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Ubiquitous Presence in Mammalian Cells of Enzymatic Activity Specifically Cleaving 8-Hydroxyguanine-containing DNA

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Here we report the finding of enzymatic activity that specifically cleaves DNA containing 8-hydroxyguanine (oh^{8}Gua) residues in various mammalian cells. To detect this activity, we used a synthetic double-stranded DNA containing a single oh^{8}Gua at a defined position as the substrate, and analyzed the products of enzymatic digestion by polyacrylamide gel electrophoresis. Two cleavage sites near the oh^{8}Gua residue were detected with partially purified fractions from cow brain and rat liver, and also with preparations from all mammalian tissues examined. These results suggest that enzymatic activity for the removal of oh^{8}Gua from DNA is widely distributed in mammalian cells.

Key words: 8-Hydroxyguanine — Repair enzyme

Active oxygen species, which are generated by environmental agents or during normal aerobic metabolism,[1-4] are known to cause modifications of nuclear and mitochondrial DNAs, such as strand scissions, and formations of thymine glycol, 5-hydroxymethyluracil, 8-hydroxyadenine and 8-hydroxyguanine[6] (oh^{8}Gua). These DNA modifications are thought to be relevant to mutagenesis and/or carcinogenesis,[5-9] and the formation of oh^{8}Gua in DNA has recently received much attention. Results have shown that 1) the amount of oh^{8}Gua formation is comparable to that of thymine glycol; 2) formation of oh^{8}Gua in cellular DNA *in vivo* is easily analyzed using a sensitive electrochemical detector coupled with HPLC; 3) formation of oh^{8}Gua in cellular DNA in target organs is increased by treatment of experimental animals with chemical carcinogens forming oxygen radicals or by whole-body irradiation of mice with γ-rays or X-rays; and 4) oh^{8}Gua residue in DNA induces misreading in *in vitro* DNA synthesis as well as mutation *in vivo.*[5-9]

The existence of a process for repair of this damage in DNA has been suggested by the presence of oh^{8}Gua in the urine of humans and rodents,[10,11] and decrease of oh^{8}Gua in mouse liver DNA after whole body γ-irradiation.[12] In fact, a specific enzyme responsible cleavage of DNA containing oh^{8}Gua was isolated from *Escherichia coli,* by the use of a double-stranded (ds) oligonucleotide containing a single oh^{8}Gua residue at a specific position as a substrate.[13] This enzyme was found to release oh^{8}Gua as a free base from DNA by its glycosylase activity and then to cleave the phosphodiester bonds 5′ and 3′ to the apurinic site.[14] Therefore, we were interested in whether similar enzymatic activity is present in mammalian cells. To examine this problem, we used the same assay procedure as for isolation of the *E. coli* enzyme. Results showed that enzymatic activity for cleavage of DNA containing oh^{8}Gua was present in various organs of rats, mice and cows, and in human cancer cell lines. Thus, enzymatic activity responsible for removal of oh^{8}Gua from DNA is widely distributed, being present in both bacteria and mammals.

MATERIALS AND METHODS

Materials [γ-^{32}P]ATP (specific activity, >5000 Ci/mmol) and dideoxyadenosine 5′-[α-^{32}P]triphosphate ([α-^{32}P]ddATP) (>5000 Ci/mmol) were purchased from Amersham. T4 polynucleotide kinase was from Takara Shuzo Co., and terminal deoxynucleotidyl transferase was from Amersham.

