Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 8650–8654, November 1989
Biochemistry

Molecular analysis of O^6-substituted guanine-induced mutagenesis of *ras* oncogenes

(*O*^{6}-methylguanine/*O*^{6}-benzylguanine/*Bsp*MI cassette/gene transfer/polymerase chain reaction)

GOPA MITRA*†, GARY T. PAULY‡, RAMESH KUMAR*§, GUO K. PEI‡, STEPHEN H. HUGHES¶, ROBERT C. MOSCHEL‡, AND MARIANO BARBACID*§

*Developmental Oncology, ‡Carcinogen-Modified Nucleic Acid Chemistry, and ¶Gene Expression in Eukaryotes Sections, BRI-Basic Research Program, National Cancer Institute-Frederick Cancer Research Facility, Frederick, MD 21701

Communicated by Allan H. Conney, August 9, 1989 (received for review March 10, 1989)

ABSTRACT **We have designed an Ha-*ras*/thymidine kinase (TK) cassette that permits the incorporation of chemically synthesized adducts within specific domains of the rat Ha-*ras* protooncogene. This cassette has been used to evaluate the mutagenicity of O^6-substituted guanine residues, including *O*^{6}-methylguanine and *O*^{6}-benzylguanine, incorporated within the 12th codon of this locus. Mutations were monitored by the ability of these modified Ha-*ras* DNAs to transform Rat4 TK$^-$ cells. Our results indicate that both types of O^6-substituted guanines are substantially mutagenic, although the methyl substituent induced a 2-fold higher percentage of transformed Rat4 TK$^+$ colonies than its bulkier benzyl analogue. Interestingly, the mutagenicity of both O^6-substituted guanines was found to be independent of their relative position within codon 12, therefore suggesting that the specific activation of Ha-*ras* oncogenes by GGA → GAA mutations in tumors induced by methylating carcinogens might be due to differences in the accessibility of these guanine residues to the carcinogen rather than to a differential rate of repair. Molecular analysis of the mutations induced by these O^6-substituted guanines indicated that *O*^{6}-methylguanine exclusively induced G → A transitions. In contrast, *O*^{6}-benzylguanine produced G → C and G → T transversions in addition to G → A transitions. These results suggest that *O*^{6}-methylguanine and its bulkier analogue *O*^{6}-benzylguanine may induce mutagenesis by different mechanisms.**

Accumulating evidence indicates that *ras* oncogenes play an important role in chemical carcinogenesis (1, 2). Today, we know of more than two dozen carcinogen-induced animal tumor systems in which members of the *ras* gene family are reproducibly activated. One such model system involves the induction of mammary carcinomas in pubescent rats by a single intravenous injection of *N*-nitroso-*N*-methylurea (NMU), a direct-acting carcinogen (3). Almost 90% of these tumors possess Ha-*ras* oncogenes, each of them reproducibly activated by the same miscoding mutation, a G → A transition in the second base of their 12th codon (4, 5). G → A transitions are the most common mutations induced by the initiating carcinogen NMU, a result of its ability to produce *O*^{6}-methylguanine residues (6–8). We therefore hypothesized that *ras* oncogene activation might result from the direct interaction of NMU with Ha-*ras* sequences (4). This proposal gained support when we showed that replacement of the initiating carcinogen by a nonmethylating agent such as 7,12-dimethylbenz[*a*]anthracene resulted in the activation of Ha-*ras* oncogenes that no longer carried missense G → A mutations (5). Since then, similar results have been obtained in other tumor systems, particularly in liver carcinomas of B6C3 F$_1$ mice induced by a variety of carcinogens (9, 10). In each case, the Ha-*ras*-activating mutation exhibited a striking parallel with the expected mutagenic properties of the corresponding carcinogen (9, 10), thus providing further support to the concept that *ras* genes are likely to be targeted by the initiating carcinogen.

To understand the molecular mechanisms by which *ras* oncogenes become activated by chemical carcinogens, it is necessary to determine the mutagenic properties of carcinogen-induced adducts within those critical residues required for their malignant activation. To this end, we have designed a cassette vector that will accept carcinogen-modified DNA sequences in place of the normal residues encompassing the critical codon 12 of the Ha-*ras* gene. We have incorporated two different O^6-substituted guanine residues, *O*^{6}-methyl- and *O*^{6}-benzylguanine. *O*^{6}-Methylguanine is known to induce G → A transitions as a consequence of its mispairing properties (6–8). The bulkier *O*^{6}-benzylguanine, a product of benzylating (11–14) or potential benzylating (15) carcinogens, was included to assess the impact of the increasing size of the O^6 substituent on the mutagenic potency of the modified guanine. The mutagenicity of these O^6-substituted guanines, as a function of their location within the 12th codon of the Ha-*ras* gene, has been monitored by measuring the transforming activity of these adduct-carrying Ha-*ras* DNAs in gene transfer assays. Characterization of the donor Ha-*ras* sequences in the resulting transformed cells by the polymerase chain reaction (PCR) technique (16) has allowed us to precisely identify the type of mutation induced by each of these two O^6-substituted guanines.

