β misinsert dGMP and dAMP and dGMP, respectively. We also report that OH^{8}Ade can activate the c-Ha-*ras* gene by a point mutation with A→G and that the modified base induces an A→C transversion as well.

MATERIALS AND METHODS

Enzymes

Recombinant rat DNA polymerase β was a gift from Dr Akio Matsukage. Mouse DNA polymerase α–primase complex was isolated from FM3A cells, as previously described (28). λ exonuclease was purchased from BRL. Snake venom phosphodiesterase was obtained from Boehringer Mannheim. *Taq* DNA polymerase was from Perkin-Elmer Cetus. *Bcl*I, *Msc*I and *Pvu*I were from Toyobo. *Eag*I was from New England Biolabs. *Taq*I was from Nippon Gene and BRL. Other enzymes were from Takara.

Synthesis of oligonucleotides with OH^{8}Ade

The preparation of *N*^{6}-acetyl-7,8-dihydro-8-oxo-5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxyadenosine will be reported elsewhere (H.Inoue, manuscript in preparation). Briefly, *N*^{6}-acetyl-7,8-dihydro-8-oxo-2'-deoxyadenosine was synthesized from 8-bromo-2'-deoxyadenosine by reaction with sodium acetate/acetic anhydride and by subsequent sodium methoxide treatment. This compound was then converted to the tritylated derivative. Phosphitylation with 2-cyanoethyl *N*,*N*'-diisopropylchlorophosphoramidite yielded the building block.

The oligonucleotide with OH^{8}Ade (U8, 5'-dCTTGATACCG-CAGGCCA*AG-3', A* represents OH^{8}Ade) was synthesized by the phosphoramidite method (29) in an Applied Biosystems model 380A DNA synthesizer. The oligonucleotides were purified extensively by reversed-phase and anion exchange high performance liquid chromatographies (HPLCs), as described previously (30). The purity and base composition of the

oligonucleotides were confirmed by reversed-phase HPLC, after complete digestion with snake venom phosphodiesterase and *E.coli* alkaline phosphatase.

Preparation of the *ras* template with OH^{8}Ade

An OH^{8}Ade-containing DNA template, which corresponds to the region surrounding codon 61 of a synthetic c-Ha-*ras* gene, was prepared by enzymatic joining of oligonucleotides. Namely, oligonucleotides U8 and U9 (Fig. 2a) (31) were phosphorylated in the presence of ATP and T4 polynucleotide kinase. The phosphorylated oligonucleotides were mixed with oligomers U7, L11, L12 and L13 and the mixture was annealed and treated with T4 DNA ligase. Single-stranded DNA with OH^{8}Ade (Fig. 2a) was purified by 20% polyacrylamide gel electrophoresis (PAGE) in the presence of 8 M urea and was then extracted from the gel. The DNA obtained was passed through NAP-5 (Pharmacia) to remove the urea.

DNA polymerase reactions and analysis of incorporated nucleotides

The template DNA with OH^{8}Ade was annealed with primer L11 (Fig. 2a) and the primed template was used in the polymerase reactions described below.

