In Vitro Replication Study of Modified Bases in *ras* Sequences

Hiroyuki KAMIYA,[a] Tomoki SAKAGUCHI,[a] Naoko MURATA,[a] Masahiro FUJIMURO,[a] Hiroyuki MIURA,[a] Hiroyuki ISHIKAWA,[a] Miho SHIMIZU,[a] Hideo INOUE,[a] Susumu NISHIMURA,[b] Akio MATSUKAGE,[c] Chikahide MASUTANI,[d] Fumio HANAOKA[d] and Eiko OHTSUKA*,[a]

Faculty of Pharmaceutical Sciences, Hokkaido University,[a] Kita-12 Nishi-6, Kita-ku, Sapporo 060, Japan, Biology Division, National Cancer Center Research Institute,[b] Tsukiji 5–1–1, Chuo-ku, Tokyo 104, Japan, Laboratory of Cell Biology, Aichi Cancer Center Research Institute,[c] Chikusa-ku, Nagoya 464, Japan and Cellular Physiology Laboratory, RIKEN Institute,[d] Wako, Saitama 351–01, Japan. *Received April 30, 1992*

DNA templates containing a modified base (O^6-methylguanine, 8-hydroxyguanine, xanthine or hypoxanthine) which was located in nucleotide sequences corresponding to the 12th or 61st codon of a *ras* gene were synthesized and deoxynucleotide incorporation opposite the lesions was investigated. The templates were replicated by *Taq* DNA polymerase, recombinant rat DNA polymerase β and mouse DNA polymerase α-primase complex. Sequence analysis of the replicated products indicated selective incorporation of nucleotide(s) opposite a modified base, depending on the kind of base and of DNA polymerase. This system is very useful to obtain results of *in vitro* replication of modified bases in *ras* sequences.

Keywords O^6-methylguanine; 8-hydroxyguanine; xanthine; hypoxanthine; *Taq* DNA polymerase; DNA polymerase β; DNA polymerase α; replication; *ras* gene

Introduction

Activated *ras* genes detected in human tumors and tumor cells have been found to have a point mutation at codon 12, 13, or 61 that induces substitution of an amino acid in the *ras*-encoded protein, p21.[1] Point mutations are thought to be induced in many cases by modification of a base by a mutagen and subsequent misincorporation of an "incorrect" deoxynucleotide(s) opposite the modified base by a DNA polymerase.

We have reported construction of synthetic c-Ha-*ras* genes containing a modified base (O^6-methylguanine, me^{6}Gua[2]; 8-hydroxyguanine, oh^{8}Gua[3]; xanthine, X[4] or hypoxanthine, I[5]) and their transforming activities in NIH3T3 cells. The modified genes containing me^{6}Gua or oh^{8}Gua induced significantly more foci than a normal c-Ha-*ras* gene, and the genes containing X or I showed very high transforming activity. The modified bases induced a point mutation in the modified position (me^{6}Gua→A, oh^{8}Gua→T, A and C, X→A, I→G),[2-5] and in the adjacent position in the case of oh^{8}Gua (G→A and T).[3]

It is of interest to study incorporation of deoxynucleotides into the site opposite a modified base *in vitro* as well as *in vivo* or in living cells. Many investigators have reported nucleotide incorporation opposite various modified bases (including those mentioned above; for review see ref. 6). It is possible that neighboring sequence(s) have a great effect on deoxynucleotide incorporation opposite a modified base. In order to compare the results obtained in parallel in *in vitro* and mammalian systems, it is important to know nucleotide incorporation into the site opposite the modified base (and the adjacent position in the case of oh^{8}Gua) in a *ras* sequence.

We report here analysis of deoxynucleotide incorporated *in vitro* into the site opposite the modified bases in the *ras* sequences by *Taq* DNA polymerase, which is widely used, and mouse DNA polymerase α which plays a major role in DNA replication. Rat DNA polymerase β was also used, for comparison of deoxynucleotide incorporation in DNA replication and DNA primed reaction.