MATERIALS AND METHODS

Bacteria and Plasmids. Competent *Escherichia coli* strain DH5 cells were used as recipients for transformation. Restriction digestion, DNA purification, and ligation were conducted according to standard protocols (17). Details of the preparation and properties of the O^6-substituted guanine-containing oligodeoxyribonucleotides have been reported elsewhere (18).

Gene Transfer Assays. Rat4 thymidine kinase negative (TK$^-$) cells, a derivative of the Rat1 Fischer rat embryo fibroblast cell line (19), were used. Cells were transfected with 400 ng of adduct-modified *ras* DNA constructs by the calcium phosphate precipitation technique (20) in the presence of 10 μg of carrier DNA. TK$^+$ colonies were selected in

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: NMU, *N*-nitroso-*N*-methylurea; PCR, polymerase chain reaction; TK, thymidine kinase.

†Present address: Department of Molecular Biology, Cleveland Clinical Foundation, 9500 Euclid Avenue, Cleveland, OH 44106.
§Present address: Department of Molecular Biology, Squibb Institute for Medical Research, P.O. Box 4000, Princeton, NJ 08543-4000.

hypoxanthine/aminopterin/thymidine (HAT) medium supplemented with 10% calf serum. HAT-resistant colonies exhibiting a transformed phenotype were scored after 18 days. Transformed colonies were cloned in semisolid agar prior to their use for DNA analysis.

PCR Analysis. Ten thousand HAT-resistant Rat4 cells were thoroughly washed in phosphate-buffered saline (PBS) and boiled for 10 min in distilled water, and the resulting cell lysates were submitted to amplification by the PCR technique with a thermal DNA cycler machine (Perkin–Elmer/Cetus) as described (16, 21). After 40 cycles of amplification, aliquots of DNA were hybridized to ^{32}P-labeled 19-mer oligonucleotide probes including 5′-TGGGCGCTGNAGGCGTGGG-3′ and 5′-TGGGCGCTNGAGGCGTGGG-3′ where N is any of the four deoxynucleotides (A, C, G, or T). The samples were hybridized in liquid to the respective probes under stringent conditions and subsequently characterized by the probe shift assay as described elsewhere (21).

RESULTS

Construction of an Ha-*ras*/TK Cassette. To design a *ras* cassette vector in which we could introduce chemically modified bases, we took advantage of the ability of the *Bsp*MI restriction endonuclease to cleave DNA four nucleotides downstream of the sequence ACCTGC and eight nucleotides upstream of its complementary sequence GCAGGT. This property allows the replacement of any given set of sequences, such as those encompassing the 12th codon region of the Ha-*ras* gene, by simply inserting two flanking inverted *Bsp*MI cleavage sites (Fig. 1). As a source of Ha-*ras* sequences, we used a viral Ha-*ras* DNA clone with modified codons 59 (GCA) and 60 (GGG). Removal of the viral Thr^{59} codon was required since this mutation is sufficient to confer transforming properties to the Ha-*ras* oncogene (22). The neighboring condon 60 was changed from GGT to GGG to eliminate an unwanted *Bsp*MI recognition site.

This *Bsp*MI$^-$ Ha-*ras* DNA was digested with *Sma* I and *Nhe* I, and the resulting 681-base-pair (bp) fragment, which contains the entire Ha-*ras* coding sequence, was subcloned at the unique *Sma* I site of pBR322ΔHP/S to generate pGM17. pBR322ΔHP/S is a pBR322 derivative in which its unique *Hin*dIII and *Pvu* II sites have been deleted and an *Sma* I cleavage site was introduced at the single *Eco*RI site by using an *Eco*RI/*Sma* I adaptor. pGM17 DNA was next digested with *Hin*dIII and *Pvu* II to remove the 53-bp DNA fragment containing Ha-*ras* sequences corresponding to codons 5–22. This 53-bp DNA fragment was replaced by a synthetic DNA fragment to generate pGM18 (Fig. 1). Features of this DNA fragment include, from left to right: (*i*) Ha-*ras* sequences corresponding to codons 5–9 and the first base of codon 10; (*ii*) four arbitrarily chosen deoxyribonucleotides; (*iii*) a *Bsp*MI cleavage sequence (GCAGGT); (*iv*) an *Xho* I cleavage site; (*v*) a *Bsp*MI cleavage sequence (ACCTGC); (*vi*) four arbitrarily chosen deoxyribonucleotides; and (*vii*) the second and third bases of Ha-*ras* codon 14 and codons 15–22 (Fig. 1). The unique *Xho* I cleavage site was designed to allow the insertion of a 1.3-kbp "spacer" DNA between the two *Bsp*MI sites (see pGM20 in Fig. 1) to monitor the extent of *Bsp*MI digestion of the final Ha-*ras* cassette construct.