Preparation of substrate oligodeoxynucleotides Two kinds of 21 mer oligodeoxynucleotides containing oh^{8}Gua (a) 5′-CAGCCAATCAGTG*CACCATCC-3′ and (b) 5′-CAGCCAATCAG*TGCACCATCC-3′ (G* indicates oh^{8}Gua) were synthesized as described previously,[5] except that the phosphoramidite method was employed by phosphitylating N^2-acetyl-2′-deoxy-5′-monomethoxytrityl-8-methoxyguanosine with N,N-diiso-

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[6] The favored tautomeric structure of oh^{8}Gua is the 8-keto form, 7,8-dihydro-8-oxoguanine.

propylchlorophosphoramidite.[15] The 8-O-methyl group of the purified oligonucleotide was removed by treatment with thiophenol (0.1 ml) in a mixture of H_2O-N,N-dimethylformamide-triethylamine (1:4:1, 0.6 ml) at 35°C for 2 days under N_2. The conversion was confirmed by reverse-phase HPLC. An oligonucleotide that does not contain oh^{8}Gua, and the complementary strands of these oligonucleotides were synthesized using a DNA synthesizer (Applied Biosystems, model 380A) and purified in an oligonucleotide purification cartridge (Applied Biosystems). The products were purified further by HPLC (Beckman Ultrasphere ODS column 0.46×25 cm), developed with a linear CH_3CN gradient (9–17%) in 25 ml of 0.1 *M* triethylamine acetate (pH 6.9) at a flow rate of 1 ml/min.

Labeling and annealing of oligonucleotides For labeling of the 5′ terminus, 10 pmol of oligodeoxynucleotide was incubated for 30 min at 37°C in reaction mixture (10 μl) containing 70 m*M* Tris-HCl (pH 7.4), 10 m*M* $MgCl_2$, 5 m*M* dithiothreitol, 50 μCi [γ-^{32}P]ATP, and 10 units of T4 polynucleotide kinase. The reaction was terminated by heating at 70°C, and then ammonium acetate at a final concentration of 2.5 *M* was added followed by two volumes of ethanol. The precipitate was collected, washed with 70% ethanol and dissolved in water. The 5′-labeled oligodeoxynucleotide was annealed to its complementary strand by heating at 70°C for 10 min in a solution of 100 m*M* NaCl, 10 m*M* Tris-HCl (pH 7.4) and 1.0 m*M* EDTA, and then slowly cooled to room temperature. The formation of the duplexes was confirmed by nondenaturing polyacrylamide gel electrophoresis.

For labeling the 3′-terminus, 10 pmol of oligonucleotide was incubated at 37°C for 1 h in a solution (50 μl) of sodium cacodylate (pH 7.2), $CoCl_2$, dithiothreitol, 50 μCi [α-^{32}P]ddATP and 10 units of terminal deoxynucleotidyl transferase, using a 3′-end labeling kit from Amersham. The reaction was terminated by heating the mixture for 10 min at 70°C and then oligonucleotides were precipitated with ethanol and annealed as described above.

Enzyme nicking assay Total crude extract and fractions obtained after various purification procedures were incubated with end-labeled, double-stranded DNA substrates (0.5 pmol) in 100 μl of 50 m*M* Tris-HCl (pH 7.4), containing 50 m*M* KCl and 5 m*M* EDTA at 25°C for 1 h. Then the solution was mixed with 200 μl of 0.3 *M* sodium acetate containing 1 m*M* EDTA and yeast tRNA (0.4 mg/ml), treated with 900 μl of cold ethanol and kept for 30 min at −70°C. The resulting precipitate was dissolved in 200 μl of 0.3 *M* sodium acetate, reprecipitated with ethanol and washed with cold 80% ethanol. The pellet was dried *in vacuo,* dissolved in 10 μl of loading buffer (80% v/v formamide, 10 m*M* NaOH, 1 m*M* EDTA, 0.1% w/v xylene cyanol, 0.1% w/v bromophenol blue) and denatured by heating at 90°C for 3 min. A sample of 2 μl was applied to 20% denaturing polyacrylamide gel for electrophoresis (PAGE) to analyze the DNA fragment cleaved at the position of oh^{8}Gua.

