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Transforming Activity of a Synthetic c-Ha-*ras* Gene Containing O^6-Methylguanine in Codon 12

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A mutagenic DNA-adduct, O^6-methylguanine, was introduced into codon 12 of the synthetic c-Ha-*ras* gene by cassette mutagenesis. Transfection of this modified *ras* gene into normal NIH3T3 cells by the calcium phosphate procedure resulted in significant induction of focus formation. The *ras* gene inserted into the transformed cells was found to have a G to A transition at the position of the modified base. These results indicate that an O^6-methylguanine residue in DNA may lead to a mutation and be one cause of activation of the *ras* gene.

Key words: c-Ha-*ras* — O^6-Methylguanine — Transformation — Mutation — Cassette mutagenesis

Point-mutational activation of *ras* family genes (c-Ha-*ras*, c-Ki-*ras* and N-*ras*) is one of the most frequently found activations of proto-oncogenes in human cancers as well as experimental tumors, and is believed to be involved in carcinogenesis and progression of cancers.[1,2] Activated *ras* genes have a point mutation in codon 12, 13, 61 or some other codon that results in substitution of an amino acid in the *ras*-encoded protein, termed p21.[1,2] Activated forms of p21 have been shown to have lost both their intrinsic capacity to hydrolyze GTP and their GAP-mediated GTPase activity, and therefore are always in the signal "on" state. This prolongs transmission of the growth signal, resulting in unregulated cell growth.[1] Introduction of activated forms of *ras* genes into normal NIH3T3 cells by transfection is known to induce *in vitro* transformation of the cells.[1]

O^6-Methylguanine (O^6-MeG)[5] is one of the modified bases produced in DNA when cells are exposed to methylating agents such as *N*-nitroso-*N*-methylurea (NMU), which is a strong mutagen and carcinogen.[3] Many *in vitro* and *in vivo* experiments have indicated that formation of O^6-MeG in DNA is involved in mutagenesis and carcinogenesis.[3–7] The isolation of a specific repair enzyme for O^6-MeG, O^6-methylguanine-DNA methyltransferase, also supports this idea.[8–11] In a previous study mammary carcinomas developed in NMU-treated rats, and an activated c-Ha-*ras* gene with a point mutation in codon 12 (GGA→GAA; Gly→Glu) was detected in almost all the tumors.[6] Moreover, DNA polymerases were shown to insert TTP as well as dCTP that paired with O^6-MeG residues in DNA in *in vitro* DNA synthesis, suggesting that the formation of O^6-MeG leads to a G to A transition in DNA.[4,5]

Mitra *et al.* recently introduced an O^6-MeG residue into the first and second positions of codon 12 of the rat c-Ha-*ras* proto-oncogene and transfected it into Rat4 TK^- cells.[7] They found that the *ras* gene containing O^6-MeG induced focus formation, and that the *ras* genes present in the transformed cells had a mutation with a G to A transition. These results suggested that formation of O^6-MeG in a critical position of the rat c-Ha-*ras* gene is involved in activation of the gene. Similar point-mutational activation of the *ras* gene (a G to A transition; Gly→Asp) has also been found in many human cancers.[2] The nucleotide sequences around codon 12 differ in the rat and human c-Ha-*ras* genes,[6,12] and this difference may influence the mutational activity of an O^6-MeG residue in the 12th codon. Therefore, we examined whether O^6-MeG can induce a G to A transition in the human c-Ha-*ras* gene.

In this work, we induced site-specific replacement of guanine by O^6-MeG in codon 12 of a synthetic c-Ha-*ras* gene. The resulting human c-Ha-*ras* gene containing O^6-MeG was found to be active in transfection assay with normal NIH3T3 cells, and the c-Ha-*ras* genes present in the transformed cells were shown to possess a mutation with a G to A transition.

MATERIALS AND METHODS

Enzymes *Bss*HII, *Aat*II and *Nar*I were purchased from New England Biolabs, Toyobo Co. and Nippon Gene, respectively. Snake venom phosphodiesterase was obtained from Boehringer Mannheim. Other enzymes were from Takara Shuzo Co.

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[5] Abbreviations: O^6-MeG, O^6-methylguanine; NMU, *N*-nitroso-*N*-methylurea; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography.