Digestion of pGM20 with *Sma* I released a 1994-bp *Sma* I DNA fragment that contained the entire Ha-*ras* coding sequence. This fragment was next introduced into pGM19 to generate pGM21 (Fig. 1). pGM19 is a pUC18 derivative in which we had previously inserted a 725-bp *Eco*RI/*Sma* I DNA fragment containing the Moloney murine sarcoma virus long terminal repeat and a 243-bp *Bam*HI DNA fragment containing a bidirectional simian virus 40 polyadenylylation signal (Fig. 1). In addition, we had removed the *Bsp*MI site present in the pUC18 polylinker (residues 433–438) by deleting those sequences contained between the *Sal* I and *Hin*dIII polylinker sites. The final step in the generation of the Ha-*ras* cassette vector pGM22, involved the addition of a 1980-bp *Cla* I/*Bgl* II DNA fragment encompassing a functional chicken TK that served as a selectable marker (23).

O^6-Substituted Guanine-Containing Ha-*ras* Genes. To generate Ha-*ras* genes containing O^6-substituted guanine adducts in their 12th codon, pGM22 DNA was digested with *Bsp*MI, fractionated by agarose gel electrophoresis, and ligated to 16-bp DNA fragments that reconstituted the Ha-*ras* coding sequences (Fig. 2). O^6-substituted guanine residues

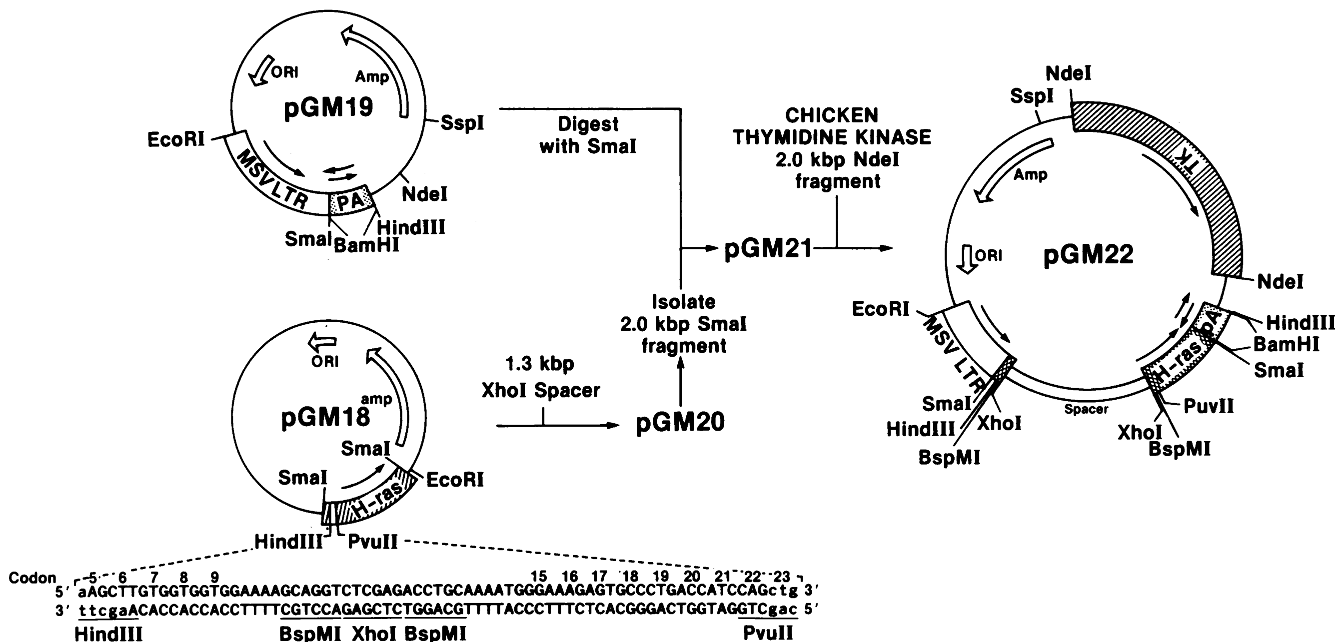

FIG. 1. Schematic diagram of the strategy used to construct the *Bsp*MI-derived Ha-*ras*/TK cassette pGM22. See text for details. MSV LTR, Moloney murine sarcoma virus long terminal repeat; PA, polyadenylylation signal. Ha-*ras* codons 5–9 and 15–23 present in the synthetic oligonucleotide are indicated.

