Preferential Carcinogen−DNA Adduct Formation at Codons 12 and 14 in the Human *K-ras* Gene and Their Possible Mechanisms[†]

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ABSTRACT: In the *ras* gene superfamily, codon 12 (-TGGTG-) of the *K-ras* gene is the most frequently mutated codon in human cancers. Recently, we have found that bulky chemical carcinogens preferentially form DNA adducts at codons 12 and 14 (−CGTAG-) in the *K-ras* gene in normal human bronchial epithelial (NHBE) cells. Furthermore, DNA adducts formed at codon 12 of the *K-ras* gene are poorly repaired compared with those at other codons including codon 14. These results suggest that targeted carcinogen−DNA adduct formation is a major reason for the observed high mutation frequency at codon 12 of the *K-ras* gene in human cancers. This preferential carcinogen−DNA adduct formation at codons 12 and 14 could result from effects of (1) primary sequences of these codons and their surrounding codons in the *K-ras* gene, (2) the chromatin structure, and/or (3) epigenetic factors such as C5 cytosine methylation or other DNA modifications at these codons and their surrounding codons. To distinguish these possibilities, we have introduced modifications with benzo[*a*]pyrene diol epoxide, *N*-hydroxy-2-aminofluorene, and aflatoxin B1 8,9-epoxide in (1) naked intact genomic DNA isolated from NHBE cells, (2) fragmented genomic DNA digested by restriction enzymes, and (3) in vitro synthesized DNA fragments containing the *K-ras* gene exon 1 sequence with or without methylation of the cytosines at CpG sites and the cytosines pairing with the guanines of codons 12 and 14. The distribution of carcinogen−DNA adducts in the *K-ras* gene was mapped at the nucleotide sequence level using the UvrABC nuclease incision method with or without the ligation-mediated polymerase chain reaction technique. We have found that carcinogens preferentially form adducts at codons 12 and 14 in the *K-ras* gene exon 1 in intact as well as in fragmented genomic DNA. In contrast, this preferential DNA adduct formation at codons 12 and 14 was not observed in PCR-amplified DNA fragments containing the *K-ras* gene exon 1 sequence. Methylation of the cytosine at the CpG site of codon 14, or the cytosine pairing with guanine of codon 14, greatly enhanced carcinogen−DNA adduct formation at codon 14 but did not affect carcinogen−DNA adduct formation at codon 12. Methylation of the cytosine pairing with the guanine of codon 12 also did not enhance carcinogen−DNA adduct formation at codon 12. Furthermore, we found that the cytosine at the CpG site of codon 14 is highly methylated in NHBE cells. These results suggest that cytosine methylation at the CpG site is the major reason for the preferential DNA damage at codon 14 and that epigenetic modification(s) other than cytosine methylation may contribute to the preferential DNA damage at codon 12 of the *K-ras* gene.

The *ras* gene superfamily consists of three functional genes, *K-ras*, *N-ras*, and *H-ras*, coding for the highly homologous protein P21, a GTPase, which plays a pivotal role in modulating various intracellular signal transduction pathways governing cell growth and differentiation, apoptosis, membrane trafficking, and transcriptional regulation (*1*−*4*). Mutations in *ras* genes have been found in a wide range of human cancers and also in animal models, which occur almost exclusively at codons 12, 13, and 61 of *ras* genes (*5*, *6*). Mutations at these three positions can reduce the intrinsic GTPase activity of the *ras* gene product, impairing its ability to bind to the GTPase activating protein, thus confining P21 in a GTP-bound activated mode (*2*). Activated P21 is thought to trigger the kinase cascade and thus stimulate cell growth or differentiation (*4*, *7*). Although any mutation at codons 12, 13, and 61 of these three *ras* genes can activate their oncogenic function (*2*), intriguingly, mutation at codon 12 of the *K-ras* gene is the most common mutation found among the three *ras* genes in human cancers. For example, 90% of pancreatic cancers, 50% of colon cancers, and over 30% of smoking-related lung cancers have a mutation exclusively at codon 12 of the *K-ras* gene (*5*, *6*, *8*−*12*).

Recently, using the UvrABC nuclease incision method in combination with the ligation-mediated polymerase chain

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reaction (LMPCR)[1] technique, we have found that various bulky chemical carcinogens form DNA adducts preferentially at codons 12 and 14 of *K-ras* in normal human bronchial epithelial (NHBE) cells compared with codons 13 and 61 and other codons in the *K-ras* gene and codons in the *H-* or *N-ras* genes (*13*). Furthermore, DNA adducts formed at codon 12 of the *K-ras* gene are poorly repaired compared with those formed at other codons including codon 14 (*13*). These findings suggest that the preferential DNA damage and poor repair of DNA damage may contribute to the high mutation frequency at codon 12 of the *K-ras* gene in human cancers.

It remains unclear why codons 12 and 14 in the *K-ras* gene are preferential DNA damage sites. The selectivity of carcinogen−DNA adduct formation at codons 12 and 14 of the *K-ras* gene could be due to (1) the special primary sequences of these two codons and their surrounding codons, (2) the local chromatin structure in human cells resulting from protein−DNA associations, and/or (3) epigenetic modifications, such as C5 cytosine methylation and other DNA modifications, at these two codons and surrounding codons in the *K-ras* gene. In this study we examined these possibilities. Using the UvrABC nuclease incision method in combination with LMPCR technique, we have mapped the distribution of DNA adducts induced by various bulky chemical carcinogens along exon 1 of the *K-ras* gene in naked genomic DNA isolated from NHBE cells, as well as in in vitro synthesized DNA fragments containing the *K-ras* gene exon 1 sequence with or without methylation of the cytosines at CpG sites or at the cytosines pairing with the guanines of codons 12 and 14 of the *K-ras* gene, respectively. We have also characterized the C5 cytosine methylation pattern in exon 1 of the *K-ras* gene in NHBE cells.

MATERIALS AND METHODS

Materials. NHBE cells were obtained from Clonetics (San Diego, CA). Benzo[*a*]pyrene diol epoxide (BPDE) was purchased from Chemsyn Science Laboratories (Lenexa, KA). *N*-Acetoxy-2-(acetylamino)fluorene (NAAAF) and *N*-hydroxy-2-aminofluorene (N-OH-AF) were obtained from the National Cancer Institute Repository (Midwest Research Institute, Kansas City, MO). Aflatoxin B1 8,9-epoxide (AFB1-DE) was synthesized as described in the literature (*14*). Restriction enzymes (*Pst*I, *Pvu*II, *Sca*I, and *Xba*I), *Sss*I methylase, and *S*-adenosylmethionine (SAM) were obtained from New England Biolabs (Beverly, MA). T4 polynucleotide kinase and Taq DNA polymerase were purchased from Promega (Madison, WI). [α-^{32}P]dCTP and [γ-^{32}P]ATP (specific activity ~3000Ci/mmol) were purchased from NEN (Boston, MA). Primers were synthesized by Midland Certified Reagent Co. (Midland, TX).

Cell Culture and Carcinogen Treatment. NHBE cells were cultured in medium provided by the supplier (Clonetics, San Diego, CA). For carcinogen treatment, cells were grown until they were 50−70% confluent, washed with phosphate-buffered saline [68 mM NaCl, 1.94 mM KCl, 1.07 mM KH_2PO_4 (pH 7.4)], and treated with 2 μM BPDE for 30 min, 10 μM NAAAF, or 50 μM AFB1-DE for 60 min, at 37 °C in the dark. The carcinogens were prepared freshly before use. After treatment, the cells were washed with phosphate-buffered saline to remove unreacted carcinogens. NHBE cells with or without carcinogen treatment were then harvested for genomic DNA isolation.