DNA sequencing Unmodified single-stranded oligonucleotides were sequenced by the method of Maxam and Gilbert.[16] Sequence ladders were used to identify the cleavage sites of the oligonucleotides containing oh^{8}Gua. The cleavage sites were also identified by comparison with the product of hot piperidine treatment. On treatment of single-stranded oligonucleotide containing oh^{8}Gua with 10% piperidine at 90°C for 30 min, strand breaks occur on the 3′- and 5′-sides of oh^{8}Gua (M. H. Chung *et al.,* unpublished results).

Assays of the enzyme in various mammalian tissues Freshly removed cow brain (100 g) was kept in ice and then homogenized with three volumes of ice-cold buffer A [50 m*M* Tris-HCl (pH 7.4), 50 m*M* KCl, 3 m*M* EDTA, 5 m*M* magnesium acetate, 3 m*M* β-mercaptoethanol] containing protease inhibitors (5 μg/ml each of leupeptin, antipain, pepstatin and chymostatin) in a Potter-Elvehjem type homogenizer. The homogenate was centrifuged at 25,000*g* for 30 min and the resultant supernatant (crude extract) was mixed with solid ammonium sulfate to give 30% saturation. Precipitated protein was removed by centrifugation at 25,000*g* for 20 min, the supernatant fraction was adjusted to 50% saturation of ammonium sulfate, and the precipitate was collected. This precipitate was suspended in 10 ml of buffer A containing protease inhibitors and dialyzed against the same buffer. The active fraction precipitated with 30 to 50% saturation of ammonium sulfate was loaded onto a DEAE cellulose column (2.6×20 cm, DE52) equilibrated with buffer A, and the column was developed with a linear gradient of 0 to 0.4 *M* KCl in buffer A. The same procedure was used for the study of enzyme activity in rat liver. The activity in crude extracts from rat brain, kidney, spleen, heart and lung, and mouse liver, brain, kidney, stomach and spleen, and cow liver was recovered by the same procedure.

RESULTS

Detection of enzymatic activity for the removal of oh^{8}Gua from DNA (oh^{8}Gua excision repair enzyme) in cow brain extract When 5′-labeled oh^{8}Gua-containing 21 mer duplex was incubated with a crude extract of cow brain and the product was analyzed by PAGE, specific cleavage at the site of the oh^{8}Gua residue was observed (Fig. 1, lane 2). The activity responsible for this cleavage was precipitated with 30–50% saturation of ammonium sulfate and the extent of cleavage was proportional to the amount of this fraction added (Fig. 1, lanes 8 and 9). This cleavage was not observed when double-stranded

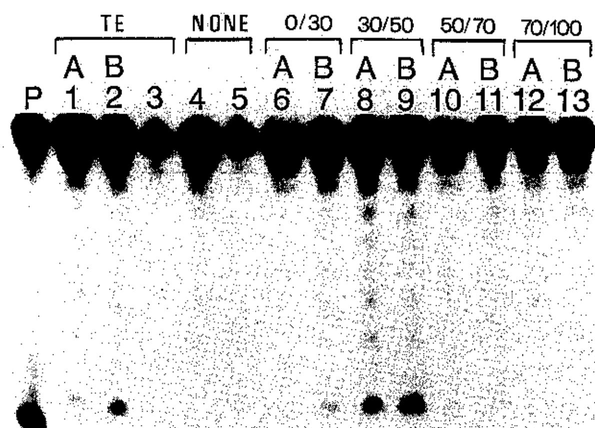

Fig. 1. Detection of enzymatic activity for the removal of oh⁸Gua in a crude extract of cow brain with 5′-end-labeled double-stranded (ds) oh⁸Gua-containing oligonucleotide (a) as the substrate. P; 5′-end-labeled oh⁸Gua-containing single-stranded oligonucleotide was treated with piperidine at 90°C for 30 min. TE, total enzyme extract (protein content: about 3.5 mg/ml); NONE, no enzyme fraction; 0/30, 30/50, 50/70 and 70/100, enzyme fractions precipitated with these concentrations of ammonium sulfate (the protein content of each fraction used was about 8–10 mg/ml). Lanes A and B, 15 μl and 30 μl of the specified enzyme fractions were added. Lanes 3 and 5, ds oligonucleotide with normal Gua in place of oh⁸Gua was incubated with 30 μl of TE fraction (lane 3) or without the enzyme (lane 5). Lane 4, ds oligonucleotide with oh⁸Gua was incubated without the enzyme. The amount of protein was estimated from the absorption at both 260 and 280 nm.[26]