The reaction with *Taq* pol was carried out in 100 µl of a solution containing 100 nM primed template, 10 mM Tris–HCl, pH 8.4, 50 mM KCl, 2.5 mM MgCl$_2$, 200 µg/ml gelatin, 200 µM deoxynucleoside triphosphates (dNTPs) and 5 U polymerase at 72°C for 10 min. The full-length product obtained in the reaction with *Taq* pol was analyzed directly by the polymerase chain reaction–restriction enzyme (PCR–RE) method, as described (3).
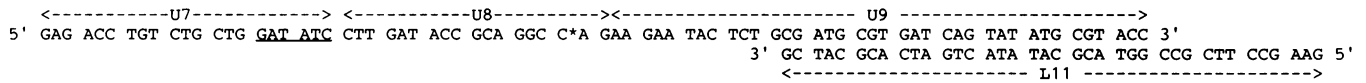
The experiments with KF were conducted in 100 µl of a solution containing 200 nM primed template, 50 mM Tris–HCl, pH 7.4, 5 mM MgCl$_2$, 2 mM 2-mercaptoethanol, 50 µM dNTPs and 2 U polymerase at 37°C for 20 min. The reactions with pol β were carried out in 100 µl of a solution containing 100 nM primed template, 50 mM Tris–HCl, pH 8.8, 7 mM MgCl$_2$, 12% glycerol, 0.17 µg/ml bovine serum albumin, 50 µM dNTPs and 100 U polymerase at 37°C for 10 min. The pol α reactions were carried out in 100 µl of a solution containing 100 nM primed template, 20 mM Tris–HCl, pH 8.0, 5 mM MgCl$_2$, 3.3 mM 2-mercaptoethanol, 0.2 mg/ml bovine serum albumin, 100 µM dNTPs and 10 U polymerase at 37°C for 60 min.

The full-length products obtained in the reactions with KF and the pol α and pol β were purified by 20% PAGE. The DNAs thus obtained were treated with *Eco*RV. This treatment generated a 5'-end with a phosphate group in the strand containing OH^{8}Ade (Fig. 2a). The cleaved DNAs were purified by 20% PAGE and then treated with λ exonuclease to obtain the single strand that lacks the modified base (complementary strand), as described (32). The single-stranded DNA was analyzed by the PCR–RE method as described (3).

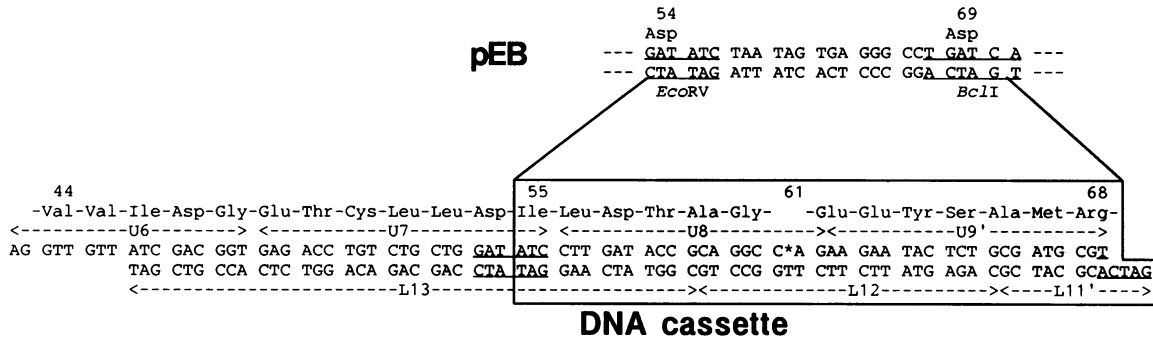### Construction of the c-Ha-*ras* gene with OH^{8}Ade and transfection of the gene into NIH 3T3 cells

Preparation of the DNA cassette and insertion into the vector was done as described previously (26,33). The vector thus obtained (100, 150 and 500 ng), with 30 µg genomic DNA isolated from NIH 3T3 cells, was transfected into NIH 3T3 cells by the calcium phosphate procedure, as described (34,35).

a)

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      <-----------U7------------->  <-----------U8---------->  <-------------------- U9 --------------------->
5' GAG ACC TGT CTG CTG GAT ATC CTT GAT ACC GCA GGC C*A GAA GAA TAC TCT GCG ATG CGT GAT CAG TAT ATG CGT ACC 3'
                                                                          3' GC TAC GCA CTA GTC ATA TAC GCA TGG CCG CTT CCG AAG 5'
                                                                             <-------------------- L11 -------------------->
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b)

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                                                      54                          69
                                                      Asp                         Asp
                                          pEB    --- GAT ATC TAA TAG TGA GGG CCT GAT C A ---
                                                 --- CTA TAG ATT ATC ACT CCC GGA CTA G T ---
                                                      EcoRV                       BclI