Genomic DNA Isolation and Restriction Enzyme Digestion. Genomic DNA was isolated from NHBE cells as previously described (*13*, *15*). Briefly, cells were washed with phosphate-buffered saline and lysed with lysing buffer [0.5% SDS, 10 mM Tris (pH 7.8), 10 mM EDTA, 10 mM NaCl, 100 μg/mL proteinase K] at room temperature for 1 h. The protein was removed by repeated phenol and then diethyl ether extractions, and the DNA was ethanol precipitated and then resuspended in TE (10 mM Tris, 1 mM EDTA, pH 7.5). RNA was removed by treatment with RNase A (50 μg/mL) at 37 °C for 2 h, followed by repeated phenol and diethyl ether extractions, and DNA was ethanol precipitated and resuspended in TE (pH 7.5). A 370 bp genomic DNA fragment containing the *K-ras* gene exon 1 was produced by cleavage of genomic DNA with restriction enzymes *Pst*I and *Sca*I. A 2420 bp genomic DNA fragment containing the *K-ras* gene exon 1 was produced by cleavage of genomic DNA with restriction enzymes *Pvu*II and *Xba*I. Complete cleavage was confirmed by observing that additional increases in enzyme concentration did not result in additional cleavage.

Detection of the Cytosine Methylation Pattern of the K-ras Gene Exon 1 of NHBE Cells by Sodium Bisulfite Treatment. Methods for sodium bisulfite treatment of genomic DNA are the same as previously described (*16*). Briefly, genomic DNA from NHBE cells (5 μg) was sheared by passing through a narrow gauge needle (size 25G1 and 1/2) and denatured by adding freshly prepared NaOH to a final concentration of 0.3 M. The denatured DNA was mixed with freshly prepared 0.5 mM hydroquinone (Sigma, St. Louis, MO) and 3.1 M sodium bisulfite (Sigma, St. Louis, MO) at pH 5.0 and then incubated at 55 °C for 16 h. After incubation, the free bisulfite was removed by adding freshly prepared NaOH (0.3 M) and incubating the sample at 37 °C for 15 min. The resultant DNA was ethanol precipitated and resuspended in distilled water for PCR amplification. Bisulfite-treated DNA (100 ng) was amplified with two methylated primers specific for the *K-ras* gene exon 1 noncoding strand: 5′-TTTTCATTATTTTTATTATAAAACCTAC-TAAAA-3′ and 5′-GTATATTAAAATAAGATTTATTTT-TATTGTTGGAT-3′. Purified PCR products were sequenced with a Beckman Coulter CEQ dye-terminator cycle sequencing kit (Beckman Coulter, Fullerton, CA) on an Beckman Coulter CEQ 2000 auto sequencer (Beckman Coulter) and manually with a Thermo sequenase ^{33}P radiolabeled terminator cycle sequencing kit (Amersham, Piscataway, NJ).

Preparation of the PCR-Amplified DNA Fragment and ^{32}P-End-Labeling. A 390 bp DNA fragment containing the *K-ras* gene exon 1 sequence was obtained by PCR amplification from NHBE genomic DNA using two oligonucleotide primers: P1, 5′-CGTCGATGGAGGAGTTTGTAAATGAA-GTA-3′, and P2, 5′-CTGTATCAAAGAATGGTCCTGCAC-CAGTA-3′. The fragment was purified by electrophoresis in a 2% agarose gel. A 390 bp 5′-^{32}P-end-labeled DNA

[1] Abbreviations: BPDE, benzo[*a*]pyrene diol epoxide; PAH, polycyclic aromatic hydrocarbon; NAAAF, *N*-acetoxy-2-(acetylamino)-fluorene; N-OH-AF, *N*-hydroxy-2-aminofluorene; AFB1-DE, aflatoxin B1 8,9-epoxide; NHBE, normal human bronchial epithelial; SAM, *S*-adenosylmethionine; LMPCR, ligation-mediated polymerase chain reaction; bp, base pair; C, coding; NC, noncoding; RI, relative intensity.

fragment containing the *K-ras* gene exon 1 sequence was obtained by PCR amplification using primers P1 and P2, with P1 5′-end-labeled with [γ-^{32}P]ATP according to the method described previously (*17*). The 5′-^{32}P-end-labeled DNA fragment was purified by electrophoresis in an 8% polyacrylamide gel as described previously (*17*).

C5 Cytosine Methylation at CpG Sites in the DNA Fragment. The 5′-^{32}P-end-labeled DNA fragments were subjected to *Sss*I methylase treatment in the presence of SAM to methylate all cytosines at CpG sites as previously described (*18*).

Preparation of Chemically Synthesized K-ras Gene Exon 1 DNA Fragments Containing Methylation at the Cytosine Which Pairs with the First Guanine of Codon 12 or the Guanine of Codon 14. The oligonucleotide of the *K-ras* gene exon 1 coding (C) strand (100-mer) and three complementary oligonucleotides of the *K-ras* gene exon 1 noncoding (NC) strand (100-mer) with or without C5 cytosine methylation at particular positions were chemically synthesized. The first NC strand (NC-1) has no methylation at any cytosine, the second NC strand (NC-2) has C5 methylation at the cytosine (5′-AC^{m}C-3′; the superscript m indicates the methylation site) which pairs with the first guanine of the *K-ras* gene codon 12 (5′-GGT-3′), and the third NC strand (NC-3) has C5 methylation at the cytosine (5′-TA^{m}C-3′) which pairs with the guanine of codon 14 (5′-GTA-3′). To construct double-stranded 100 bp DNA fragments of the *K-ras* gene containing different percentages of methylation at the cytosine pairing with the first guanine of the *K-ras* gene codon 12 or the guanine of codon 14, NC strands with different ratios of NC-1:NC-2 and NC-1:NC-3 were mixed and annealed with the C strand.

Carcinogen Modification of Genomic DNA or DNA Fragments. For BPDE modification, freshly prepared BPDE (in dimethyl sulfoxide) was added to purified genomic DNA or in vitro synthesized DNA fragments (in TE, pH 7.5) to a final concentration of 2 μM, and the reaction was carried out at room temperature for 2 h (*13*, *19*, *20*). For N-OH-AF modification, freshly prepared N-OH-AF (in argon-purged ethanol) was added to purified genomic DNA or PCR-amplified DNA fragments (in 4 mM sodium acetate buffer, pH 5.5) to a final concentration of 10 μM, and the reaction was carried out at room temperature for 3 h (*21*). For AFB1-DE modification, freshly prepared AFB1-DE (in 10% acetone) was added to purified genomic DNA or PCR-amplified DNA fragments (in TE, pH 7.5) to a final concentration of 50 μM, and the reaction was carried out at room temperature for 1 h (*22*). The unreacted carcinogens were removed by repeated phenol and diethyl ether extractions, and the resultant DNA was ethanol precipitated and resuspended in TE (pH 7.5).

Purification of UvrA, UvrB, and UvrC Proteins. The UvrA, UvrB, and UvrC proteins, the nucleotide excision repair proteins of *Escherichia coli*, were isolated from the *E. coli* K12 strain CH296 carrying plasmids pUNC 45 (*uvrA*), pUNC21 (*uvrB*), or pDR3274 (*uvrC*), respectively. These plasmids and the *E. coli* strain CH296 were kindly provided by Dr. A. Sancar (University of North Carolina, Chapel Hill, NC). The purification procedures were the same as previously described (*23*, *24*).

Cleavage of DNA Adducts by UvrABC Nuclease. Carcinogen-modified genomic DNA and DNA fragments were reacted with UvrABC nuclease, the nucleotide excision repair protein complex, as described previously (*13*, *24*). Briefly, a 10-fold molar excess (the molar concentration of genomic DNA was calculated on the basis of the assumption that the size of genomic DNA is 10 kb) of UvrABC nuclease was added to DNA in a reaction buffer containing 100 mM KCl, 1 mM ATP, 10 mM $MgCl_2$, 10 mM Tris (pH 7.5), and 1 mM EDTA. Under this condition, the UvrABC nuclease incises the carcinogen−DNA adduct specifically and quantitatively, which can reflect the extent of carcinogen−DNA adduct formation (*18*−*21*). The reactions were carried out at 37 °C for 60 min and stopped by phenol and diethyl ether extractions, followed by ethanol precipitation of the DNA.