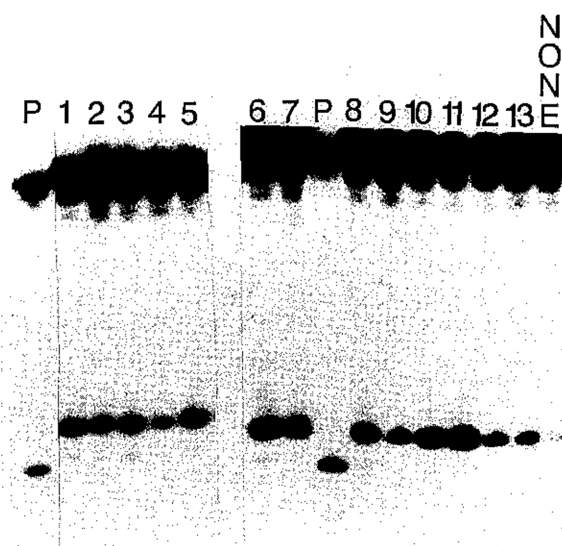

Fig. 2. Presence of oh⁸Gua excision repair enzyme in crude extracts from various mammalian tissues. The assay procedure was as described in the legend of Fig. 1. The duplex with the oligonucleotide (a) containing oh⁸Gua was used. Lane 1, mouse kidney; lane 2, mouse brain; lane 3, mouse liver; lane 4, mouse stomach; lane 5, mouse spleen; lane 6, cow brain; lane 7, cow liver; lane 8, rat brain; lane 9, rat spleen; lane 10, rat liver; lane 11, rat kidney; lane 12, rat lung; lane 13, rat heart. Lane P and NONE were as for Fig. 1. The protein content of each extract was about 3–5 mg/ml.

oligonucleotide with normal Gua in place of oh⁸Gua was used as the substrate (Fig. 1, lane 3).

Presence of oh⁸Gua excision repair enzyme in various mammalian tissues Next we examined whether the oh⁸Gua excision repair enzyme is present in other organs of cows, and in other mammals. As shown in Fig. 2, the enzymatic activity was also found in cow liver, mouse kidney, brain, liver, stomach and spleen, and rat brain, spleen, liver, kidney, lung and heart. These results indicated that the oh⁸Gua excision repair enzyme is widely distributed in various organs of the cow, mouse and rat.

Sites of incision by mammalian oh⁸Gua excision repair enzyme Fig. 2 also shows that the cleavage product derived from the 5′-end-labeled oligonucleotide catalyzed by mammalian oh⁸Gua excision repair enzyme differs from that produced by hot piperidine treatment. The mobility of the cleavage product was slightly slower than that of the product formed by hot piperidine treatment, being located between the bands of oh⁸Gua and the neighboring base, Cyt, in the Maxam-Gilbert sequence ladder. On the contrary, when 3′-end-labeled oh⁸Gua-containing 21 mer duplex was used as the substrate, the cleavage product produced by DEAE fractionation of cow brain migrated at exactly the same position as that produced by piperidine treatment on PAGE, indicating that the mammalian enzyme also cuts the phosphodiester bond 3′ to the oh⁸Gua residue (Fig. 3).