     44
     -Val-Val-Ile-Asp-Gly-Glu-Thr-Cys-Leu-Leu-Asp-|Ile-Leu-Asp-Thr-Ala-Gly-  -Glu-Glu-Tyr-Ser-Ala-Met-Arg-
     <----------U6--------> <-----------U7----------|--> <-----------U8---------><-----------U9'---------->
     AG GTT GTT ATC GAC GGT GAG ACC TGT CTG CTG GAT|ATC CTT GAT ACC GCA GGC C*A GAA GAA TAC TCT GCG ATG CGT
        TAG CTG CCA CTC TGG ACA GAC GAC CTA|TAG GAA CTA TGG CGT CCG GTT CTT CTT ATG AGA CGC TAC GCACTAG
        <-----------------------L13-------|----------------><------------L12---------><----L11'---->
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DNA cassette

Figure 2. (a) DNA template corresponding to the c-Ha-*ras* fragment surrounding codon 61 and primer L11. The positions of the OH[8]Ade and the *Eco*RV sequence are indicated by asterisks and underlining, respectively. (b) Generation of a synthetic c-Ha-*ras* gene with OH[8]Ade at the second position of codon 61. DNA cassettes with OH[8]Ade in codon 61 were inserted into pEB, which had been digested with *Eco*RV and *Bcl*I. The positions of the OH[8]Ade and the restriction enzyme sites are indicated by asterisks and underlining, respectively.

Analysis of c-Ha-*ras* genes present in transformed cells

c-Ha-*ras* genes present in transformed cells were analyzed by the PCR–RE method described previously (8,26). Briefly, the *ras* gene fragments surrounding codon 61 (204 bp) were amplified by the polymerase chain reaction (PCR) (36) using U5 (5'-dGAAG-ACTCTTACCGTAAGC-3', which corresponds to the region from codon 37 to 43 of the *ras* gene) and L9 (5'-dTTTAACGCG-TTTGATCTGTTCACGGTATTGATGGATGTC-TTC-3', which corresponds to the region from codon 91 to 104) (31) as primers and the PCR–RE method was carried out. To analyze the second position of codon 61, the mutagenic primers 5'-dGATATCCTTG-ATACCGCNGGC-3' (which corresponds to the *ras* gene from codon 54 to 60, except for the bold N) and L9 were used in the second PCR. The amplified DNA (153 bp) was then incubated with *Stu*I (N = A), *Apa*I (N = G), *Eag*I (N = C) or *Msc*I (N = T) (26). For *Taq*I digestion, the mutagenic primer 5'-dGATATCCT-TGATACCGCAGGTC-3' (the bold T is the mismatched base) was used in the second PCR. To analyze the third position of codon 61, the mutagenic primers 5'-dATCACGCATCGCAGA-GTATNGATC-3' (which corresponds to the region from codon 62 to 69, except for the bold NGA) and U5 were used in the second PCR and the PCR products (99 bp) were treated with *Bam*HI (N = G), *Bcl*I (N = T), *Bgl*II (N = A) or *Pvu*I (N = C) (26). To analyze the first position of codon 61 of the *ras* gene, the mutagenic primers 5'-dGATATCCTTGATACCGNNNNN-3', which correspond to codons 54–60 of the gene, except for the bold Ns, were employed in the second PCR. The PCR products (153 bp) were digested with a restriction enzyme (*Sna*BI, *Pvu*II and *Alu*I for detection of C→A, G and T respectively and *Hae*III for the normal sequence) (8). After restriction enzyme digestion, the PCR products were analyzed by 10% PAGE.

In some cases the results obtained by the PCR–RE method were confirmed by direct sequencing of the PCR products. The primer 5'-dAAAAGATTTGGTGTTGTTAA-3', which corresponds to codons 84–90 of the gene, was used for analysis of codon 61 by direct sequencing in an Applied Biosystems model 373A DNA sequencer, using a *Taq* DyeDeoxy Cycle Sequencing kit (Applied Biosystems).