Materials and Methods

Materials *Alu*I and *Eco*RV were obtained from Takara. *Taq*I and λ exonuclease were purchased from Nippon Gene and BRL, respectively. The other restriction enzymes, T4 polynucleotide kinase and T4 DNA ligase were essentially the same as described previously.[2-5,7] Rat DNA polymerase β was prepared as described.[8] Mouse DNA polymerase α-primase complex was purified from FM3A cells as described.[9]

Oligonuceotides were prepared by the phosphoramidite method using an Applied Biosystems model 380A DNA synthesizer and purified as described previously.[2-5,7]

Preparation of DNA Templates Phosphorylation of oligonucleotides was performed as described.[2-5,7] DNA templates containing either me^{6}Gua oh^{8}Gua or X were prepared by enzymatic joining of HRU1, HRU2 and HRU3 using HRL1[2] as a splint. A DNA template containing I was synthesized in a similar way using U7, U8 and U9 for the template and L11, L12 and L13[5] as splints. The ligation products were purfied by denaturing (8 M urea) 20% polyacrylamide gel electrophoresis (PAGE).

DNA Replication DNA templates were annealed with a primer (Seq 2 or L11, Fig. 1) and a DNA polymerase was added. Conditions for replication reactions in a total volume of 100 μl were essentially the same as described.[10]

Preparation of Single Strand DNA for Sequence Analysis Replicated DNA by recombinant rat DNA polymerase β or mouse DNA polymerase α-primase complex was digested with *Alu*I for me^{6}Gua, oh^{8}Gua and X, or with *Eco*RV for I. After purification by PAGE, they were treated with λ exonuclease as described.[11]

Analysis of Replicated Products Incorporated nucleotide(s) opposite me^{6}Gua, oh^{8}Gua and X (corresponding to the second position of codon 12 of a c-Ha-*ras* gene) were analyzed by the polymerase chain reaction-restriction enzyme (PCR-RE) method as described previously (Table 1).[2-4] The replicated products by *Taq* DNA polymerase or the single strand DNA obtained by treatment with λ exonuclease were amplified by the PCR[10a] in which a mutagenic primer and Seq 2 were used. The amplified DNAs were digested with an appropriate restriction enzyme as described previously.[2-4] Analysis of the 5'-flanking site of oh^{8}Gua (corresponding to the first position of codon 12 of a c-Ha-*ras* gene) was done in a similar way using restriction enzymes as previously described (Table I).[3]

The PCR-RE method was also used for analysis of nucleotide(s) opposite I (corresponding to the second position of codon 61). The mutagenic primers used for the PCR were dGATATCCTTGATACCGC*N*GGC, which correspond to synthetic c-Ha-*ras* sequence from codon 54 to codon 60, except that the third position of codon 59 (N) was replaced by T, A or G to detect incorporation of dTMP, dAMP or dGMP, respectively: a primer dGATATCCTTGATACCGCAGG*TC* was used in the case of dCMP. The PCR was carried out using L11 and one of the mutagenic primers in the reaction mixture. Restriction enzymes, *Msc*I, *Stu*I, *Apa*I and *Taq*I were used for detection of incorporated dTMP, dAMP, dGMP and dCMP, respectively (Table I).

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

a)

```
                                                12
5' CGAT ATG ACG GAA TAT AAG CTG GTG GTG GTG GGC GCC GNC GGT GTG GGC AAG AGCGCGCTGTAGG 3'  template
                                                    3' CCG TTC TCGCGCGACATCC 5'  Seq 2

                                  N = me6Gua, oh8Gua, X
```

b)

```
                                                61
5' GAG ACC TGT CTG CTG GAT ATC CTT GAT ACC GCA GGC CNA GAA GAA TAC TCT GCG ATG CGT GAT CAG TAT ATG CGT ACC 3'  template
                                                    3' GC TAC GCA CTA GTC ATA TAC GCA TGG CCG CTT CCG AAG 5'  L11
```

Fig. 1. Nucleotide Sequences of DNA Templates and Primers for me^{6}Gua, oh^{8}Gua and X (a) and I (b)

a) A template corresponding to a c-Ha-*ras* fragment around codon 12 and a primer, Seq 2 are shown. An *Alu*I sequence is indicated by shading. b) A template corresponding to a c-Ha-*ras* fragment around codon 61 and a primer, L11 are shown. An *Eco*RV sequence is indicated by shading.