Synthesis and purification of oligonucleotides For the synthesis of an O^6-MeG-containing oligonucleotide, a modified nucleotide unit [O^6-methyldeoxyguanosine phosphoramidite, DMT-O^6-Me-dG-(N-iBu)-P-(OCH_2CH_2CN)-N-$(iPr)_2$] was obtained from American Bionetics. The oligonucleotides were synthesized by the phosphoramidite method in an Applied Biosystems model 380A DNA synthesizer. All oligonucleotides were deblocked under standard conditions (55°C, 6 h) except that containing O^6-MeG (62°C, 50 h). The oligonucleotides were purified as described previously.[13, 14] Namely, purification by reverse-phase HPLC was performed before and after detritylation with 80% acetic acid, and, when necessary, the resulting preparations were purified further by ion exchange HPLC. The purity and base composition of the oligonucleotide containing O^6-MeG were confirmed by reverse-phase HPLC after complete digestion with snake venom phosphodiesterase and *E. coli* alkaline phosphatase.

Construction of vectors for DNA transfection Phosphorylation and ligation for DNA cassette construction were carried out by the methods described previously.[13, 14] The DNA cassettes thus obtained were phosphorylated, and joined with a plasmid pRSV-rg12[15] which was previously digested with *Cla*I and *Bss*HII. The DNAs were then treated with *Bss*HII and subjected to a ligation reaction to obtain the vectors for transfection. Aqueous solutions of the vectors were treated with phenol and chloroform. The vectors were then precipitated with ethanol, and quantitated with DNA DipStick (InVitrogen).

DNA transfection The vectors were transfected into NIH3T3 cells by the calcium phosphate procedure as described previously[15, 16]: 50 or 150 ng of the vector DNA and 30 μg of genomic DNA isolated from NIH3T3 cells were used for each transfection assay.

Analysis of the mutation found in the synthetic c-Ha-*ras* The sequence around codon 12 of the synthetic human c-Ha-*ras* present in the transformed NIH3T3 cells was amplified by PCR[17] in a DNA Thermal Cycler (Perkin Elmer Cetus). The primers used for the PCR were HR U1 (see Fig. 1) and L10[13] (dAAAAGATTTGGTGTTGTTGATAGCGAAAACGCACAG). The following reaction conditions were used for the PCR: 94°C 0.5 min, 55°C 1 min and 72°C 2 min; 45 cycles. The amplified PCR products were purified by agarose gel electrophoresis and subjected to nucleotide sequence analysis. A mutagenic primer, dAAGCTGGTGGTGGTGGGCG*N*CG, which corresponds to the human c-Ha-*ras* sequence from codon 5 to codon 11 except that the second position of codon 11 (N) was replaced by T, A or G was used in the second PCR together with L12[13] (dCAGAGTATTCTTCTTGGCCTG).[18] The products were then incubated with *Sal*I (for detection of a mutation to A), *Aat*II (for a mutation to T) or *Nar*I (for a mutation to C), and analyzed by PAGE.

RESULTS

Synthesis and purification of an oligonucleotide containing O^6-methylguanine for cassette mutagenesis We previously reported the total synthesis of the genes for human c-Ha-*ras* p21.[13, 14] These synthetic genes were then used in an *E. coli* host-vector system for large-scale isolation of normal and activated p21s for X-ray crystallography.[19-22] Moreover, when joined with the Rous sarcoma virus long terminal repeat, these synthetic c-Ha-*ras* genes with a mutation in codon 12 (Gly to Val) or codon 61 (Gln to Leu or Arg) were shown to have transforming activity in transfection assay with NIH3T3 cells as recipients.[15] The synthetic c-Ha-*ras* genes have several unique restriction enzyme sites. Therefore, site-directed introduction of an O^6-MeG residue into codon 12 of c-Ha-*ras* to analyze the mutagenicity of O^6-MeG with transforming activity can easily be achieved by exchanging the DNA cassette.

Fig. 1 shows a DNA cassette for insertion of an O^6-MeG residue into the second position of codon 12 of the synthetic c-Ha-*ras*. The DNA cassette possessing an

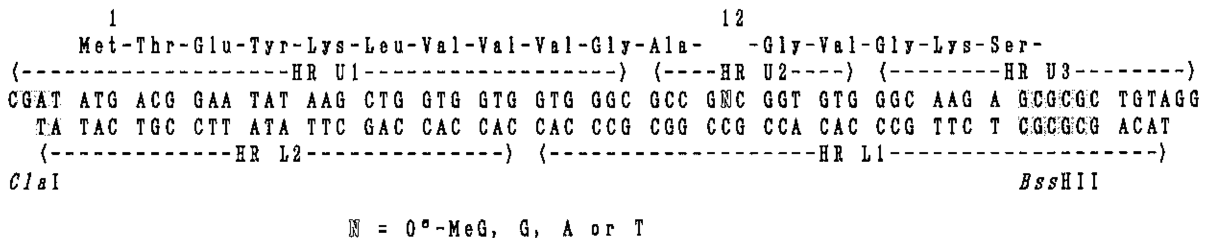

Fig. 1. DNA cassettes for site-specific modification of codon 12. Their nucleotide sequences are according to that of a human c-Ha-*ras*-1 gene. They have a *Cla*I end and an internal *Bss*HII site (indicated by shading).