RESULTS

Incorporation of nucleotides opposite OH[8]Ade in the *ras* template by DNA polymerases

First, we analyzed nucleotide incorporated opposite OH[8]Ade in the *ras* sequence by various DNA polymerases. PCR–RE analysis of the full-length products produced by KF, *Taq* pol, pol β and pol α are shown in Figure 3a–d respectively. Cleavage was detected when the PCR product that was obtained with KF was treated with the restriction enzyme *Msc*I (Fig. 3a, lane M+). This means that dTMP was incorporated opposite OH[8]Ade by KF. Exclusive incorporation of dTMP was detected with *Taq* pol (Fig. 3b). The DNA amplified from the product by pol β was cut by *Stu*I and *Apa*I, as well as by *Msc*I (Fig. 3c, lanes S+, A+ and M+). It was determined that the nucleotides were incorporated in the order dTMP > dGMP > dAMP into the site opposite OH[8]Ade. In the case of pol α a cleaved band was produced when the PCR product was digested with *Apa*I (Fig. 3d, lane A+), in addition to *Msc*I. This means that pol α inserted dGMP in addition to dTMP, although the incorporation of dGMP was much less than that of dTMP. Therefore, it was concluded that dTMP was incorporated opposite OH[8]Ade with the DNA polymerases used and that pol α and pol β allowed the misincorporation of dGMP (and dAMP). It is noteworthy that the prokaryotic DNA polymerases (KF and *Taq* pol) exclusively inserted a 'correct' nucleotide, while the eukaryotic DNA polymerases (α and β) incorporated an 'incorrect' nucleotide(s) in addition.
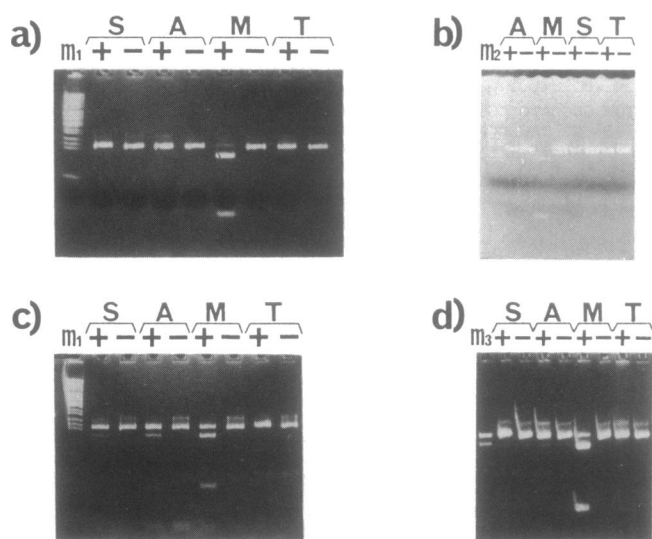
Figure 3. Sequence analysis of full-length products with Klenow fragment (a), *Taq* DNA polymerase (b), rat DNA polymerase β (c) and mouse DNA polymerase α (d). Incorporation of dAMP, dGMP, dTMP and dCMP is indicated by cleavage of the PCR products by *Stu*I (S), *Apa*I (A), *Msc*I (M) and *Taq*I (T) respectively. To confirm the cleavage, the controls, which were not digested with the enzymes, are also shown (samples with restriction enzyme treatment are indicated as S+ and those without treatment are shown as S–). m1; pBR322 *Msp*I digest, m2; pBR322 *Hae*III digest, m3; marker DNA (75 and 55 bp).