were introduced in the first and/or second positions of codon 12 (GGA) as described (18). Since the overhand ends of the Ha-*ras*/TK cassette could not ligate to each other, the ligation reaction favors the formation of properly regenerated Ha-*ras* coding sequences (Fig. 2). Nevertheless, ligated DNA was linearized with *Ssp* I, and those DNA molecules exhibiting the expected size (6.3 kbp) were purified by gel electrophoresis to ensure that only properly ligated DNA was used in subsequent biological assays.

Gene Transfer Assays. To assess the mutagenic properties of adduct-containing Ha-*ras* genes in Rat4 cells, we first had to determine the transforming properties of Ha-*ras* oncogenes carrying different mutations in codon 12. For this purpose, we generated a series of plasmids (pGM24–pGM31) in which *Bsp*MI-cleaved pGM22 DNA was ligated to oligonucleotides containing either wild-type codon 12 (GGA) or all possible mutations resulting from replacement of one of the two deoxyguanosines (Fig. 2).

A representative experiment is shown in Table 1. pGM24, a plasmid that contains a normal Ha-*ras* gene (GGA/Gly^{12}), failed to morphologically transform any of the HAT-resistant (TK^+) Rat4 cells. These results indicate that Rat4, unlike NIH 3T3 cells, are resistant to transformation by high levels of expression of the normal Ha-*ras* gene, thus eliminating undesirable levels of background transformation. pGM25, a plasmid that harbors the Ha-*ras* oncogene found in all NMU-induced mammary carcinomas (GAA/Glu^{12}), induced the morphologic transformation of 60% of the transfected TK^+ Rat4 colonies (Table 1). Similar results were obtained with pGM27 (AGA) and pGM30 (CGA), two plasmids encoding Arg^{12} Ha-*ras* gene products. pGM28 (CTA/Val^{12}) and pGM29 (GCA/Ala^{12}) were also found to contain transforming genes, albeit somewhat less potent (Table 1). Finally, pGM31, a plasmid that carries a G $\rightarrow$ T mutation that generates a stop codon (TGA), failed to induce detectable morphologic transformation. These results indicate that transfection of Rat4 TK^- cells with Ha-*ras*/TK DNA constructs is a suitable system to monitor adduct-induced mutagenicity in *ras* oncogenes.

Transformation Induced by Ha-*ras* Genes Carrying O^6-Substituted Guanine Residues. A series of oligonucleotides carrying O^6-methyl- and O^6-benzylguanine residues in positions corresponding to the first and second base of the 12th codon of the rat Ha-*ras* gene were ligated to *Bsp*MI-digested pGM22 DNA that had been previously purified from the spacer sequences (Fig. 1). The resulting DNAs, designated GM-A–GM-E (Table 1), were subsequently used to transfect Rat4 TK^- cells. As controls, 16-bp oligonucleotides carrying either a normal (GM-N) or a transforming (GM-T) Ha-*ras* codon 12 were ligated to *Bsp*MI-digested pGM22 DNA under the same experimental conditions.

Table 2 shows results from a representative experiment. Whereas GM-N DNA failed to induce morphologic transformation of Rat4 cells, GM-T DNA was capable of transforming as much as 52% of the TK^+ colonies. Ha-*ras* DNA containing an O^6-methylguanine in the second position of

Table 1. Relative transforming efficiencies of Ha-*ras* oncogenes carrying different activating mutations in codon 12 in Rat4 TK^- cells

			TK^+ Rat4 colonies		Relative
Plasmid	Codon 12	Amino acid	Total	Transformed	transforming efficiency
pGM24	GGA	Gly	3350	0 (0%)	0.00
pGM25	GAA	Glu	1200	720 (60%)	1.00
pGM27	AGA	Arg	2600	1510 (58%)	0.97
pGM28	GTA	Val	2320	1020 (44%)	0.73
pGM29	GCA	Ala	2040	410 (20%)	0.33
pGM30	CGA	Arg	1960	1100 (56%)	0.93
pGM31	TGA	Stop	2160	0 (0%)	0.00

Mutated bases are underlined.