Mapping DNA Adduct Distribution in 5′-^{32}P-End-Labeled DNA Fragments with Direct UvrABC Nuclease Incision and Gel Separation. The 5′-^{32}P-end-labeled DNA fragments were sequenced by the Maxam−Gilbert sequencing reaction (*25*). The 5′-^{32}P-end-labeled DNA fragments, resulting from UvrABC nuclease incision as described above or the Maxam−Gilbert sequencing reaction, were resuspended in sequencing tracking dye (90% v/v deionized formamide, 0.1% xylene cyanol, and 0.1% bromophenol blue), heated at 90 °C for 5 min, and separated by electrophoresis in 8% denaturing polyacrylamide gels. The gels were dried and initially exposed to a Cyclone PhosphorImager (Packard, Meriden, CT) and then to Kodak X-ray films. The intensity of UvrABC incision bands was quantified by the Cyclone PhosphorImager (Packard). The relative intensity (RI) was calculated on the basis of RI = I_j/I_{max}, where I_j is the intensity of each UvrABC incision band and I_{max} is the UvrABC incision band with the highest intensity for each carcinogen in an autoradiograph.

Mapping DNA Adduct Distribution in PCR-Amplified DNA Fragments or Genomic DNA with UvrABC Nuclease Incision in Combination with LMPCR. The distribution of DNA adducts along exon 1 of the *K-ras* gene was mapped by UvrABC nuclease incision in combination with LMPCR. The genomic DNA or DNA fragments incised with UvrABC nuclease or resulting from the Maxam−Gilbert sequencing reaction were subjected to LMPCR (*26*, *27*). The resultant PCR products were separated by electrophoresis in 8% denaturing polyacrylamide gels, then electrotransferred to nylon membranes, and hybridized with the ^{32}P-labeled DNA probe specific for *K-ras* gene exon 1. The membranes were exposed initially to a Cyclone PhosphorImager (Packard) and then to films. The blot hybridization method and LMPCR method and primers used in this study were the same as those described previously (*26*, *27*). The intensity of UvrABC nuclease incision bands was quantified by the Cyclone PhosphorImager. The RI was calculated on the basis of RI = I_j/I_{max}, where I_j is the intensity of each UvrABC incision band and I_{max} is the UvrABC incision band with the highest intensity for each carcinogen in an autoradiograph.

RESULTS

Codons 12 and 14 of the K-ras Gene in Naked Human Genomic DNA Are Preferential Adduct Formation Sites for Bulky Chemical Carcinogens. Recently, we have found that, in NHBE cells, codons 12 and 14 of the *K-ras* gene are preferential DNA−adduct formation sites for various bulky chemical carcinogens. Furthermore, we have found that

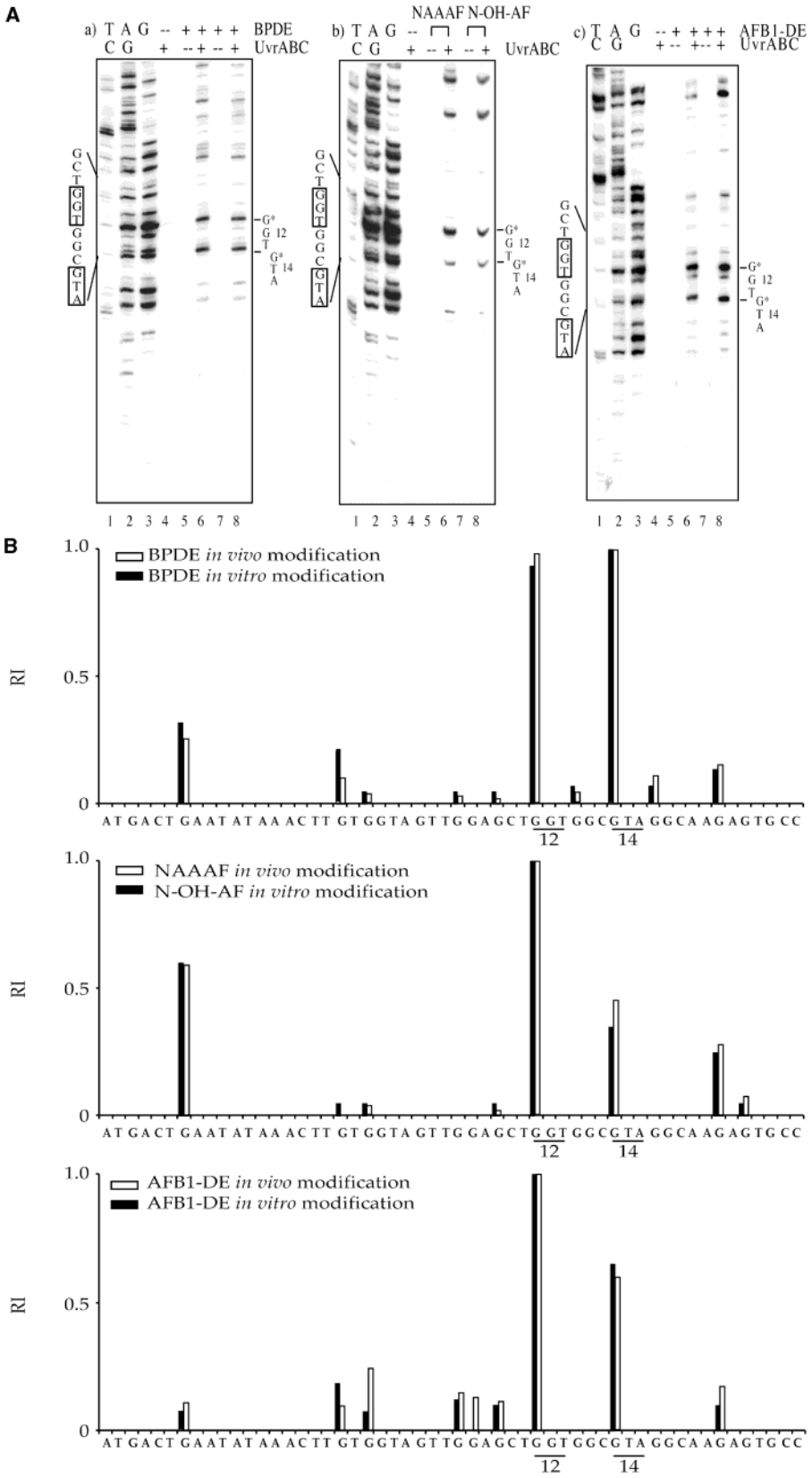

FIGURE 1: Carcinogens preferentially bind at codons 12 and 14 of the *K-ras* gene in naked human genomic DNA as well as in exposed cells. DNA adduct distribution in exon 1 of the *K-ras* gene was mapped by the UvrABC incision method in combination with LMPCR as described in Materials and Methods. (A) Typical autoradiographs. The sequences of codons 12 and 14 (boxed) and their neighbors are indicated on the left-hand side; the guanine residues bound by carcinogens in these codons are indicated by G* on the right-hand side. Lanes 5 and 6 are genomic DNA isolated from NHBE cells treated with (a) 2 μM BPDE, (b) 10 μM NAAAF, or (c) 50 μM AFB1-DE. Lanes 7 and 8 are genomic DNA, isolated from untreated NHBE cells, modified with (a) 2 μM BPDE, (b) 10 μM N-OH-AF, or (c) 50 μM AFB1-DE in vitro. Lanes 1−3 are the Maxam−Gilbert T + C, A + G, and G sequencing reactions. Lanes 4 are unmodified genomic DNA. DNAs in lanes 4, 6, and 8 were reacted with UvrABC nuclease, and DNAs in lanes 5 and 7 were with mock treatment. (B) Quantifications. Quantification methods were described in Materials and Methods. The relative intensity (RI) was calculated on the basis of RI = I_j/I_{max}, where I_j is the intensity of each UvrABC incision band and I_{max} is the UvrABC incision band with the highest intensity of each lane for each carcinogen in an autoradiograph.