Enzymatic properties of mammalian oh⁸Gua excision repair enzyme Next we examined whether the oh⁸Gua excision repair enzyme cleaves oligonucleotide duplexes containing oh⁸Gua with various mismatches against the oh⁸Gua residue. The oligonucleotides used as complementary strands of oligonucleotide (b) were 5′-GGATGGTGCANTGATTGGCTG-3′ where N represents A, G or T as well as C. Each of these complementary oligonucleotides was annealed with oligonucleotide containing oh⁸Gua or normal Gua instead of oh⁸Gua, and used as the substrate for the enzymatic cleavage. As shown in Fig. 4, all the duplexes with or without mismatch of the oh⁸Gua residue were cleaved (lanes 1, 2, 3 and 4), but cleavage preference was different with each

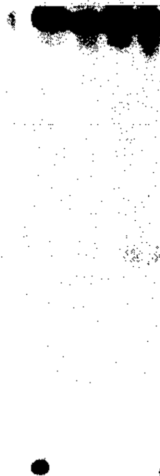

Fig. 3. Cleavage of 3′-end-labeled oh[8]Gua-containing oligonucleotide duplex by cow brain oh[8]Gua excision repair enzyme. The oligonucleotide (a) was used. Lane P, 3′-end-labeled oh[8]Gua-containing single-stranded oligonucleotide was treated with the hot piperidine. Lanes 2 and 4, oh[8]Gua-containing oligonucleotide (b) without or with the enzyme. Lanes 1 and 3, oligonucleotide with normal Gua in place of oh[8]Gua without or with the enzyme. Samples of about 250 μg (30 μl) of the DEAE fraction were used.

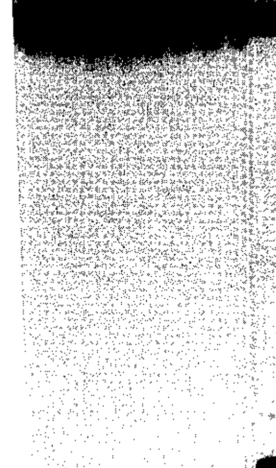

Fig. 4. Actions of rat liver oh[8]Gua excision repair enzyme in various mismatched duplexes having oh[8]Gua or normal Gua. The oligonucleotide (b) was used. Lane P, hot piperidine treatment. ENZYME (+) and (−) indicate reactions with or without the enzyme. G*, oh[8]Gua. The bases shown above lanes, such as G/T and G*/T indicate mismatched base T in the complementary strand with the oligonucleotide containing Gua or oh[8]Gua. Samples of about 250 μg (30 μl) of the DEAE fraction were used.

duplex. In order to quantitate the extent of the cleavage, the intensity of the band generated in each duplex was measured by using a Fujix 2000 Bio-Image Analyser. The ratio of the radioactivity of the cleaved band to that of uncleaved product was found to be 0.021, 0.109, 0.034 and 0.044 for the duplexes of oh[8]Gua:Ade, oh[8]Gua:Cyt, oh[8]Gua:Gua, and oh[8]Gua:Thy, respectively. Namely the mammalian oh[8]Gua excision repair enzyme seems to cleave most preferentially the duplex having an oh[8]Gua:Cyt base pair. The duplex with oh[8]Gua:Ade was found to be most resistant to the enzymatic cleavage. This order of preference for mismatched duplexes is different from that of *E. coli* oh[8]Gua specific endonuclease as shown in Fig. 5. In the case of *E. coli* enzyme, the ratios of these values were 0.084, 0.288, 0.674 and 0.101 for the duplexes of oh[8]Gua:Ade, oh[8]Gua:Cyt, oh[8]Gua:Gua, and oh[8]Gua:Thy, respectively, showing that the mismatched duplex with oh[8]Gua:Gua was more easily cleaved than the duplex with oh[8]Gua:Cyt.

It should be noted that none of the mismatched duplexes with normal Gua was cleaved by the mammalian enzyme (Fig. 4, lanes 5, 6, 7 and 8). It is also noteworthy that single-stranded oligonucleotide containing oh[8]Gua was not cleaved (data not shown).