codon 12 (GM-B DNA) was able to induce the morphologic transformation of ≈1% of the transfected TK^+ Rat4 cells. Preincubation of an identical sample of GM-B DNA with a 12-fold molar excess (1.25 units) of *E. coli* O^6-methylguanine DNA methyltransferase (Applied Genetics) for 1 hr at 37°C completely eliminated its transforming activity. Mock-incubated GM-B DNA, retained about one-half (8–10 transformed TK^+ colonies of Rat4 cells per 400 ng of DNA) of the transforming activity of untreated GM-B DNA (Table 2). These results indicate that the observed transforming activity of O^6-methylguanine-modified Ha-*ras* DNA is a direct consequence of the presence of premutagenic O^6-methylguanine residues in the donor DNA.

Interestingly, the transforming properties of these O^6-methylguanine-containing Ha-*ras* DNAs were independent of whether the methylated adducts were located in the first (m^{6}GGA, GM-A DNA) or second (Gm^{6}GA, GM-B DNA) residue of codon 12 (Table 2), therefore suggesting that the observed *in vivo* preference for mutagenesis of Ha-*ras* genes in the second residue of codon 12 must be a direct consequence of its better accessibility to the methylating carcinogen. Transfection of Rat4 TK^- cells with Ha-*ras* DNAs containing O^6-benzylguanines provided similar results (Table 2). GM-D DNA (bzl^{6}GGA) and GM-E DNA (Gbzl^{6}GA) transformed TK^+ Rat4 colonies with comparable relative efficiencies. However, the total number of transformants obtained with these O^6-benzylguanine derivatives was somewhat lower than that obtained with their O^6-methylated counterparts. As previously observed with O^6-methylguanine residues, transformation of Rat4 cells by Ha-*ras* genes carrying O^6-benzylguanine adducts was independent of their relative position within codon 12 (Table 2).

Analysis of O^6-Substituted Guanine-Induced Mutations. Representative TK^+ colonies of transformed cells arising from each adduct-carrying Ha-*ras* DNA were selected to determine their harboring mutations. The results of such analysis are summarized in Table 3. DNAs amplified from Rat4 transformants derived from GM-A DNA, which contained an O^6-methylguanine in the first residue of codon 12, efficiently hybridized to the AGA-, but not to the CGA-containing 19-mer oligonucleotide probe, thus indicating that

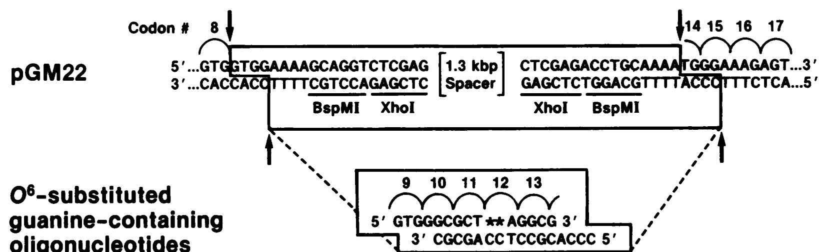

FIG. 2. Generation of Ha-*ras* genes carrying chemically synthesized O^6-substituted guanine residues (indicated by asterisks) in their 12th codons. Ha-*ras* codons 8–17 are indicated.

Table 2. Mutagenic efficiency of O^6-substituted guanine adducts incorporated in the 12th codon of the rat Ha-*ras* protooncogene

Donor DNA	Codon 12	TK$^+$ Rat4 colonies Total	Transformed	Relative mutagenicity
GM-N	GGA CCT	3350	0 (0%)	0.000
GM-T	G<u>A</u>A C<u>T</u>T	3600	1860 (51.7%)	1.000
GM-A	m^{6}GGA CCT	2120	24 (1.1%)	0.042
GM-B	Gm^{6}GA CCT	2000	21 (1.0%)	0.038
GM-C	m^{6}Gm^{6}GA CCT	3400	72 (2.1%)	0.081
GM-D	bzl^{6}GGA CCT	4520	24 (0.5%)	0.019
GM-E	Gbzl^{6}GA CCT	5950	35 (0.6%)	0.023

Four hundred nanograms of donor DNA was used to transfect 10^6 Rat4 TK$^-$ cells. Mutated bases are underlined. Mutagenicity is relative to an Ha-*ras* oncogene carrying an activating mutation in only one of the two DNA strands.

each of the O^6-methylguanine-derived transformants contained a G → A transition (Fig. 3). The presence of putative G → T transversions was not examined since such mutations would yield a terminator codon and therefore a truncated, nontransforming Ha-*ras* product. G → A transitions were also the only mutations observed in each of 12 transformants induced by GM-B, a DNA that contained an O^6-methylguanine as the middle base of codon 12 (Table 3).