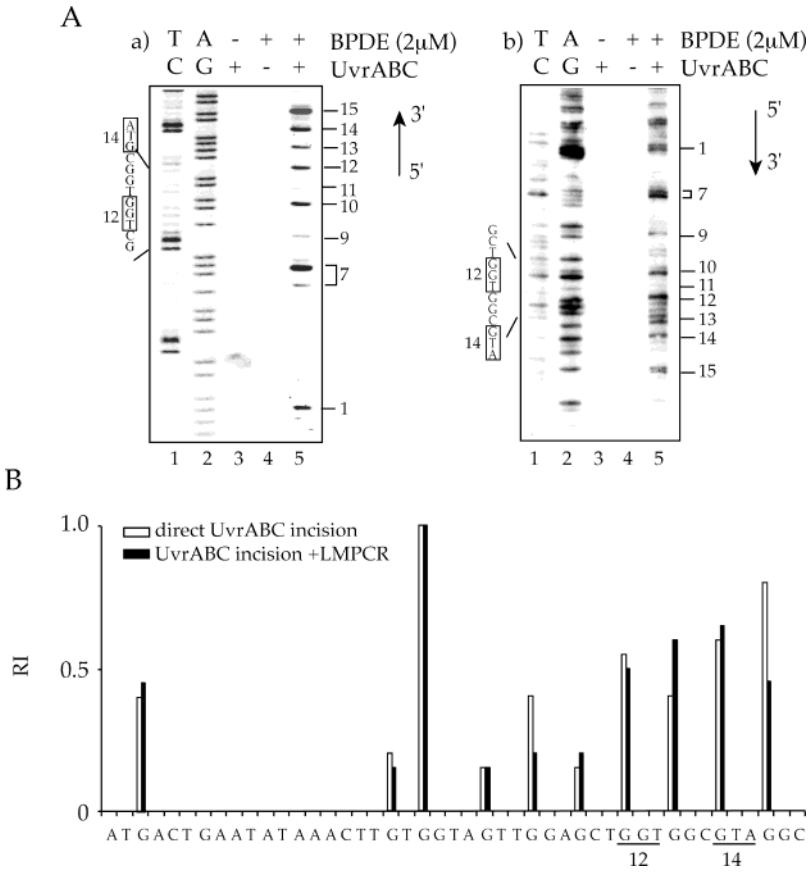

FIGURE 2: BPDE−DNA adduct distribution in exon 1 of the *K-ras* gene in PCR-amplified DNA fragment mapped by the (A-a) UvrABC nuclease incision method or (A-b) UvrABC nuclease incision in combination with LMPCR. (A) Typical autoradiographs. A-a: The 5′-^{32}P-end-labeled PCR-amplified fragment (390 bp) containing exon 1 of the *K-ras* gene was modified with (lanes 4 and 5) or without (lane 3) 2 μM BPDE and then reacted with (lanes 3 and 5) or without (lane 4) UvrABC nuclease. The resultant DNAs were separated by electrophoresis in a denaturing gel as described in Materials and Methods. A-b: The PCR-amplified fragment (390 bp) containing exon 1 of the *K-ras* gene was modified with (lanes 4 and 5) or without (lane 3) 2 μM BPDE and reacted with or without UvrABC nuclease, followed by LMPCR. The sequences of codons 12 and 14 (boxed) and their neighbors are indicated on the left-hand side; the codon numbers corresponding to the UvrABC incision bands are depicted on the right-hand side. T + C (lane 1) and A + G (lane 2) are the Maxam−Gilbert sequencing reactions. (B) Quantification. Quantification methods were described in Materials and Methods. The relative intensity (RI) was calculated on the basis of RI = I_j/I_{max}, where I_j is the intensity of each UvrABC incision band and I_{max} is the UvrABC incision band with the highest intensity in an autoradiograph.

BPDE−DNA adducts formed at codon 12 of the *K-ras* gene are poorly repaired as compared to adducts formed at other codons including codon 14 (*13*). These results suggest that preferential DNA damage contributes greatly to shape the mutational hot spots in *ras* genes. To determine whether the chromatin structure in human cells plays any role in causing this high susceptibility of carcinogen−DNA adduct formation at codons 12 and 14 of the *K-ras* gene, we have mapped DNA adduct formation in exon 1 of the *K-ras* gene (1) in NHBE cells treated with different carcinogens and (2) in purified NHBE genomic DNA reacted with different carcinogens directly. The carcinogens used for genomic DNA modification were BPDE, AFB1-DE, and N-OH-AF, and the carcinogens used for treating cells were BPDE, AFB1-DE, and NAAAF. It is known that modifying DNA with N-OH-AF produces the same major DNA adduct, *N*-(deoxyguanosin-8-yl)-2-aminofluorene, as treating cells with NAAAF (*21*). These carcinogens were chosen not only because of their carcinogenic potency but also because they form adduct at different sites of guanine residues: BPDE forms adduct at the N2 position of the exocyclic amino group; AFB1-DE forms adduct at the N7 position; and N-OH-AF and NAAAF form adduct at the C8 position of the guanine residues. The DNA−adduct distribution was mapped at the nucleotide sequence level using UvrABC nuclease incision in combination with the LMPCR technique. In this study, various concentrations of each carcinogen were used for DNA modifications (data not shown). The objective of using different concentrations for DNA modifications is to find concentrations which will render one or less than one modification (average modification/DNA fragment) in the interested DNA fragment, so that we can quantify the relative adduct formation using the UvrABC incision method (*18*−*21*, *24*). We found that qualitatively the carcinogen−DNA adduct distribution patterns were not affected by the concentrations of carcinogen used in this study. The kinetics of DNA adduct formation at different modification times were also performed, and we found that the carcinogen−DNA adduct distribution patterns were very similar, if not identical, at different time points. These results indicate that our modification conditions achieve one or less than one carcinogen−DNA adduct formation per fragment and that the levels of adduct formed at different sequences in the DNA fragment can be quantified by the UvrABC incision method. For the sake of clarity, we only present data produced by one concentration and one modification time point for each carcinogen. Results in Figure 1 show that treating genomic DNA directly with these carcinogens produces very similar

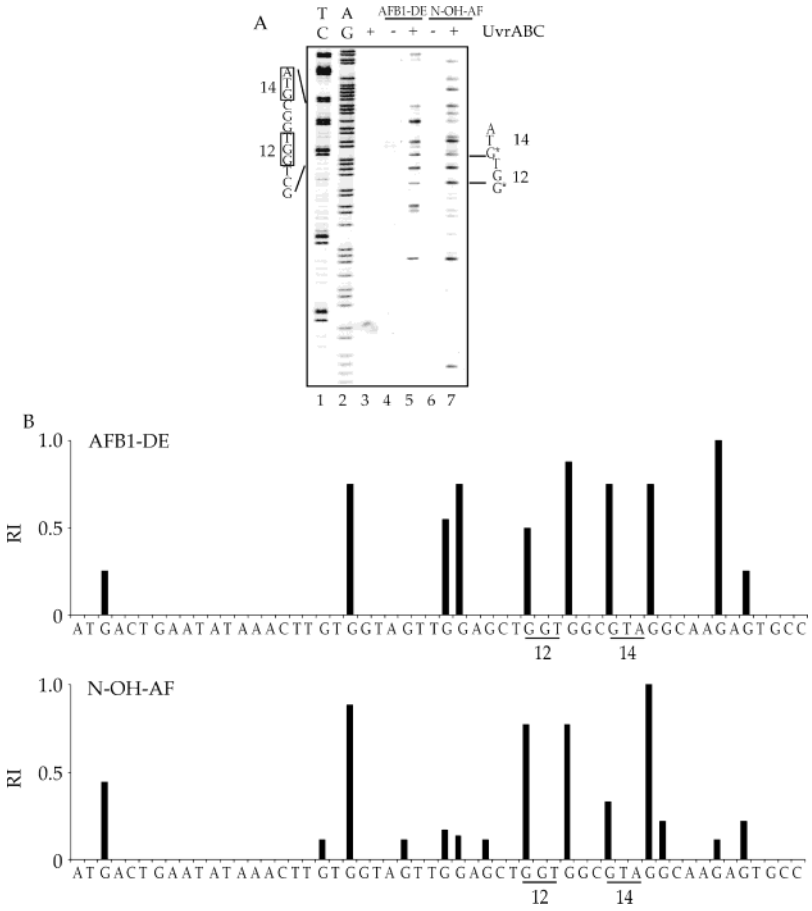

FIGURE 3: N-OH-AF− and AFB1-DE−DNA adduct distribution in exon 1 of the *K-ras* gene in the PCR-amplified DNA fragment. (A) Typical autoradiograph. The 5′-^{32}P-end-labeled DNA fragment (390 bp) containing exon 1 of the *K-ras* gene was modified with 50 μM AFB1-DE (lanes 4 and 5) or 10 μM N-OH-AF (lanes 6 and 7), reacted with UvrABC nuclease (lanes 5 and 7), and separated by electrophoresis in a denaturing gel. Lanes 1 and 2 are the Maxam−Gilbert T + C and A + G sequencing reactions. Lane 3 is the unmodified DNA fragment reacted with UvrABC nuclease. (B) Quantification. Quantification methods were described in Materials and Methods. The relative intensity (RI) was calculated on the basis of RI = I_j/I_{max}, where I_j is the intensity of each UvrABC incision band and I_{max} is the UvrABC incision band with the highest intensity for each carcinogen in an autoradiograph.