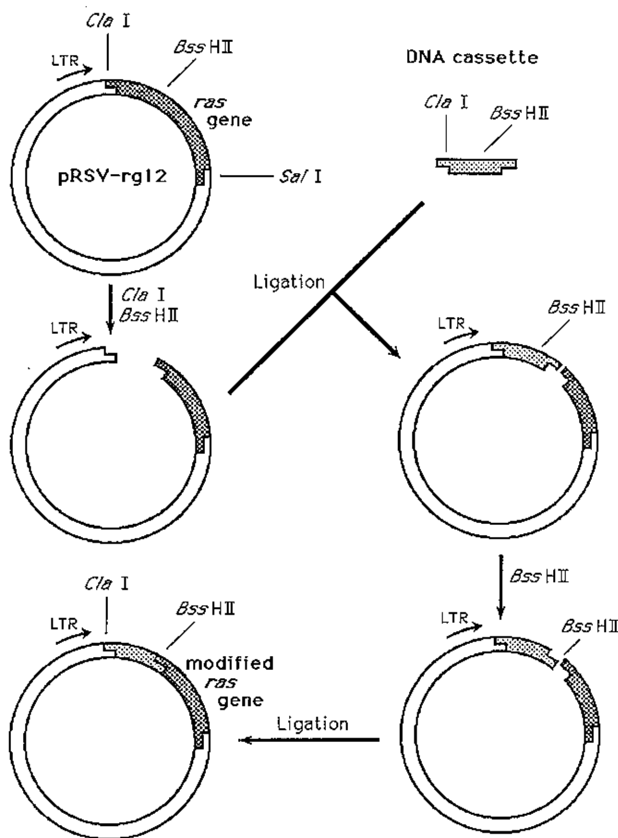

Fig. 2. Construction of vectors for transfection. pRSV-rg12 was digested with *Cla*I and *Bss*HII and joined with a phosphorylated DNA cassette. The DNA obtained was then treated with *Bss*HII and joined by T4 DNA ligase.

O^6-MeG residue was designed to have the same nucleotide sequence from codon 1 to codon 16 as that of a human c-Ha-*ras* gene; namely, unlike the original synthetic human c-Ha-*ras* gene,[13, 14] the same codon usage as that of human c-Ha-*ras* was taken.[12] An O^6-MeG residue was introduced into the second position of codon 12, since the c-Ha-*ras* gene from rat mammary carcinomas induced by NMU treatment had a mutation at this position,[6] and the activated *ras* gene containing the same mutation has often been found in human cancers.[2]

The oligonucleotide containing an O^6-MeG residue was synthesized by the phosphoramidite method with commercially available O^6-methyldeoxyguanosine phosphoramidite. The oligonucleotide was purified extensively as described in "Materials and Methods," because oligonucleotides of interest should be as pure as possible for use in mutational analysis. Complete digestion of an O^6-MeG-containing oligonucleotide, and subsequent HPLC analysis, showed that the purified oligonucleotide did not contain any components other than G, C, T and O^6-MeG, and was more than 99% pure. The oligonucleotide containing O^6-MeG was found to be stable under the conditions in which the DNA cassette was constructed.

Table I. Number of Foci Induced by c-Ha-*ras* Genes

	Experiment 1[a]	Experiment 2[b]	Experiment 3[a]	Experiment 4[b]
Gly-12 (normal)	1	2	0	2
O^6-MeG	6	6	2	4
Asp-12 (Activated)	140	362	ND	ND
Val-12 (Activated)	ND	ND	76	163

a) 50 ng of DNA was used.
b) 150 ng of DNA was used.
ND; not determined.

Construction of vector containing O^6-MeG The vector containing O^6-MeG for DNA transfection assay was constructed by the procedure outlined in Fig. 2 to avoid inserting a DNA cassette as a trimer. Vectors with the codon 12 sequence GGC (normal, Gly), GTC (activated, Val) or GAC (activated, Asp) were also constructed by the same procedure.