Construction of a *ras* gene with OH⁸Ade

We then investigated the mutation inducibility and spectrum of OH^8Ade in mammalian cells. We introduced OH^8Ade into the second position of codon 61 (CA*A, A* = OH^8Ade) of the c-Ha-*ras* gene, instead of the second adenine residue in the normal gene (CAA, Fig. 2b). A point mutation at this position has been shown to activate the c-Ha-*ras* gene (34). A DNA cassette with OH^8Ade was prepared by enzymatic joining of oligonucleotides, according to the method described previously (26,33). The cassette was phosphorylated and inserted into the *Eco*RV and *Bcl*I sites of the vector pEB (Fig. 2b) (18). The normal and the activated (Leu61, codon 61 is CTA) *ras* genes were constructed in the same way.

Focus-forming activity of the c-Ha-*ras* gene with OH⁸Ade

The *ras* vector constructs were transfected into NIH 3T3 cells by the calcium phosphate procedure (34,35). Table NO TAG shows the relative focus-forming activity of the c-Ha-*ras* gene with

OH^8Ade. The focus-forming activity of the normal c-Ha-*ras* gene was ~2%, as compared with an activated Leu61 c-Ha-*ras* gene (Table NO TAG). This background transformation may be elicited by overproduction of the normal c-Ha-*ras* protein (37). The c-Ha-*ras* gene with OH^8Ade at the second position of codon 61 induced more foci than the normal c-Ha-*ras* gene, but to a much lower degree as compared with the Leu61 c-Ha-*ras* gene (~3%, Table NO TAG). Thus the corrected relative focus-forming activity was ~1%.

This modified base is a very poor substrate for the *E.coli* OH^8Gua endonuclease (also known as FPG or MutM protein) (38,39). It would be interesting to know whether the mammalian repair enzymes for OH^8Gua (40) act upon OH^8Ade. If NIH 3T3 cells lack repair enzymes for OH^8Ade, the relative transforming efficiency of the c-Ha-*ras* gene with OH^8Ade may reflect the ratio of misincorporation opposite the modified base.

Mutations detected in the c-Ha-*ras* genes in transformed cells

The cells that formed a focus were isolated and the sequence in the region of codon 61 of the c-Ha-*ras* gene present in transformants was analyzed by the PCR-RE method (8,26). Figure 4a shows an example of the analysis. The PCR products were cleaved by *Taq*I (lane T+). This indicates that dCMP was incorporated and that this clone contained an activated (Arg61) c-Ha-*ras* gene. Another example of the analysis is shown in Figure 4b. The cleavage occurred when the DNAs were treated with *Apa*I, *Msc*I and *Taq*I (lanes A+, M+ and T+). This indicates that dGMP and dCMP, in addition to dTMP, were incorporated. This clone contained both mutated (Arg61 and Pro61) and normal genes. The results obtained with the PCR-RE method were confirmed by direct sequencing of PCR products (Fig. 4c). Of 33 clones analyzed, 21 clones possessed mutated gene(s). Eighteen clones have a mutation to G (Gln61→Arg61), three clones mutated to G plus C (Arg61 and Pro61) and three clones mutated to T (Leu61). All of the clones carrying the mutant gene with C have the gene with G. A point mutation to C changes amino acid 61 to Pro and does not lead to *ras* gene activation (41). It appears that cells containing both the Pro61 gene and an activated *ras* gene, such as Arg61, can form foci, although more clones have a mutated gene with C. The mutation to C may be more frequent than the mutation to G, when we consider the weak, if any, transforming activity of the Pro61 *ras* gene (41). One clone contained a point mutation at the first position of codon 61, the 5′-flanking position of OH^8Ade. No mutations at the third position of codon 61 were detected. Twelve of the 33 clones analyzed had a normal c-Ha-*ras* gene.