Results

Replication of Template DNA Containing a Modified Base Figure 1 shows sequences of template DNA and primers used in this study. me^{6}Gua, oh^{8}Gua and X, which are derived from guanine, are located in the site corresponding to the second position of codon 12 of the synthetic c-Ha-*ras* gene (Fig. 1a). An adenine-derived I base is in the second position of codon 61 (Fig. 1b). DNA templates containing the modified base were prepared by enzymatic ligation of 3 oligonucleotide fragments which are parts of the synthetic c-Ha-*ras* gene.[2-5] After purification by denaturing gel electrophoresis, they were annealed with a primer and then replicated by DNA polymerases. Obtained duplex DNAs were purified by PAGE. In the case of *Taq* DNA polymerase, the replication products were analyzed directly by the PCR-RE method[2-4] (Fig. 2, method A). Those by recombinant rat polymerase β and mouse FM3A cell-derived polymerase α-primase complex were digested with *Alu*I for me^{6}Gua, oh^{8}Gua and X, or *Eco*RV for I to generate 5'-phosphorylated blunt ends. After purification, they were treated with λ exonuclease which preferentially recognizes a 5'-phosphorylated blunt end and hydrolyzes the strand possessing 5'-phosphate. The single strands which did not contain a modified base were analyzed (Fig. 2, method B) by the PCR-RE method.

Analysis of Incorporated Nucleotide The incorporated nucleotide was investigated by the PCR-RE method (Table I).[2-4] This method has been proven very useful to detect a point mutation in a definitive site. The purified duplex DNA or the single strand DNA was subjected to PCR, in which mutagenic primer was employed. DNA was then treated with adequate restriction enzymes and analyzed together with controls by PAGE.

Figure 3 shows results of analysis of deoxynucleotides incorporated into the site opposite me^{6}Gua by DNA polymerases. All of the DNA polymerases inserted both dTMP and dCMP at different ratios. *Taq* polymerase and polymerase α preferred dTMP to dCMP, while polymerase β incorporated dCMP and dTMP at a similar level. Incorporation of other nucleotides (dGMP and dAMP) into the opposite site was not detected in any case (Fig. 3).

Nucleotide incorporation opposite oh^{8}Gua, X and I was investigated in a similar way. Figure 4 shows the results in the case of polymerase α and Table II summarizes the results: dAMP and dCMP were incorporated opposite oh^{8}Gua by *Taq* polymerase and polymerase β, while dAMP was exclusively inserted by polymerase α; dCMP was

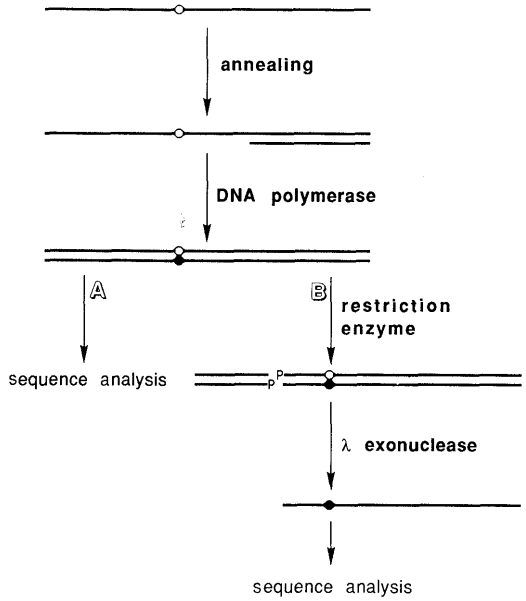

Fig. 2. Schemes for Replication of a DNA Template Containing a Modified Base and Analysis of the Product

A DNA template was annealed with a primer (Seq 2 or L11) and replicated by a DNA polymerase. In the case of *Taq* DNA polymerase, replicated DNA was directly analyzed (method A). Replicated DNA by recombinant rat DNA polymerase β or mouse DNA polymerase α-primase complex was digested with *Alu*I for me^{6}Gua, oh^{8}Gua and X, or with *Eco*RV for I. After purification by PAGE, it was treated with λ exonuclease (method B). Open circles and closed circles indicate modified positions and the opposite positions, respectively. P indicates 5'-phosphate groups.