Each of five transformants generated by Ha-*ras* DNA (GM-D DNA) carrying an O^6-benzylguanine residue as the first base of codon 12, also exhibited G → A transitions (Table 3). However, more than one type of mutation was observed in the DNA of transformants derived from Ha-*ras* genes containing O^6-benzylguanine at the second position (GM-E DNA). As shown in Fig. 4, G → A transitions were observed in four of the five clones examined. In addition, G → T and G → C transversions were present in four and three of these clones, respectively. The appearance of more than one type of mutation in a single clone of transformed cells is not unexpected since multiple adduct-containing Ha-*ras* molecules are likely to be introduced into the transfected Rat4 cells. These results suggest that the more bulky O^6-benzylguanine residue may induce mutagenesis by a mechanism different from that of the less obtrusive O^6-methyl analogue.

DISCUSSION

To further our understanding of the mechanisms involved in the mutational activation of *ras* oncogenes in mammalian cells, we have designed an Ha-*ras*/TK cassette that permits the site-specific incorporation of carcinogen-modified DNA bases in the region encompassing the critical 12th codon of the Ha-*ras* protooncogene. Stone *et al.* (24) have recently described their construction of a similar *ras* cassette to conduct site-directed mutagenesis studies on the putative effector domain of *ras* proteins. We have utilized our Ha-*ras*/TK cassette to analyze the transforming/mutagenic properties in Rat4 fibroblasts of O^6-methyl- and O^6-

Table 3. Analysis of mutations induced by O^6-substituted guanine-containing Ha-*ras* genes

Donor DNA	Codon 12	Observed mutation	Positive colonies/ total tested
GM-A	m^{6}GGA	<u>A</u>GA	9/9
GM-B	Gm^{6}GA	G<u>A</u>A	12/12
GM-D	bzl^{6}GGA	<u>A</u>GA	5/5
GM-E	Gbzl^{6}GA	G<u>A</u>A	4/5
		G<u>C</u>A	3/5
		G<u>T</u>A	4/5

Mutated bases are underlined. In gene transfer assays multiple adduct-containing Ha-*ras* molecules are likely to be transfected into each of the transformed TK$^+$ Rat4 colonies.

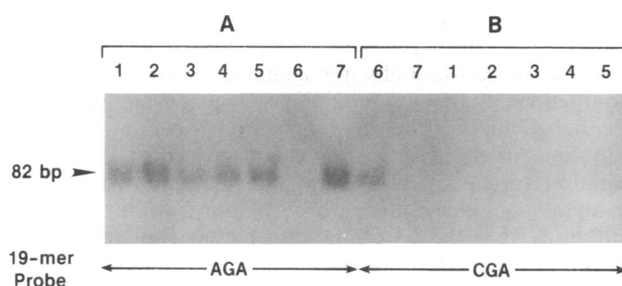

FIG. 3. Analysis of O^6-methylguanine-induced mutations in the first residue of the 12th codon of the Ha-*ras* oncogene. Lanes: 1–5, cells derived from five independent clones of Rat4 cells transformed by GM-A (m^{6}GGA) DNA; 6, Rat4 cells transformed by pGM30 DNA (Ha-*ras* oncogene activated by a GGA → CGA transversion); 7, Rat4 cells transformed by pGM27 DNA (Ha-*ras* oncogene activated by a GGA → AGA transition). Amplified DNAs were hybridized to 19-mer oligonucleotide probes carrying either G → A (*A*) or G → C (*B*) substitutions in the nucleotide corresponding to the first base of Ha-*ras* codon 12 and analyzed by gel electrophoresis. Gels were exposed to Kodak XAR film for 4 hr. The migration of the amplified DNAs (82 bp) is indicated by arrowhead.