Table 1. Focus-forming activity of c-Ha-*ras* gene with oh⁸Ade[a]

Codon 61	DNA transfected (ng)						
	100	150			500		
		Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
CAA (Gln-61, normal)	1 (2.1)	2 (2.4)	2 (3.6)	5 (1.7)[b]	4 (1.7)	0 (0.0)	17 (3.7)
CA*A[c] (OH⁸Ade)	2 (4.2)	5 (6.0)	1 (1.8)	10 (3.5)[b]	7 (2.9)	4 (2.0)	10 (2.2)
CTA (Leu-61, activated)	48 (100)	84 (100)	55 (100)	290 (100)[b]	242 (100)	204 (100)	459 (100)

[a]Number of foci induced by a c-Ha-*ras* gene. Number in parentheses represents percentage of control (Leu-61 c-Ha-*ras* gene) values.
[b]Total number of duplicate experiments.
[c]A* represents OH⁸Ade.
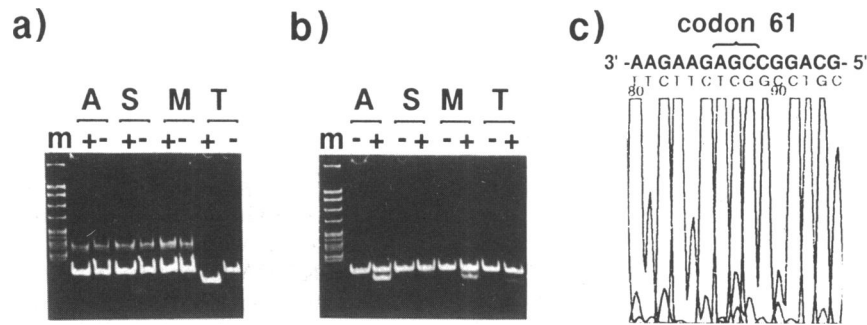
Figure 4. Sequence analysis of c-Ha-*ras* genes present in transformed NIH 3T3 cells. (**a**) Analysis of a clone containing c-Ha-*ras* with a mutation to G. (**b**) Analysis of a clone containing c-Ha-*ras* with mutations to G and C. Mutations to T, C, A (normal) and G are indicated by cleavage of the PCR products by *Apa*I (A), *Stu*I (S), *Msc*I (M) and *Taq*I (T) respectively. To confirm the cleavage, the controls, which were not digested with the enzymes, are also shown (samples with restriction enzyme treatment are indicated as A+ and those without treatment are shown as A–). m; pBR322 *Msp*I digest. (**c**) Direct sequencing of the PCR product in the region surrounding codon 61 of the c-Ha-*ras* gene.

On the other hand, we analyzed 10 focus-forming clones obtained by transfection of the normal c-Ha-*ras* gene and found that all of them contained the normal sequence (data not shown). The existence of a normal c-Ha-*ras* gene in the transformants obtained by transfection of either the *ras* gene with OH^8Ade or the normal gene may be explained by focus formation caused by overproduction of a normal c-Ha-*ras* protein (37).

Thus most of the detected mutations appeared to be induced by OH^8Ade and were not spontaneous. It is likely that dGMP and dCMP were incorporated primarily opposite OH^8Ade (except for dTMP) in NIH 3T3 cells.

DISCUSSION

Gamma irradiation of DNA produces OH^8Ade (11). The modified base is analogous to another DNA lesion, OH^8Gua (1), which is produced by oxygen radicals and which is mutagenic in prokaryotic (5,6) and mammalian cells (7,8). To investigate whether OH^8Ade is mutagenic, we studied the incorporation of deoxynucleotides opposite the modified base *in vitro* and its mutation inducibility in mammalian cells when introduced into a hotspot of the c-Ha-*ras* gene.

First we studied deoxynucleotide incorporation opposite OH^8Ade in *in vitro* DNA synthesis using various DNA polymerases. We analyzed the full-length products obtained by *in vitro* DNA synthesis in the presence of the four dNTPs. It was demonstrated that KF and *Taq* pol did not incorporate any 'incorrect' nucleotides (Fig. 3a and b). However, we found that pol β inserted dGMP and dAMP, in addition to dTMP (Fig. 3c). Moreover, pol α allowed the incorporation of dGMP, in addition to dTMP (Fig. 3d). Therefore, it was concluded that the deoxynucleotide incorporated opposite OH^8Ade most frequently was dTMP and that pol α and pol β misinserted dGMP (and dAMP), at least under these conditions and with this *ras* template.