TABLE I. Restriction Enzymes Used in the PCR-RE Method

DNA lesion	Enzyme	Recognition sequence[a]	Incorporated nucleotide, detectable[b]
me^{6}Gua	*Hap*II	5' CCGG 3'	C
oh^{8}Gua	*Aat*II	5' GACGTC 3'	A
X	*Sal*I	5' GTCGAC 3'	T
	*Bbe*I	5' GGCGCC 3'	G
oh^{8}Gua[c]	*Hap*II	5' CCGG 3'	C
	*Stu*I	5' AGGCCT 3'	A
	*Msc*I	5' TGGCCA 3'	T
	*Apa*I	5' GGGCCC 3'	G
I	*Taq*I	5' TCGA 3'	C
	*Stu*I	5' AGGCCT 3'	A
	*Msc*I	5' TGGCCA 3'	T
	*Apa*I	5' GGGCCC 3'	G

a) Investigated positions and altered positions by mutagenic primers are underlined and italicized, respectively. *b*) Nucleotides which form the recognition sites when incorporated are shown. *c*) Position opposite 5'-flanking site of oh^{8}Gua.

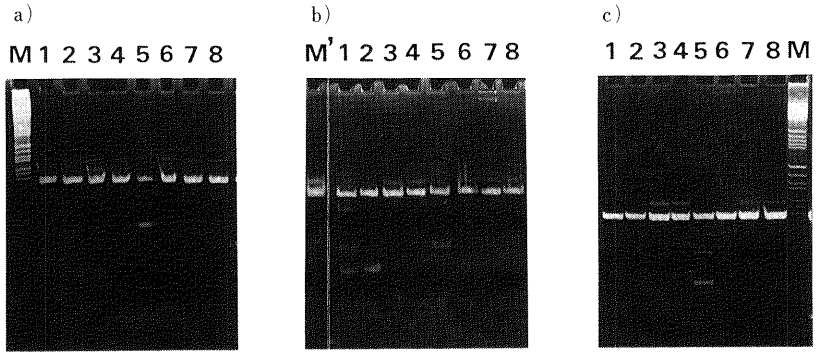

Fig. 3. Sequence Analysis of Replicated Products of me^{6}Gua by *Taq* DNA Polymerase (a), Rat DNA Polymerase β (b) and Mouse DNA Polymerase α (c)

The PCR-RE method used was described previously.[2-4] Incorporation of dCMP, dAMP, dTMP and dGMP were indicated by cleavage of the PCR products by *Hap*II, *Aat*II, *Sal*I and *Bbe*I, respectively (shown as *Hap*II +, for example). To confirm the cleavage, the controls which were omitted from digestion with the enzyme are also shown (as *Hap*II −, for example). Lane 1, *Hap*II +; lane 2, *Hap*II −; lane 3, *Aat*II +; lane 4, *Aat*II −; lane 5, *Sal*I +; lane 6, *Sal*I −; lane 7, *Bbe*I +; lane 8, *Bbe*I −. M and M′ indicate marker DNA (pBR322-*Hae*III digests) and an uncleaved PCR product, respectively.