DNA adduct distribution patterns in exon 1 of the *K-ras* gene as treating NHBE cells does. All of these carcinogens form DNA adducts preferentially at codon 12. Both AFB1-DE and BPDE also form DNA adducts preferentially at codon 14; N-OH-AF and NAAAF, however, form DNA adducts only moderately at codon 14. These results suggest that the preferential DNA damage at codons 12 and 14 of the *K-ras* gene is not due to local chromatin structure in human cells and may be related to the intrinsic property of genomic DNA in this region.

Codons 12 and 14 in the PCR-Amplified DNA Fragment Containing the K-ras Gene Sequence Are Not Preferential DNA Adduct Formation Sites for Bulky Chemical Carcinogens. Since the preferential carcinogen−DNA adduct formation at codons 12 and 14 of the *K-ras* gene was found not only in cells treated with carcinogens but also in purified genomic DNA modified with carcinogens directly, this result raises the possibility that the preferential carcinogen−DNA adduct formation at these two codons could be due to the characteristic of primary sequences of these two codons and their surrounding codons. If this is the case, codons 12 and 14 in the PCR-amplified DNA fragment containing the *K-ras* gene exon 1, which, unlike genomic DNA, have no epigenetic modifications, should be also preferential adduct formation sites for BPDE as well as other carcinogens. To test this possibility, a 390 bp DNA fragment containing the exon 1 sequence of the *K-ras* gene was produced by PCR amplification and then modified with BPDE. The BPDE−DNA adduct distribution in this PCR-amplified DNA fragment containing exon 1 of the *K-ras* gene was then mapped by two methods: (1) by the UvrABC nuclease incision method and (2) by the UvrABC nuclease incision method in combination with LMPCR. Results of the BPDE−DNA adduct distribution along the *K-ras* gene in the PCR-amplified fragment in Figure 2 show that neither codon 12 nor codon 14 of the *K-ras* gene is a preferential DNA adduct formation site for BPDE as compared with other codons. These results (Figure 2B) also show that the BPDE−DNA adduct distribution pattern mapped by both methods is very similar and demonstrate that LMPCR amplification does not change the original UvrABC nuclease incision pattern which represents the DNA−adduct distribution pattern. These results exclude a trivial possibility that LMPCR amplification may render the false strong signal at codons 12 and 14. We further mapped the DNA−adduct distribution in the PCR-amplified DNA fragments modified with N-OH-AF or AFB1-DE by the UvrABC nuclease incision method. The results in Figure 3 demonstrate that codons 12 and 14 of the *K-ras* gene in the PCR-amplified DNA fragment are not preferential adduct formation sites for N-OH-AF or AFB1-

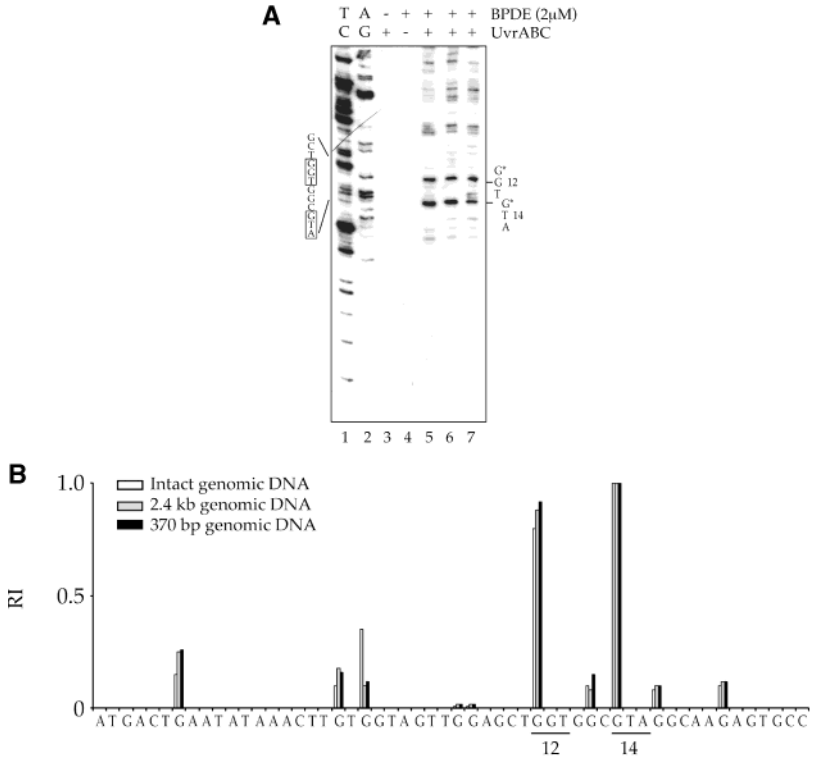

FIGURE 4: BPDE−DNA adduct distribution in exon 1 of the *K-ras* gene in restriction enzyme-digested genomic DNA. (A) Typical autoradiograph. Genomic DNA, isolated from untreated NHBE cells, was digested with (lanes 6 and 7) or without (lane 5) restriction enzyme (lane 6, *Pvu*II/*Xba*I; lane 7, *Pst*I/*Sca*I) and then modified with 2 μM BPDE. The BPDE−DNA adduct distribution was mapped by UvrABC nuclease incision in combination with LMPCR as described in Materials and Methods. The sequences of codons 12 and 14 (boxed) and their neighbors are indicated on the left-hand side; the guanine residues bound by BPDE in these codons are indicated by G* on the right-hand side. Lanes 1 and 2 are the Maxam−Gilbert T + C and A + G sequencing reactions. Lane 3 is unmodified genomic DNA reacted with UvrABC nuclease, and lane 4 is intact genomic DNA modified with BPDE but without UvrABC nuclease reaction. (B) Quantification. Quantification methods were described in Materials and Methods. The relative intensity (RI) was calculated on the basis of $RI = I_j/I_{max}$, where I_j is the intensity of each UvrABC incision band and I_{max} is the UvrABC incision band with the highest intensity of each lane in an autoradiograph.

DE, either. These results strongly suggest that preferential carcinogen−DNA adduct formation at codons 12 and 14 of the *K-ras* gene in the NHBE cells does not originate with the primary DNA sequence of exon 1 of the *K-ras* gene.

BPDE Preferentially Forms DNA Adducts at Codons 12 and 14 of the K-ras Gene in Fragmented Human Genomic DNA. The results that bulky carcinogens preferentially form DNA adducts at codons 12 and 14 of the *K-ras* gene in the naked intact human genomic DNA but not in the 390 bp PCR-amplified DNA fragment raise the possibilities that either the size of DNA and/or the modifications inborn with genomic DNA may play important roles in determining the susceptibility of codons 12 and 14 of the *K-ras* gene to carcinogen−DNA adduct formation. To test these possibilities, genomic DNA isolated from NHBE cells was fragmented with restriction enzyme digestion. *Pst*I/*Sca*I digestion was used to produce a 370 bp fragment containing exon 1 of the *K-ras* gene, and *Pvu*II/*Xba*I digestion was used to produce a 2.4 kb fragment containing exon 1 of the *K-ras* gene. Both fragmented genomic DNAs were then modified with BPDE, and the DNA adduct distribution was mapped by the UvrABC nuclease incision method in combination with LMPCR. The results in Figure 4 show that codons 12 and 14 of the *K-ras* gene remain the preferential BPDE−DNA adduct formation sites in both fragmented genomic DNA and, furthermore, that the BPDE−DNA adduct distributions in these fragmented genomic DNAs are almost identical to that in the intact genomic DNA. These results indicate that the factor(s) which cause(s) the high susceptibility of codons 12 and 14 of the *K-ras* gene to bulky chemical carcinogens is (are) unrelated to the size of DNA and reside-(s) in the genomic DNA within the *Pst*I/*Sca*I 370 bp fragment.