It is interesting that the prokaryotic DNA polymerases (KF and *Taq* pol) exclusively inserted a 'correct' nucleotide, while the eukaryotic DNA polymerases (α and β) incorporated an 'incorrect' nucleotide(s). This may be a reason for the lack of detectable mutation inducibility of OH^8Ade in bacteria (17).

Guschbauer *et al.* reported that *Taq* pol inserted dTMP opposite OH^8Ade (42). Their results are consistent with our present results obtained with the same DNA polymerase. Shibutani *et al.* reported that KF and the pol α and pol β incorporated dTMP

opposite OH^8Ade almost exclusively, although they observed the incorporation of small amounts of other nucleotides (dGMP and dAMP with KF and dGMP with the pol α and pol β) in the presence of a single dNTP (16). They analyzed the full-length products obtained with all the polymerases tested and found that the products contained T opposite OH^8Ade, although they used different templates in the two experiments (16). Their results obtained when a single dNTP was used in the reactions are consistent with our present findings, that pol α and pol β misincorporated dGMP opposite OH^8Ade. We detected small amounts of incorporation of dAMP by pol β, while Shibutani *et al.* detected the incorporation of dAMP by KF. Their results on the full-length products obtained using a different oligonucleotide template are in contrast to our present findings. The reason(s) for the discrepancies between our results and theirs is unknown, but differences in the nucleotide sequences employed would possibly affect nucleotide incorporation, as reported with other modified bases (4,43).

When transfected into NIH 3T3 cells, the c-Ha-*ras* gene with OH^8Ade induced focus formation. The relative focus-forming activity of the c-Ha-*ras* gene with OH^8Ade was ~3%, while the activity of the normal gene was ~2% (Table NO TAG). Focus formation by the normal gene may be due to overproduction of the normal c-Ha-*ras* protein (37). Thus the corrected relative focus-forming activity was ~1%.

Sequence analysis of *ras* genes present in transformed cells revealed that all possible substitutions were induced, i.e. an A→G transition (18 clones, Gln61→Arg61) and A→C (three clones, Gln61→Pro61) and A→T (three clones, Gln61→Leu61) transversions. It should be noted that an A→C transversion at the second position of codon 61 does not activate the gene (41). All clones possessing a gene with an A→C mutation contained another mutated (A→G) gene. It is probable that the cells containing both the Pro61 gene and an activated *ras* gene, such as Arg61, can form foci, although more clones have a mutated gene with C. One clone had a gene mutated at the 5′-flanking position of OH^8Ade. Mutations at the 5′-flanking site of OH^8Ade occurred less frequently than those induced by OH^8Gua (8). It was unclear whether the mutation was induced by OH^8Ade or was produced spontaneously.

It is possible that the mutation to C was more frequent than the mutation to G, when we consider the weak, if any, transforming activity of the Pro61 *ras* gene (41). Therefore, it appeared that the

a)

b)

c)

d)