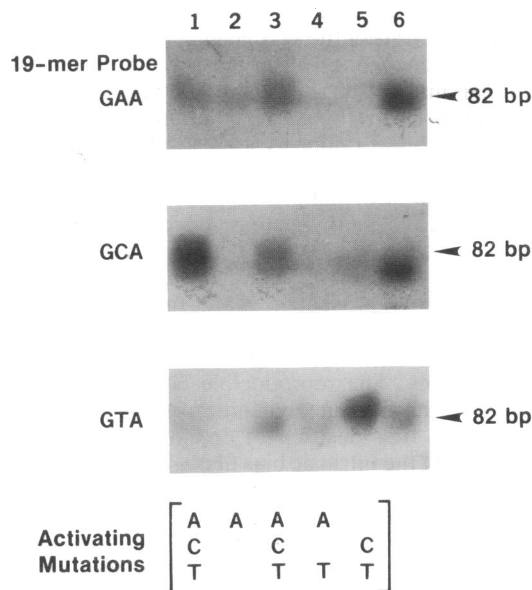

FIG. 4. Analysis of O^6-benzylguanine-induced mutations in the second residue of the 12th codon of the Ha-*ras* oncogene. Lanes: 1–5, cells derived from five independent clones of Rat4 cells transformed by GM-E (Gbzl^{6}GA) DNA; 6 (*Top*), control Rat4 cells transformed by pGM25 DNA (Ha-*ras* oncogene activated by a GGA → GAA transition); 6 (*Middle*), control Rat4 cells transformed by pGM29 DNA (Ha-*ras* oncogene activated by a GGA → GCA transversion); 6 (*Bottom*), control Rat4 cells transformed by pGM28 DNA (Ha-*ras* oncogene by a GGA → GTA transversion). Amplified DNAs were hybridized to 19-mer oligonucleotide probes carrying G → A (*Top*), G → C (*Middle*), or G → T (*Bottom*) substitutions in the nucleotide corresponding to the second base of Ha-*ras* codon 12 and analyzed by gel electrophoresis. Gels were exposed to Kodak XAR film for 4 hr. The migration of the amplified DNAs (82 bp) is indicated by arrowheads. The observed mutated bases in each of the Rat4 clones transformed by GM-E DNA are tabulated at the bottom.

benzylguanine residues within the 12th codon of the rat Ha-*ras* gene. Both types of O[6]-substituted guanines have proven to be substantially mutagenic in these rat fibroblasts. Modified Ha-*ras* genes containing a single *O*[6]-methylguanine in either the first or the second position of codon 12 induced mutations in at least 1% of the transformed cells. This mutation frequency was even higher when two adjacent *O*[6]-methylguanine residues were positioned within codon 12. Mutations in ≈0.5% of the transfected Rat4 cells were observed when *O*[6]-benzylguanine residues were incorporated at either the first or second position of codon 12.

One of the most intriguing aspects of the activation of *ras* oncogenes in NMU-induced tumors is the highly selective mutagenesis of the second residue of codon 12 (1, 2, 5). Mutagenesis studies in *E. coli* (25, 26) and in human cells (27) have indicated that NMU preferentially mutates the second guanine in the sequence 5′-purine-G-3′, yielding the expected G → A transitions. Indirect evidence suggests that this preferential mutagenesis may reflect formation of premutagenic *O*[6]-methylguanine adducts (25, 26). In contrast, other authors have reported that such preferential mutagenesis may be a consequence of inefficient repair (28, 29). In these bacterial studies, the consensus sequence surrounding poorly repaired *O*[6]-methylguanine adducts closely resembled that surrounding the second residue of the 12th codon of the rat Ha-*ras* locus (28). Our results in Rat4 fibroblasts support the preferential alkylation hypothesis since Ha-*ras* molecules carrying *O*[6]-methylguanine residues in either the first or second position of codon 12 exhibit the same rate of G → A mutagenesis. Interestingly, Dolan *et al.* (30) have recently shown that *in vitro* NMU preferentially modifies the second deoxyguanosine residue in 5′-NGGN-3′ sequences. These observations, taken together, strongly suggest that the exclusive mutagenesis of the second guanine residue in the 12th codon of *ras* oncogenes observed *in vivo* results from the preferential interaction of this base with NMU to yield premutagenic *O*[6]-methylguanine adducts. These findings lend further support to our hypothesis that NMU is directly responsible for the malignant activation of the Ha-*ras* locus during initiation of mammary carcinogenesis in rats (5).

As expected, *O*[6]-methylguanine residues located at either the first or second position of codon 12 produced G → A transitions (8), the type of mutation observed in each of the Ha-*ras* oncogenes present in NMU-induced mammary tumors (4, 5). In contrast, the bulkier *O*[6]-benzylguanine residue produced G → C and G → T transversions in addition to G → A transitions. The reproducible G → A mutations observed when the *O*[6]-benzylguanine residues became incorporated in the first position of codon 12 is likely to be a consequence of the intrinsic bias of our experimental system, in which G → T transitions cannot be phenotypically monitored since they generate TGA terminator codons. Whether *O*[6]-benzylguanine residues induce G → C transversions in this position will require additional experiments. In any case, our results clearly indicate that the bulky *O*[6]-benzyl substituent can produce a wider variety of substitutions, thus suggesting that *O*[6]-benzylguanine residues are likely to induce mutagenesis by a mechanism(s) different from that utilized by its *O*[6]-methyl analogue.