Transformation of NIH3T3 cells by O^6-MeG-containing c-Ha-*ras* genes DNA transfection was performed by the calcium phosphate procedure.[15, 16] As shown in Table I, the synthetic human c-Ha-*ras* gene with O^6-MeG at the second position of codon 12 induced significantly more foci than the normal c-Ha-*ras* gene. The extent of focus formation by the O^6-MeG-containing c-Ha-*ras* gene was about 2 to 4% of those with activated (Asp or Val) c-Ha-*ras* genes.

Analysis of mutations in codon 12 of the c-Ha-*ras* present in transformed cells The sequence in the region of codon 12 of the c-Ha-*ras* present in the transformed cells was analyzed by the procedure of Haliassos *et al.*[18] A mutagenic primer dAAGCTGGTGGTGGTGGGC-G*T*CG that corresponded to codon 5 to codon 11 of a human c-Ha-*ras* sequence except for T (italicized) in the second position of codon 11 was used as a primer in the PCR reaction. When the second position of codon 12 is replaced by A, the PCR product should contain the sequence of ...G*T*CG*A*C... (the italicized A corresponds to the second position of codon 12) and, therefore, should be susceptible to cleavage by *Sal*I. To detect other types of mutations at the second position of codon 12, we used other combinations of mutagenic primers and restriction enzymes, *Aat*II (for a mutation to T) and *Nar*I

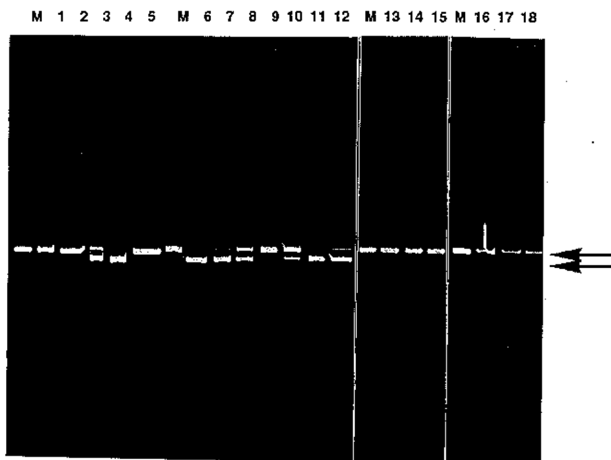

Fig. 3. Restriction enzyme digestion of the PCR products. *Sal*I digestion, lanes 1–12; *Aat*II digestion, lanes 13–15; *Nar*I digestion, lanes 16–18. Lanes 1, 13 and 16, clone 1; lanes 2, 14 and 17, clone 2; lanes 3, 15 and 18, clone 3; lanes 4–12, clone 4–12. M: uncleaved PCR product. The upper and lower arrows indicate the uncleaved and cleaved products, respectively.

(for a mutation to C) as described in "Materials and Methods." Strict specificity of cleavage was confirmed in a preliminary experiment in which standard DNAs having A, G, C and T at the second position of codon 12 were amplified and digested with the appropriate restriction enzymes (data not shown).

PCR products were made from the transformed cells derived from 12 clones which were isolated after transfection with c-Ha-*ras* containing O^6-MeG. As shown in Fig. 3, *Sal*I digestion of the PCR products clearly indicated that 8 of 12 clones had a G to A mutation in the c-Ha-*ras* gene (lanes 1–12). Other analyses with different primers and different restriction enzymes showed that none of the clones had a G to T or G to C mutation (Fig. 3; lanes 13–18). The four clones (clone 1, 2, 5 and 9) that did not have a mutation seemed to contain a c-Ha-*ras* gene with the GGC sequence at codon 12. This conclusion was supported by the susceptibility of the PCR products to digestion with *Hap*II (data not shown). This may be explained by the fact that normal (Gly-12) c-Ha-*ras* induced focus formation as shown in Table I, although the frequency was lower than that of c-Ha-*ras* containing O^6-MeG. Since the primers used amplified the synthetic c-Ha-*ras* gene, but not the endogenous mouse c-Ha-*ras* gene, it is possible that in these cases a mutation had occurred spontaneously in some position other than codon 12 in the synthetic c-Ha-*ras*, not involving O^6-MeG. Alternatively, this background may be derived from transformation of cells by overproduction of normal c-Ha-*ras* p21.[23] Our present system is inferior in terms of background level to the result obtained by Mitra *et al.*[7] Some of the clones contained both normal (Gly-12) and activated (Asp-12) c-Ha-*ras*. As several copies of the c-Ha-*ras* gene are known to be inserted into the cells on transfection, mutational activation of one of the c-Ha-*ras* genes inserted into the cells was probably sufficient for transformation of these clones.