e)
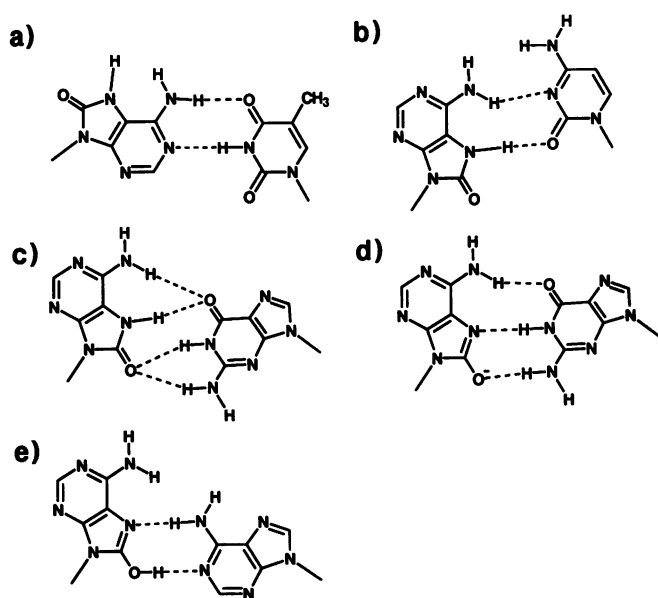
Figure 5. Base pairs involving OH^8Ade. (a) OH^8Ade:T pair elucidated in the NMR study (42). (b) Postulated OH^8Ade:C pair. (c) OH^8Ade:G pair detected in the crystal (48). (d) Another possible form of the OH^8Ade (enolate):G pair. (e) Postulated OH^8Ade (enol):A pair.

A→C and A→G mutations were primarily induced by OH^8Ade in NIH 3T3 cells.

Although dGMP was inserted opposite OH^8Ade by pol α and pol β *in vitro*, the incorporation of dCMP was not detected during *in vitro* DNA synthesis (Fig. 3c and d). The discrepancy between the *in vitro* and the semi-*in vivo* results may be explained by the possible involvement of other DNA polymerase(s) in replication. DNA polymerase δ or ε may insert dCMP more frequently than pol α. Protein(s) and other factor(s) may alter the fidelity of DNA polymerase(s). Indeed, replication protein A affects DNA polymerase fidelity (44). Moreover, the fidelities of isolated DNA polymerases are lower than those of the human replication machinery (45). Although accessory protein(s) and other factor(s) usually contribute to the accuracy of replication, they may promote the incorporation of dCMP opposite OH^8Ade. A proof-reading mechanism(s) may eliminate the nucleotide(s) other than dCMP that was inserted by DNA polymerase(s).

8-Substituted purine nucleosides are apt to adopt the *syn* conformation (46). When we consider the tautomerism between the keto-enol forms and dissociation of the N-7 proton of OH^8Ade (47), several structures can be postulated (Fig. 1). Base pairs containing OH^8Ade are shown in Figure 5. An NMR study using an oligonucleotide with OH^8Ade (42) revealed that it paired with T in a Watson–Crick manner and in the keto form (Fig. 5a). The OH^8Ade base can pair with C in the *syn*-keto form (Fig. 5b). It can pair with G by two reverse three-center hydrogen bonding systems (Fig. 5c), which were found in the crystal (48), or by three hydrogen bonds in the ionized enol form (Fig. 5d). It was reported that the pK_a of the N-7 proton is 8.7 in the riboside of OH^8Ade (47). By analogy with the riboside, the ionized form in DNA can exist in cells. Although the neutral enol form is at a disadvantage, an OH^8Ade:adenine pair can be considered (Fig. 5e).

We previously introduced OH^8Gua into the first position of codon 61 in the antisense strand of the c-Ha-*ras* gene (8). The relative focus-forming activity of the gene with OH^8Gua was ~1%. Although the mutations, and thus the amino acid alterations, induced by the two DNA lesions were different, this value is comparable with that obtained in this study (Table NO TAG). Therefore, it appears that OH^8Ade has a mutation inducibility similar to OH^8Gua in mammalian (NIH 3T3) cells.

In this paper we reported that the DNA polymerases tested incorporated dTMP opposite OH^8Ade *in vitro* and that pol β and pol α inserted dGMP as well. DNA polymerase β also incorporated dAMP. In NIH 3T3 cells OH^8Ade induced A→G and A→C mutations, suggesting the incorporation of dGMP and dCMP. We conclude that OH^8Ade is mutagenic, in contrast to previous findings (16,17).

ACKNOWLEDGEMENTS

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