These studies illustrate the feasibility of using molecular approaches to analyze the mutagenic properites of chemically defined DNA adducts in genes relevant to the onset of human cancer. A systematic analysis of the mutagenic properties of these adducts should provide relevant information regarding the fate of critical residues involved in the generation of oncogenes in mammalian cells exposed to chemical carcinogens.

We thank Linda Dunn for excellent technical assistance and T. Y. Shih and M. Perucho for making their plasmids available to us. This research was sponsored by the National Cancer Institute, Department of Health and Human Services, under Contract N01-CO-74101 with Bionetics Research, Inc.

1. Barbacid, M. (1987) *Annu. Rev. Biochem.* **56**, 779–827.
2. Balmain, A. & Brown, K. (1988) *Adv. Cancer Res.* **51**, 147–182.
3. Gullino, P. M., Pettigrew, H. M. & Grantham, F. H. (1975) *J. Natl. Cancer Inst.* **54**, 401–414.
4. Sukumar, S., Notario, V., Martin-Zanca, D. & Barbacid, M. (1983) *Nature (London)* **306**, 658–661.
5. Zarbl, H., Sukumar, S., Arthur, A. V., Martin-Zanca, D. & Barbacid, M. (1985) *Nature (London)* **315**, 382–385.
6. Singer, B. & Kusmierek, J. T. (1982) *Annu. Rev. Biochem.* **51**, 655–693.
7. Pegg, A. E. (1984) *Cancer Invest.* **2**, 223–231.
8. Basu, A. K. & Essigmann, J. M. (1988) *Chem. Res. Toxicol.* **1**, 1–18.
9. Reynolds, S. H., Stowers, S. J., Patterson, R. M., Maronpot, R. R., Aaronson, S. A. & Anderson, M. W. (1987) *Science* **237**, 1309–1316.
10. Wiseman, R. W., Stowers, S. J., Miller, E. C., Anderson, M. W. & Miller, J. A. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 5825–5829.
11. Ivancovic, S. (1978) *Z. Krebsforsch.* **91**, 63–68.
12. Greim, H. & Wolff, T. (1984) in *Chemical Carcinogens*, Monograph 182, ed. Searle, C. E. (Am. Chem. Soc., Washington, DC), 2nd Ed., Vol. 1, pp. 525–575.
13. Moschel, R. C., Hudgins, W. R. & Dipple, A. (1980) *J. Org. Chem.* **45**, 533–535.
14. Moschel, R. C., Hudgins, W. R. & Dipple, A. (1986) *J. Org. Chem.* **51**, 4180–4185.
15. Druckrey, H., Preussmann, R., Irankovic, S. & Schmäl, D. (1967) *Z. Krebsforsch.* **69**, 103–201.
16. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988) *Science* **239**, 487–491.
17. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
18. Pauly, G. T., Powers, M., Pei, G. K. & Moschel, R. C. (1988) *Chem. Res. Toxicol.* **1**, 391–397.
19. Topp, W. C. (1981) *Virology* **113**, 408–411.
20. Graham, F. L. & van der Eb, A. J. (1973) *Virology* **52**, 456–467.
21. Kumar, R. & Barbacid, M. (1988) *Oncogene* **3**, 651–657.
22. Fasano, O., Aldrich, T., Tamanoi, F., Taparowski, E., Furth, M. & Wigler, M. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 4008–4012.
23. Perucho, M., Hanahan, D., Lipsich, L. & Wigler, M. (1980) *Nature (London)* **285**, 207–210.
24. Stone, J. C., Vass, W. C., Willumsen, B. M. & Lowy, D. R. (1988) *Mol. Cell. Biol.* **8**, 3565–3569.
25. Burns, P. A., Gordon, A. J. E. & Glickman, B. W. (1987) *J. Mol. Biol.* **194**, 385–390.
26. Burns, P. A., Gordon, A. J. E. & Glickman, B. W. (1988) *Carcinogenesis* **9**, 1607–1610.
27. DuBridge, R. B., Tang, P., Hsia, H. C., Leong, P.-M., Miller, J. H. & Calos, M. P. (1987) *Mol. Cell. Biol.* **7**, 379–387.
28. Topal, M. D., Eddie, J. S. & Conrad, M. (1986) *J. Biol. Chem.* **261**, 9879–9885.
29. Topal, M. D. (1988) *Carcinogenesis* **9**, 691–696.
30. Dolan, M. E., Oplinger, M. & Pegg, A. E. (1988) *Carcinogenesis* **9**, 2139–2143.