DISCUSSION

Alkylating agents such as NMU are strong mutagens and carcinogens, and are known to produce a variety of DNA adducts *in vitro* and *in vivo*. Of these DNA adducts, O^6-MeG is of particular interest, since there is much experimental evidence that it is involved in mutagenesis and carcinogenesis.[3–7] Babacid and co-workers showed that NMU-induced mammary carcinomas in female rats contained an activated c-Ha-*ras* gene with a mutation of G to A in codon 12,[6] suggesting that NMU directly modifies a guanine residue in codon 12 of c-Ha-*ras* to form an O^6-MeG residue, thereby inducing a mutation at this position that activates the c-Ha-*ras* gene.

The present study showed that a c-Ha-*ras* gene with O^6-MeG at the second position of codon 12 in fact induced significant focus formation (Table I). Furthermore, on sequence analysis of the c-Ha-*ras* present in the transformants, the only mutation found was from G to A at the position of O^6-MeG. This is in accordance with previous *in vivo* and *in vitro* studies showing that O^6-MeG mispairs with T as well as C.[4, 5]

The focus-forming activity of a c-Ha-*ras* gene carrying O^6-MeG was about 2 to 4% of those of activated c-Ha-*ras* genes (Table I). O^6-Methylguanine-DNA methyltransferase is known to remove a methyl group from O^6-MeG in DNA. The *E. coli* gene *ada* that codes for this enzyme is involved in the repair process.[8] Similar enzymes have been found in other organisms[8] including humans,[9–11] indicating that methylation (alkylation) of the O^6-position of G is critical for living organisms in general, and that the repair enzyme is important for eliminating the methyl group from an O^6-MeG residue in DNA. Therefore, most of the O^6-MeG in the c-Ha-*ras* gene introduced into NIH3T3 cells was probably repaired before replication. It would be interesting to examine whether the focus-forming activity of c-Ha-*ras* containing O^6-MeG is higher in cells without O^6-methylguanine-DNA methyl transferase or NIH3T3 cells pretreated with methylating agents to inactivate the methyl transferase.

DNA polymerases are known to incorporate TTP as well as dCTP in the position opposite to O^6-MeG in a template.[4, 5] Singer *et al.*, using a template with C on the 3′ side of O^6-MeG, found that Klenow fragment and

Drosophila DNA polymerase α incorporate more than 50 times more T than C at the position opposite to O^6-MeG.[5] The nucleotide on the 3′ side of O^6-MeG was also C in our study, but it is not yet known whether a DNA polymerase(s) in NIH3T3 cells prefers TTP to dCTP in recognizing O^6-MeG in the sequence of c-Ha-*ras*.

Recently, Mitra *et al.* described site-specific modification of a rat c-Ha-*ras* gene in the 12th codon and its transforming activity.[7] They introduced an O^6-MeG residue into the first and second positions of codon 12 of a rat c-Ha-*ras* gene by DNA cassette mutagenesis, and on transfection of this *ras* gene containing O^6-MeG into Rat4 cells they observed similar increased transformation to that observed in our study. They also demonstrated a mutation with G to A transition at the position of O^6-MeG. Thus, our findings are consistent with these results by Mitra *et al.* There were several differences in the experimental conditions in these two studies. First, they used Rat4 cells and we used mouse-derived NIH3T3 cells as recipient cells. Second, they modified the rat c-Ha-*ras* gene, while we used a synthetic c-Ha-*ras* gene with the human c-Ha-*ras* sequence around codon 12; namely, GCT GNA GGC versus GCC GNC GGT (codon 11 to codon 13). Thus, a sequence difference around O^6-MeG, especially on the 3′ side of O^6-MeG, probably does not appreciably affect the transforming efficiency. G to A transition via O^6-MeG may be a common phenomenon in different species and in the nucleotide sequence around the positions modified by methylating agents.

In this paper, we describe an easy method for inserting an O^6-MeG residue into codon 12 of the synthetic c-Ha-*ras* gene. The synthetic c-Ha-*ras* gene has several unique restriction enzyme sites, and so will be convenient for inserting various modified bases into not only codon 12, but also other positions such as codons 13 and 61. Thus, this procedure will be very useful in evaluating mutagenicities of other types of DNA lesions. Studies along these lines are in progress.

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