Effect of Cytosine Methylation on Carcinogen−DNA Adduct Formation at K-ras Gene Codons 12 and 14. Previously, we have found that various bulky chemical carcinogens form DNA adducts preferentially at methylated CpG sites in the human *p53* gene and that this preferential adduct formation is due to C5 cytosine methylation of CpG sequences (*19*, *28*−*32*). We have also found that C5 cytosine methylation at CpG sites not only greatly increases carcinogen−DNA adduct formation at CpG sites but also affects carcinogen−DNA adduct formation at surrounding sequences (*31*, *33*). Although the sequence of codon 12 (-TGGTG-) of the *K-ras* gene does not contain a CpG site, its neighbor codon 14 (-CGTAG-) does. These results together raise a possibility that the preferential carcinogen−DNA adduct formation at codons 12 and 14 in the *K-ras* gene could be due to C5 cytosine methylation at the CpG site in codon 14. To test this possibility, all CpG sequences of the 390 bp PCR-amplified DNA fragments containing exon 1 region of the *K-ras* gene were methylated with *Sss*I methylase, which specifically methylates C5 cytosine at CpG sites. The fragments were then modified with BPDE, N-OH-AF, or AFB1-DE, respectively. The carcinogen−DNA adduct distribution along these fragments was mapped by the direct

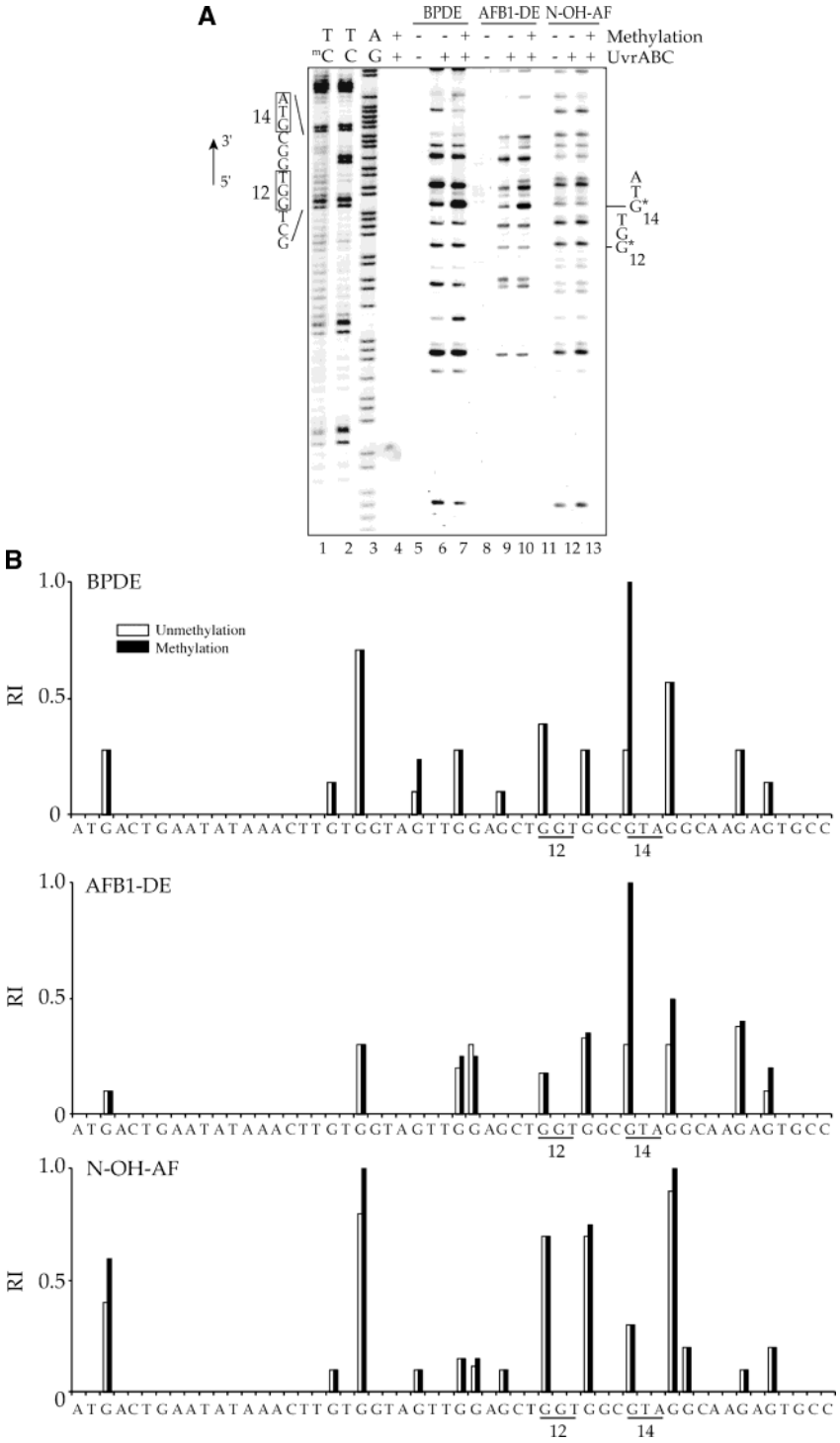

FIGURE 5: Effect of C5 cytosine methylation at CpG sites on carcinogen−DNA adduct formation in exon 1 of the *K-ras* gene. (A) Typical autoradiograph. The 5′-^{32}P-end-labeled PCR-amplified DNA fragments (390 bp) containing exon 1 of the *K-ras* gene were treated with *Sss*I and SAM to methylate the cytosines at the CpG sites. DNA fragments, with (lanes 1, 7, 10, and 13) or without (lanes 2−6, 8, 9, 11, and 12) *Sss*I treatment, were modified with 2 μM BPDE (lanes 5−7), 50 μM AFB1-DE (lanes 8−10), or 10 μM N-OH-AF (lanes 11−13), reacted with UvrABC nuclease (lanes 4, 6, 7, 9, 10, 12, and 13), and separated by denaturing gel electrophoresis. Lane 1 is the Maxam−Gilbert T + C sequencing reaction of the fragment with methylation treatment, lanes 2 and 3 are the Maxam−Gilbert T + C and A + G sequencing reaction of the fragment without methylation treatment. The sequences of codons 12 and 14 (boxed) and their neighbors are indicated on the left-hand side; the guanine residues bound by BPDE in these codons are indicated by G* on the right-hand side. (B) Quantification. Quantification methods were described in Materials and Methods. The intensity of UvrABC incision bands was quantified with a PhosphorImager, normalized by the amount of DNA applied in the gel. The relative intensity (RI) was calculated on the basis of RI $= I_j/I_{max}$, where I_j is the intensity of each UvrABC incision band and I_{max} is the UvrABC incision band with the highest intensity for each carcinogen in an autoradiograph.

UvrABC nuclease incision method. The extent of cytosine methylation at CpG sites was determined by the Maxam−Gilbert chemical cleavage reaction (*25*). Hydrazine is unable to modify C5-methylated cytosines; consequently, both the 5′ and 3′ phosphodiester bonds of each methylated cytosine are refractory to piperidine hydrolysis, and no cytosine ladders are observed at methylated cytosines (*34*). Figure 5A shows that, under our methylation conditions, all of the

CpG sites, including codon 14, in the DNA fragment were methylated. The results in Figure 5 show that cytosine methylation dramatically enhances BPDE− and AFB1-DE−DNA adduct formation at the CpG site of codon 14 and makes it a strong adduct formation site in the methylated *K-ras* gene exon 1 DNA fragment; however, it has no significant effect on adduct formation at surrounding non-CpG sites, such as codon 12. Results (Figure 5B) also show that the cytosine methylation at CpG sites has no significant effect on N-OH-AF-induced DNA adduct formation at either CpG (such as codon 14) or non-CpG (such as codon 12) sites, which may explain why codon 14 is not a very strong adduct formation site for NAAAF in human cells or N-OH-AF in naked genomic DNA (Figure 1). These results confirm that the preferential DNA damage induced by BPDE and AFB1-DE at codon 14 of the *K-ras* gene is mainly caused by C5 cytosine methylation at CpG sites, and the preferential carcinogen−DNA adduct formation at codon 12 is not caused by cytosine methylation at CpG sites.