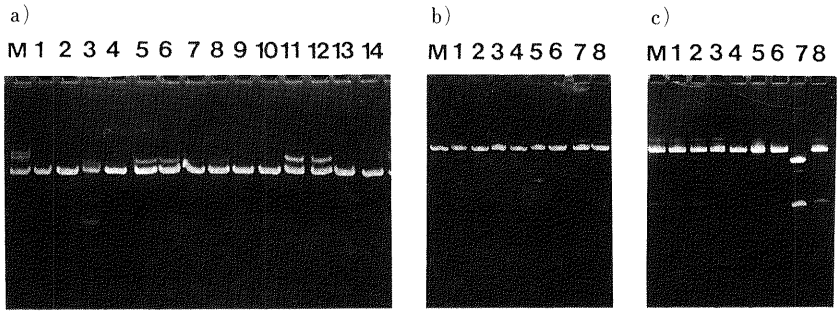

Fig. 4. Sequence Analysis of Replicated Products of oh^{8}Gua (a), X (b) and I (c) by Mouse DNA Polymerase α

a) Incorporation of dAMP, dTMP and dGMP opposite oh^{8}Gua are indicated by cleavage of the PCR products by *Aat*II, *Sal*I and *Bbe*I, respectively. Incorporation of dAMP, dTMP and dGMP opposite 5′-flanking site of oh^{8}Gua are indicated by cleavage of the PCR products by *Stu*I, *Msc*I and *Apa*I, respectively. *Hap*II cleavage was carried out for detection of dCMP incorporation at both sites. Lane 1, *Hap*II +; lane 2, *Hap*II −; lane 3, *Aat*II +; lane 4, *Aat*II −; lane 5, *Sal*I +; lane 6, *Sal*I −; lane 7, *Bbe*I +; lane 8, *Bbe*I −; lane 9, *Stu*I +; lane 10, *Stu*I −; lane 11, *Msc*I +; lane 12, *Msc*I −; lane 13, *Apa*I +; lane 14, *Apa*I −. M, an uncleaved PCR product. b) Incorporation of dCMP, dAMP, dTMP and dGMP opposite X are indicated by cleavage of the PCR products by *Hap*II, *Aat*II, *Sal*I and *Bbe*I, respectively. Lane 1, *Hap*II +; lane 2, *Hap*II −; lane 3, *Aat*II +; lane 4, *Aat*II −; lane 5, *Sal*I +; lane 6, *Sal*I −; lane 7, *Bbe*I +; lane 8, *Bbe*I −. M, an uncleaved PCR product. c) Incorporation of dAMP, dGMP, dTMP and dCMP opposite I are indicated by cleavage of the PCR products by *Stu*I, *Apa*I, *Msc*I and *Taq*I, respectively. Lane 1, *Stu*I +; lane 2, *Stu*I −; lane 3, *Apa*I +; lane 4, *Apa*I −; lane 5, *Msc*I +; lane 6, *Msc*I −; lane 7, *Taq*I +; lane 8, *Taq*I −. M, an uncleaved PCR product.

TABLE II. Nucleotides Incorporated by DNA Polymerases

	me^{6}Gua	oh^{8}Gua	X	I
Taq	T≫C	A, C	C	C
β	T, C	A<C	C	C
α	T≫C	A	T, C	C≫T

incorporated into the site opposite X by *Taq* polymerase and polymerase β, while both dTMP and dCMP were inserted by polymerase α; dCMP was selected opposite I and dTMP was also incorporated by polymerase α. Incorporation of incorrect deoxynucleotides opposite the 5′-flanking site of oh^{8}Gua was not observed with any of the DNA polymerases (Fig. 4a).

Discussion

When a synthetic c-Ha-*ras* gene containing me^{6}Gua in the second position of codon 12 was transfected into NIH3T3 cells and the c-Ha-*ras* genes present in transformed cells were analyzed, we detected exclusive mutations to A.[2] This implied that dTMP was incorporated by DNA polymerase(s) in NIH3T3 cells. Our *in vitro* study using the DNA template having the same nucleotide sequence as that of the c-Ha-*ras* gene revealed that dTMP and dCMP were incorporated opposite me^{6}Gua: *Taq* DNA polymerase and mouse DNA polymerase α showed selective incorporation of dTMP while rat DNA polymerase β inserted both dTMP and dCMP at a similar level (Fig. 3). Incorporation of dTMP and dCMP opposite me^{6}Gua, the results obtained in this study, agreed with our previous results.[2] They are also consistent with those reported by others using site-directed mutagenesis studies in *in vitro*,[12] procaryotic[13] and mammalian[12b,14] systems.