The enzymatic activity was not affected by the addition of 10 m*M* EDTA, indicating that it did not require divalent cations for activity. Furthermore, addition of KCl or NaCl inhibited the activity: 0.35 *M* NaCl or KCl inhibited the enzymatic activity almost completely. The enzyme was heat-labile, being inactivated by heating at 55°C for 10 min (data not shown).

DISCUSSION

In this study, enzymatic activity for cleavage of DNA at the position of oh[8]Gua was found in all mammalian tissues examined. Similar activity has also been found in various tissue culture cells [human colon cancer cells (HCT-116), mouse lymphoma cells (L5178Y, M10), and human glioma cells (U87, U215, U343) (unpublished results)], and in human polymorphonuclear neutrophils (PMNs).[17] The enzyme in PMNs seems to be somewhat different from the enzyme we found in vanous mammalian tissues, because it requires divalent cation and it is precipitated with 70 to 80% saturation of ammonium sulfate. Thus mammalian cells may contain more than

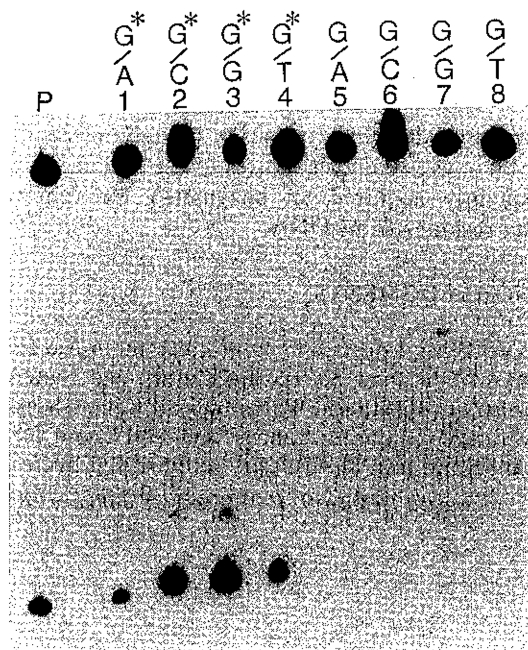

Fig. 5. Actions of *E. coli* oh^{8}Gua-specific endonuclease in various mismatched duplexes having oh^{8}Gua or normal Gua. The oligonucleotide (b) was used. *E. coli* oh^{8}Gua-specific endonuclease was prepared as described by Chung *et al.*[13] and approximately 2 μg of the purified fraction was used for each experiment.

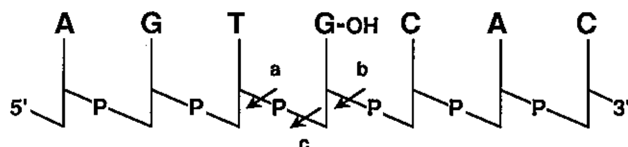

Fig. 6. Sites of cleavage of DNA containing oh^{8}Gua by mammalian oh^{8}Gua excision repair enzyme. The cleavage sites are indicated by arrows a and b. Arrows b and c indicate the cleavage site by *E. coli* oh^{8}Gua-specific endonuclease.

one enzyme with specific activity for cleavage of DNA containing oh^{8}Gua.

The sites of incision by mammalian oh^{8}Gua excision repair enzyme observed in this study differed from those of an enzyme previously isolated from *E. coli.*[13] With 5′-end-labeled oligonucleotide containing oh^{8}Gua as the substrate, the cleavage product obtained by treatment with the mammalian oh^{8}Gua excision repair enzyme moved more slowly on PAGE than that obtained by hot piperidine treatment, though the product of *E. coli* oh^{8}Gua-specific endonuclease behaved in exactly the same way as that of hot piperidine treatment.[13] Since hot piperidine treatment results in cleavage of the phosphodiester bond between the oh^{8}Gua residue and the phosphate group (arrow c in Fig. 6), the site of cleavage by the mammalian oh^{8}Gua excision repair enzyme must be the phosphodiester bond between the phosphate group and the neighboring base on the 5′-side of oh^{8}Gua, as shown by arrow a in Fig. 6, because the absence of the 5′-terminal phosphate in the DNA fragment results in a species that migrates more slowly on PAGE than that containing the terminal phosphate.[18, 19] In the case of the 3′-end-labeled oligonucleotide, the cleavage product was found to be the same as that on hot piperidine treatment. Thus, the cleavage site of mammalian oh^{8}Gua excision repair enzyme at the 3′-side of oh^{8}Gua was the same as that produced by *E. coli* oh^{8}Gua-specific endonuclease, as shown by arrow b in Fig. 6. Namely the mammalian oh^{8}Gua excision repair enzyme cuts two phosphodiester bonds adjacent to the oh^{8}Gua residue simultaneously, as shown by the arrows a and b in Fig. 6.