Using hemimethylated constructs, Johnson et al. (*35*) have shown that at CpG sites the C5 cytosine methylation enhances mitomycin C−guanine adduct formation at the CpG site of the opposite strand but does not enhance mitomycin C−guanine adduct formation at the CpG site of the same strand (*35*). Recently, it has also been reported that the enhancing effect of C5 cytosine methylation on BPDE−DNA adduct formation at guanine of the CpG sites is mainly due to the presence of the C5 methylation at the cytosine pairing with the targeted guanine (*36*). These results suggest that the guanines which pair with methylated cytosines may be more susceptible to adduct formation by some carcinogens. Although methylation of cytosine in mammalian DNA mainly occurs symmetrically at CpG sites in both DNA strands, C5 cytosine methylation can also occur at non-CpG sites (*37*, *38*). It is possible that even though codon 12 of the *K-ras* gene does not contain a CpG sequence, the preferential adduct formation of carcinogens at the first guanine of this codon could be caused by the methylation of cytosine which pairs with the first guanine of this codon. To unambiguously determine the effect of C5 cytosine methylation at codon 12 of the *K-ras* gene on carcinogen−DNA adduct formation, we constructed 100 bp DNA fragments of the *K-ras* gene exon 1 containing different percentages of methylation at the cytosine which pairs with the first guanine of codon 12 or the guanine of codon 14 of the *K-ras* gene. These DNA fragments were modified with BPDE, and the BPDE−DNA adduct distribution was then mapped by the UvrABC nuclease incision method. The results in Figure 6 show that C5 methylation at the cytosine which pairs with the guanine of CpG site at codon 14 does greatly enhance BPDE−DNA adduct formation at the guanine of codon 14, and the enhancement of BPDE−DNA adduct formation at this codon is proportional to the amount of cytosine methylation. However, methylation at the cytosine which pairs with the first guanine of codon 12 has no significant effect on adduct formation at guanines on the opposite DNA strand including codon 12.

The CpG Site at Codon 14 of the K-ras Gene in Normal Human Bronchial Epithelial Cells Is Methylated. The results in Figure 6 suggest that the guanine at codon 14 which pairs with methylated cytosines is more susceptible to carcinogen−DNA adduct formation. If this is the case, then the

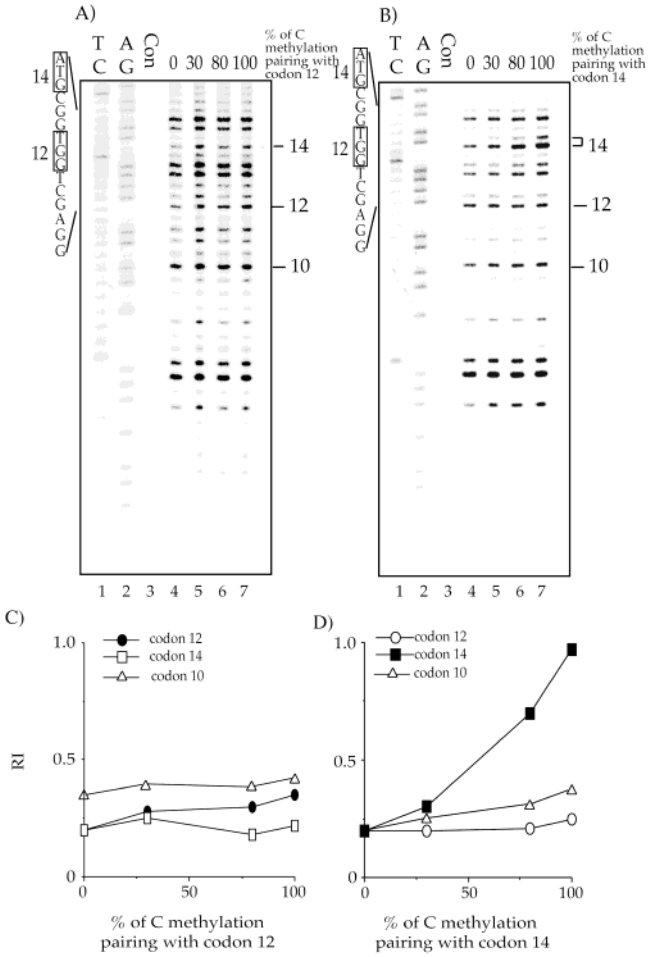

FIGURE 6: Effect of methylation at the cytosines pairing with the first guanine of codons 12 and 14 on BPDE−DNA adduct formation. The 100-mer C and NC strands (NC-1, NC-2, and NC-3) of exon 1 of the *K-ras* gene were chemically synthesized. NC-1 has no cytosine methylation. NC-2 has a site-specific C5-methylated cytosine opposite the first guanine of codon 12. NC-3 has a site-specific C5-methylated cytosine opposite the guanine of codon 14. Double-stranded DNA fragments containing different percentages of a site-specific C5-methylated cytosine opposite (lane 4, 0%; lane 5, 30%; lane 6, 80%; lane 7, 100%) the first guanine of codon 12 (A) or the guanine of codon 14 (B) of the coding strand were constructed as described in Materials and Methods. These DNA fragments were then modified with 2 μM BPDE (lanes 4−7), reacted with UvrABC nuclease, and separated by electrophoresis in an denaturing gel. The sequences of codons 12 and 14 (boxed) and their neighbors are indicated on the left-hand side. (A, B) Typical autoradiographs. (C, D) Quantification. Quantification methods were described in Materials and Methods.

preferential carcinogen−DNA adduct formation at codon 14 of the *K-ras* gene in NHBE cells shown in Figure 1 could have resulted from the fact that the CpG site in this codon is methylated either in the whole population or in the partial population of NHBE cells. To distinguish between these two possibilities, we characterized the C5 cytosine methylation pattern of exon 1 of the *K-ras* gene by the sodium bisulfite method (*16*). This method converts cytosine but not C5-methylated cytosine into uracil (*16*). The resultant DNA was then sequenced manually or by the automatic sequencer. Results of both sequencing methods show that all cytosines at CpG sites are methylated in *K-ras* exon 1 region, including codon 14; however, there is no detectable methylation at cytosines other than at CpG sites, including the one which