Selective incorporation of dAMP and dCMP opposite oh^{8}Gua was observed in *in vitro* study by Shibutani *et al.*[15] and *in vivo* studies using procaryotic systems showed that G→T transversions occurred in the positions where oh^{8}Gua were introduced.[16] On the other hand, we obtained different results in a mammalian system using the same oligonucleotides as those in this study.[3] Our previous report showed random incorporation of nucleotides at the modified position (oh^{8}Gua→T, A and C) although the transversion to T was most frequent. Also, we observed incorrect nucleotide incorporation at the 5′-flanking site of oh^{8}Gua (G→A and T) in the mammalian system. In this study selective incorporation of dAMP and dCMP opposite oh^{8}Gua was observed and we detected no point mutations

at the adjacent position *in vitro* (Fig. 4a). It is likely that discrepancy between *in vitro* results and our previous results is due to protein(s) involved in replication or other factor(s) in mammalian cells (NIH3T3 cells) rather than difference of nucleotide sequences.

Eritja *et al.* reported that deoxynucleotides were incorporated into the site opposite X in the order of T> C≫A, G by *Drosophila* DNA polymerase α *in vitro*.[17] Our present results are different: dCMP was incorporated by *Taq* polymerase and rat polymerase β while both dTMP and dCMP were inserted by mouse polymerase α at a similar level. Our previous report indicated that the c-Ha-*ras* gene containing X showed high transforming activity (30 to 50% of an activated *ras* gene) and transition (X→A) was observed.[4] No repair enzyme for X has been reported. If it does not exist in NIH3T3 cells, the transforming efficiency may correlate with the ratio of dTMP/dCMP incorporated by DNA polymerase(s) in NIH3T3 cells. Our present finding which shows dTMP was incorporated at a similar level to dCMP may support this idea.

Incorporation of dCMP by DNA polymerases opposite I is consistent with previous reports using Klenow fragment[18] or *Escherichia coli* system,[13d] in which exclusive I→G mutations were observed. It also agrees with our previous results of mutational analysis in the mammalian system (I→G).[5] Incorporation of dTMP, however, is a novel finding although such an event does not activate a *ras* gene.

DNA polymerase α may have lower affinity for dCTP because the ratio of dCMP/incorporated nucleotide(s) was lower than other polymerases in all of the cases investigated.

We have been investigating mutation-inducibility of DNA lesions produced by mutagens and carcinogens and have been analyzing a point mutation induced by the DNA lesions. To study mutation spectra of DNA lesions, *in vitro* replication experiments using DNA templates which have the same nucleotide sequence as studied in the mammalian system are also very important. In this work we examined nucleotide incorporation opposite the modified base (me^6Gua, oh^8Gua, X or I) *in vitro* by *Taq* DNA polymerase, rat DNA polymerase β and mouse DNA polymerase α. Similar results between *in vitro* and mammalian systems were obtained in the cases of me^6Gua, X and I, although the results on oh^8Gua were different. To study mutation-inducibility of oh^8Gua more precisely, *in vitro* systems containing proteins which are involved in DNA replication or repair should be employed. Our system reported here can be replaced by more complicated systems. This system is also useful to detect nucleotide incorporation opposite the modified base by other DNA polymerases and for analysis of other DNA lesions. Studies along these lines are in progress.

Acknowledgements This work was supported in part by Grant-in-Aids from the Ministry of Health and Welfare for the Comprehensive 10-Year Strategy for Cancer Control and from the Ministry of Education, Science and Culture of Japan.