The substrate specificity of mammalian oh^{8}Gua excision repair enzyme concerning mismatched duplexes was similar to that of *E. coli* oh^{8}Gua-specific endonuclease, but the preference for the duplexes seems to be a little different. *E. coli* oh^{8}Gua-specific endonuclease cleaved most predominantly the duplex with oh^{8}Gua:Gua pair and then that with oh^{8}Gua:Cyt. The duplex with oh^{8}Gua:Ade was most resistant to cleavage. This result is comparable to the result previously reported by Tchou *et al.*[14] In contrast, the mammalian oh^{8}Gua excision repair enzyme used in this study cleaved most preferentially the duplex with oh^{8}Gua:Cyt base pair.

Care is necessary in substrate selection for enzyme nicking assay. It was found that oligonucleotide (a) with the oh^{8}Gua residue has difficulty in forming a duplex with its complementary oligonucleotides containing mismatched bases opposite to oh^{8}Gua residue. On the other hand, the mismatched duplexes can be easily formed in the case of oligonucleotide (b) with the oh^{8}Gua residue under the same condition (25°C), although oligonucleotides (a) and (b) both have one base mismatch. It is likely that the stability of duplexes with oh^{8}Gua residue is very much influenced by neighboring bases.

Attempts to purify the mammalian oh^{8}Gua excision repair enzyme by several column chromatographic procedures have so far been unsuccessful due to loss of activity during the purification procedure. Possibly the enzyme consists of multiple components, and its activity is lost on separation of the components during the purification. Further studies on this point are needed.

The crude extract of *E. coli* produced two cleavage products from 5′-end-labeled oh^{8}Gua-containing oligonucleotide.[13] The activity that gave the band moving more slowly than that produced by hot piperidine treatment in the sequencing gel was lost during further pu-

rification, as in the case of purification of mammalian oh^{8}Gua excision repair enzyme. Possibly this activity is generated by the combined action of oh^{8}Gua endonuclease,[20] which is an oh^{8}Gua-DNA glycosylase that also has AP lyase activity,[21] and 3′-phosphatase. Another possibility is that this activity is generated by the combined actions of oh^{8}Gua-DNA glycosylase, AP endonuclease and 5′-deoxyribophosphodiesterase.[22]

Recently *E. coli* oh^{8}Gua-specific endonuclease was shown to be identical with FPG protein,[14] which is known to cleave imidazole ring-open forms of deoxyguanosine and deoxyadenosine.[23, 24] Margison and Pegg have reported the presence of a similar enzyme to FPG protein in rodent liver.[25] Thus, oh^{8}Gua excision repair enzyme described in this paper is probably identical with the enzyme previously reported by Margison and Pegg. Further study is needed to clarify whether the mammalian oh^{8}Gua excision repair enzyme first releases oh^{8}Gua as a free base like the enzyme of *E. coli*. Investigations on this point are in progress. The ubiquitous presence of oh^{8}Gua excision repair enzyme in bacteria and mammals supports the idea that formation of oh^{8}Gua residues in DNA by oxygen radicals injures living organisms, which consequently acquired an enzyme(s) for removal of oh^{8}Gua residues from DNA.

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