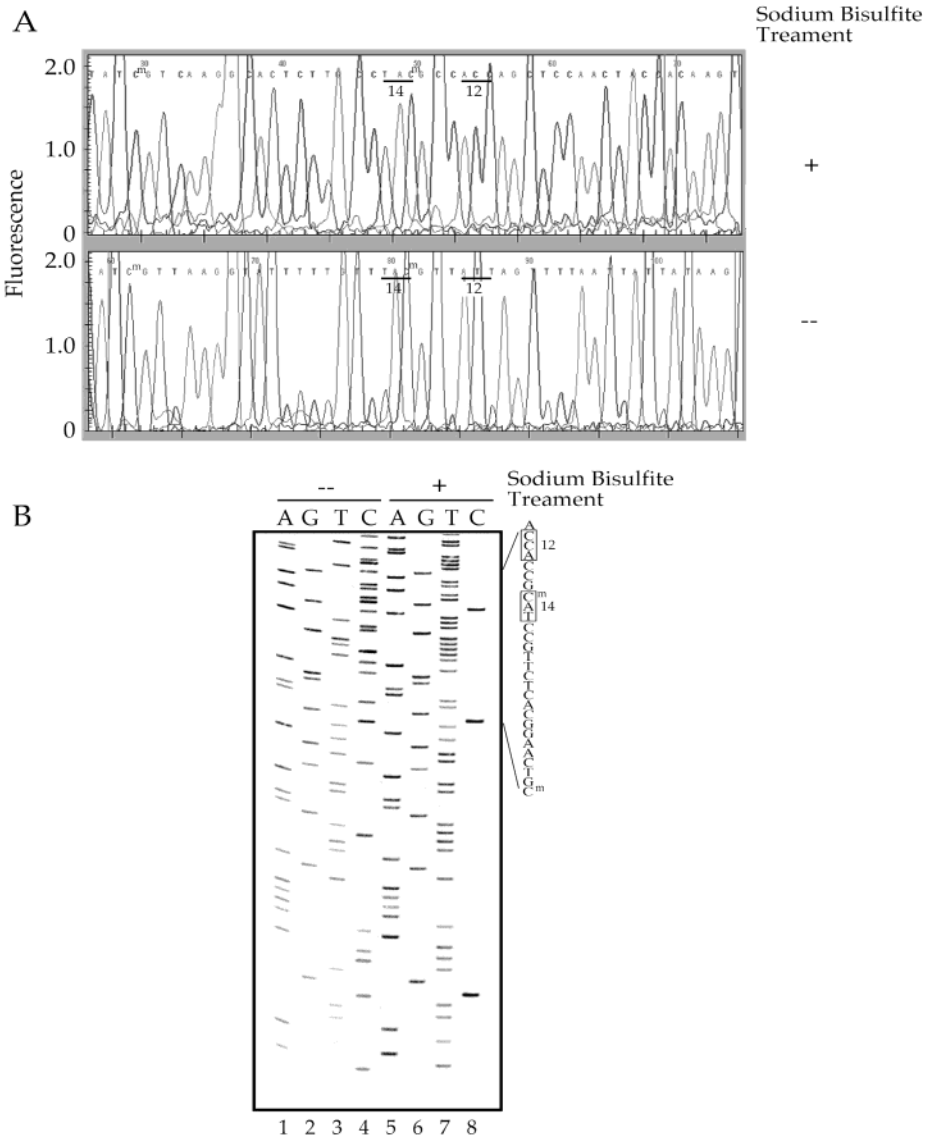

FIGURE 7: Detection of cytosine methylation in the noncoding strand of exon 1 of the *K-ras* gene in NHBE cells. Genomic DNA isolated from NHBE cells was treated with sodium bisulfite to convert all nonmethylated cytosines to uracils as described in Materials and Methods. The *K-ras* gene exon 1 region was then amplified with specific primers. The amplified fragments were then sequenced (A) by a Beckman CEQ DNA automatic sequencer or (B) manually with a Thermo sequenase ^{33}P-radiolabeled terminator cycle sequencing kit. The two ^{m}C represent the methylated cytosines, one pairing with the guanine of codon 14 and the other located in intron 1. Similar results were obtained for the coding strand of exon 1 of the *K-ras* gene (data not shown).

pairs with the first guanine of codon 12 of the *K-ras* gene (Figure 7).

These results, together, strongly suggest that cytosine methylation at the CpG site is the major reason for the preferential DNA damage at codon 14. These results also exclude the possibility that the preferential DNA adduct formation of carcinogens at codon 12 of the *K-ras* gene is caused by the C5 cytosine methylation at CpG sites or those pairing with guanines at codons 12 and 14 and suggest that the preferential carcinogen−DNA adduct formation at this position may be caused by epigenetic modifications, other than cytosine methylation, inborn in genomic DNA.

DISCUSSION

Although it has been shown that mutations at codons 12, 13, and 61 in all three *ras* genes can activate these genes to be oncogenic, mutations at codon 12 of the *K-ras* gene are far more prevalent than mutations in its counterpart at *H-* and *N-ras* genes and other mutations in these three *ras* genes in human cancers (*4*−*6*, *8*−*12*). The mechanisms underlying this intriguing phenomenon remain unclear. It is possible that mutation at codon 12 of the *K-ras* gene may give cells growth advantage, resistance to apoptosis, and an immunological surveillance system; therefore, cells with this mutation are more prone to develop to cancers (*39*, *40*). However, we have found that codon 12 of the *K-ras* gene is a preferential DNA adduct formation site for various carcinogens and, furthermore, that adducts formed at this sequence are poorly repaired (*13*). We have also found that codon 12 of *H-* and *N-ras* genes is not a preferential DNA adduct formation site for various carcinogens (*13*). These results are similar to what we have found in the *p53* gene (*28*−*30*) and suggest that targeted DNA damage in *ras* genes plays an important role in shaping the mutational hot spots in *ras* genes (*13*).

More than 200 mutation sites in the *p53* gene have been found in human cancers, and over 30% of the mutations

occur at several mutational hot spots (codons 157, 158, 175, 245, 248, 249, 273, and 282) (*41*−*44*). Except in codon 249, all of these mutational hot spots contain a CpG sequence (*41*−*44*). We have found that bulky chemical carcinogens preferentially form DNA adducts at these methylated CpG sites (*19*, *27*−*30*) and C5 cytosine methylation greatly enhances DNA adduct formation at these CpG sites and, furthermore, that the DNA adducts formed at these mutational hot spots are poorly repaired in the *p53* gene (*45*, *46*). We have proposed that the preferential adduct formation and poor repair at these CpG-containing codons may be the major reasons why these sites are mutational hot spots in human cancers.

We have found that codon 14 of the *K-ras* gene in cells or in purified genomic DNA is a preferential DNA adduct formation site for BPDE and AFB1-DE as shown in Figure 1. The first guanine of codon 14 (C*GGAG) is within a CpG site, and we have found that in human genomic DNA this CpG site is highly methylated (Figure 7); therefore, the preferential DNA adduct formation of BPDE and AFB1-DE at codon 14 might be due to C5 cytosine methylation at this CpG site. This explanation is further substantiated by the results shown in Figures 5 and 6, which demonstrate that although BPDE and AFB1-DE form DNA adduct poorly at codon 14 of the *K-ras* gene in the 390 bp PCR-amplified DNA fragments, BPDE− and AFB1-DE−DNA adduct formation at this codon can be greatly enhanced by cytosine methylation at this CpG site, which renders codon 14 as the strongest DNA adduct formation site in the methylated DNA fragment. The results in Figure 6 further demonstrate that the enhancing effect of cytosine methylation on DNA adduct formation at codon 14 is mainly due to the methylation of cytosine pairing with the guanine of codon 14, which is consistent with reports from other laboratories (*35*, *36*). We have also found that N-OH-AF does not form DNA adduct preferentially at codon 14 of the *K-ras* gene in genomic DNA (Figure 1) and that C5 cytosine methylation at the CpG sites does not significantly enhance N-OH-AF-induced DNA adduct formation at this codon (Figure 5). Our previous studies have also shown that cytosine methylation at CpG sites has much less enhancing effects on NAAAF− and 4-aminobiphenyl−DNA adduct formation in the human *p53* gene, both of which form DNA adducts at the C8 position of the guanine residue as N-OH-AF does, compared with BPDE−DNA adduct formation (*19*, *30*). It appears that the preferential DNA adduct formation at codon 14 is carcinogen dependent.

Intriguingly, unlike the majority of the mutational hot spots in the *p53* gene and codon 14 of the *K-ras* gene, codon 12 of the *K-ras* gene does not contain a CpG sequence. Our results have demonstrated that neither chromatin structure, primary sequence, nor cytosine methylation, including cytosine at the noncoding strand opposite codons 12 and 14, contributes to the preferential adduct formation at codon 12. We have also excluded a trivial possibility that somehow the UvrABC nuclease incision method in combination with the LMPCR technique renders a false strong signal at codon 12. More importantly, our results suggest that whatever factor(s) contribute(s) to the preferential carcinogen−DNA adduct formation at codon 12 of the *K-ras* gene, it resides within the *Pst*I/*Sca*I 370 bp fragment containing the *K-ras* gene exon 1 region in the human genomic DNA. Our results show that codon 12 of the *K-ras* gene is a preferential DNA adduct formation site not only for BPDE or AFB1 but also for NAAAF and N-OH-AF, suggesting that some unknown epigenetic modification(s) may provide more freedom to the first guanine of codon 12 than the C5 cytosine methylation at the CpG site to the guanine of codon 14, and consequently enables codon 12 to be bound readily by various bulky carcinogens.

Although codon 14 of the *K-ras* gene is a preferential DNA adduct formation site for bulky chemical carcinogens, very rarely has a mutation at this codon ever been reported in human cancers. This lack of mutation at codon 14 in human cancers is likely due to the fact that the *K-ras* protein with this mutation retains its wild-type function. Furthermore, previously we have found that DNA adducts formed at codon 14 are repaired more efficiently than adducts formed at codon 12, which may also contribute to the rarity of mutations at codon 14 in human cancers (*13*).

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