References

1) M. Barbacid, *Annu. Rev. Biochem.*, **56**, 779 (1987); S. Nishimura and T. Sekiya, *Biochem. J.*, **243**, 313 (1987).
2) H. Kamiya, K. Miura, N. Ohtomo, S. Nishimura and E. Ohtsuka, *Jpn. J. Cancer Res.*, **82**, 997 (1991).
3) H. Kamiya, K. Miura, H. Ishikawa, H. Inoue, S. Nishimura and E. Ohtsuka, *Cancer Res.*, **52**, 3483 (1992).
4) H. Kamiya, M. Shimizu, M. Suzuki, H. Inoue and E. Ohtsuka, *Nucleosides and Nucleotides*, **11**, 247 (1992).
5) H. Kamiya, H. Miura, H. Kato, S. Nishimura and E. Ohtsuka, *Cancer Res.*, **52**, 1836 (1992).
6) B. Singer and J. M. Essigmann, *Carcinogenesis*, **12**, 949 (1991).
7) K. Miura, Y. Inoue, H. Nakamori, S. Iwai, E. Ohtsuka, M. Ikehara, S. Noguchi and S. Nishimura, *Jpn. J. Cancer Res.*, **77**, 45 (1986).
8) T. Date, M. Yamaguchi, F. Hirose, Y. Nishimoto, K. Tanihara and A. Matsukage, *Biochemistry*, **27**, 2983 (1988).
9) R. Takada-Takayama, S. Tada, F. Hanaoka and M. Ui, *Biochem. Biophys. Res. Commun.*, **170**, 589 (1990).
10) *a*) R. K. Saiki, D. H. Gelfand, S. Stoffel, S. J. Sharf, R. Higuchi, G. T. Horn, K. B. Mullis and H. A. Erlich, *Science*, **239**, 487 (1988); *b*) Y. Ohno, D. Spriggs, A. Matsukage, T. Ohno and D. Kufe, *Cancer Res.*, **49**, 2077 (1989); *c*) M. Suzuki, T. Enomoto, C. Masutani, F. Hanaoka, M. Yamada and M. Ui, *J. Biol. Chem.*, **264**, 10065 (1989)
11) R. G. Higuchi and H. Ochman, *Nucleic Acids Res.*, **17**, 5865 (1989).
12) *a*) B. Singer, F. Chavez, M. F. Goodman, J. M. Essignmann and M. K. Dosanjh, *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 8271 (1989); *b*) K. S. Ellison, E. Dogliotti, T. D. Conners, A. K. Basu and J. M. Essigmann, *ibid.*, **86**, 8620 (1989).
13) *a*) E. L. Loechler, C. L. Green and J. M. Essigmann, *Proc. Natl. Acad. Sci. U.S.A.*, **81**, 6271 (1984); *b*) R. W. Chambers, E. Sledziewska-Gojska, S. Hirani-Hojatti and H. Borowy-Borowski, *ibid.*, **82**, 7173 (1985); *c*) O. S. Bhanot and A. Ray, *ibid.*, **83**, 7348 (1986); *d*) M. Hill-Perkins, M. D. Jones and P. Karran, *Mutat. Res.*, **162**, 153 (1986).
14) G. Mitra, G. T. Pauly, R. Kumar, G. K. Pei, S. H. Hughes, R. C. Moschel and M. Barbacid, *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 8650 (1989).
15) S. Shibutani, M. Takeshita and A. P. Grollman, *Nature* (London), **349**, 431 (1991).
16) *a*) M. L. Wood, M. Dizdaroglu, E. Gajewski and J. M. Essigmann, *Biochemistry*, **29**, 7024 (1990); *b*) M. Moriya, C. Ou, V. Bodepudi, F. Johnson, M. Takeshita and A. P. Grollman, *Mutat. Res.*, **254**, 281 (1991); *c*) K. C. Cheng, D. S. Cahill, H. Kasai, S. Nishimura and L. A. Loeb, *J. Biol. Chem.*, **267**, 166 (1992).
17) R. Eritja, D. M. Horowitz, P. A. Walker, J. P. Ziehler-Martin, M. S. Boosalis, M. F. Goodman, K. Itakura and B. E. Kaplan, *Nucleic Acids Res.*, **14**, 8135 (1986).
18) E. Ohtsuka, S. Matsuki, M. Ikehara, Y. Takahashi and K. Matsubara, *J. Biol. Chem.*, **260**, 2605 (1985).