Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 4974–4978, July 1987
Genetics

Characterization of c-Ki-*ras* oncogene alleles by direct sequencing of enzymatically amplified DNA from carcinogen-induced tumors

(aflatoxin B_1/oligonucleotide hybridization)

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Contributed by Gerald N. Wogan, April 16, 1987

ABSTRACT **Activated c-Ki-*ras* genes in liver tumors from rats exposed to the potent hepatocarcinogen aflatoxin B_1 were analyzed to determine the nature of their activation by characterization of two c-Ki-*ras* alleles present in tumor-derived NIH 3T3 mouse transformants. Using selective hybridization of synthetic oligonucleotides to transformant DNA, we have determined that a single G·C to A·T base transition in either the first or second position of the 12th codon is associated with activation of the gene. Such mutations would lead to amino acid substitutions of aspartate or serine for glycine in the mutant proteins. To confirm these findings, we applied a technique for direct sequence analysis of a 90-base-pair region of the rat c-Ki-*ras* gene produced by primer-directed enzymatic amplification. Findings produced by this approach, which provides a convenient method to characterize mutations in multiple alleles without the necessity to clone individual genes, confirmed the presence and identity of the 12th codon mutations in the activated oncogene, as initially determined by the oligonucleotide hybridization technique.**

Mutagenicity of chemical carcinogens has been implicated as an important determinant of their abilities to initiate tumorigenic processes. Findings that certain chemicals induced mutations in specific protooncogenes provide evidence that direct interaction of these chemicals with DNA may be crucial to the initiation of carcinogenesis. An accumulating body of evidence has described the characterization of single-base mutations that arise in cellular *ras* genes after administration of carcinogens representing several chemical classes (for review, see ref. 1). In some cases, it has been shown that a single dose of carcinogen may be sufficient to cause mutations that are present in the end-stage tumor (2). Zarbl *et al.* (2) have shown that mutations occur at different sites in c-Ha-*ras* genes in mammary tumors induced in rats by different chemical carcinogens. For instance, a single dose of *N*-methyl-*N*-nitrosourea (MNU) leads to G·C to A·T base transitions in the 12th codon, while similar treatment with 7,12-dimethylbenz[*a*]anthracene results in mutations of adenine residues in the 61st codon (1). This, together with related evidence, suggests that the nature of mutations arising in c-*ras* genes of various chemically induced tumors may be related to specific structural changes in DNA produced by carcinogen–DNA interactions. It is therefore of particular importance to characterize mutations induced at specific sites in oncogene sequences and to relate them to known modifications in DNA structure induced in well-documented experimental systems.

For this reason, we have been investigating oncogene activation in rat liver tumors induced by aflatoxin B_1 (AFB_1), a carcinogenesis model for which much information has been obtained with regard to the activation and interaction of the carcinogen with DNA. It has been shown that the carcinogen forms DNA adducts exclusively with guanine residues, and the primary covalent AFB_1–DNA adduct formed in rat liver (as well as bacterial and mammalian cells mutated by the compound) is 2,3-dihydro-2-(N^7-guanyl)-3-hydroxy-AFB_1 (3). A single administration of AFB_1 to rats results in maximum liver DNA adduct levels 2 hr after dosing; by 24 hr, 88% of the AFB_1–DNA adduct has been removed (4). The rapid removal of DNA adducts may be related to the requirement for multiple exposures to AFB_1 for the induction of tumors in the Fischer rat strain (5). Previously, we have shown that an oncogene of the c-Ki-*ras* gene family has been activated in liver tumors of rats chronically exposed to AFB_1 (6). Since many studies have shown that c-*ras* oncogenes have been activated by mutations in either the 12th or 61st codon of the gene, we sought to determine whether such mutations exist in AFB_1-induced liver tumors. In addition, AFB_1 has been shown to be a potent mutagen in both bacterial (7) and mammalian systems (8). Therefore, characterization of mutations in the activated c-Ki-*ras* oncogenes would serve to implicate further the mutagenicity of AFB_1 in the initiation of hepatocarcinogenesis.

We have used two methods to establish the nature of mutations in NIH 3T3 mouse transformants derived by transfection of primary liver tumor DNA. The first utilized a technique in which synthetic oligonucleotides of defined DNA sequence were hybridized to transformant DNA under conditions in which homoduplexes of known sequence were stable while single-base mismatches were unstable. The second approach involved an adaptation of the enzymatic amplification of DNA as described by Saiki *et al.* (9) combined with direct sequencing of amplified DNA derived from a specific gene region of the c-Ki-*ras* gene. Using this combined approach, we have determined that c-Ki-*ras* oncogene alleles derived from AFB_1-induced liver tumors contain G·C to A·T base transitions in either the first or second position of the 12th codon.

MATERIALS AND METHODS

NIH 3T3 Transformant DNA. To induce liver tumors, weanling male Fischer rats were injected intraperitoneally 5 days a week for 8 weeks with 25 μg of AFB_1 (Makor Chemicals, Jerusalem) as described (6). NIH 3T3 mouse fibroblasts were maintained at low cell density in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum. Transfection of tumor-derived genomic DNA into NIH 3T3 mouse fibroblasts was performed using the calcium phosphate coprecipitation method of Graham and van der Eb (10). After transfection, the cells were supplemented with DMEM containing 5% calf serum. Anchorage-independent foci were scored and subcloned between 2 and 3 weeks after transfection. DNA was isolated from cells of dispersed cell

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Abbreviations: AFB_1, aflatoxin B_1; MNU, *N*-methyl-*N*-nitrosourea.
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pellets derived from representative transformants in a buffer containing 150 mM NaCl, 10 mM Tris·HCl (pH 7.5), and 10 mM EDTA. Cells were lysed by the addition of 0.4% $NaDodSO_4$. The lysate was digested overnight with proteinase K (Sigma) and purified by phenol/chloroform extraction. DNA was made 2 M with ammonium acetate, reprecipitated with ethanol, and resuspended in 10 mM Tris·HCl, pH 8.0/1 mM EDTA prior to digestion with *Hin*dIII under conditions specified by the supplier.

Selective Oligonucleotide Hybridization. Oligomers 20 nucleotides long were synthesized using phosphoramidite methodology on an Applied Biosystems 380A DNA synthesizer. Oligomers were gel-purified and the sequence was confirmed by DNA sequencing methods (11). Radiolabeled oligomers were prepared by incubation of 80 pmol of oligomer for 1 hr at 37°C in a 15-μl reaction mixture containing 160 pmol of [γ-^{32}P]ATP (>7000 Ci/mmol; 1 Ci = 37 GBq) (New England Nuclear), 10 mM $MgCl_2$, 50 mM Tris·HCl (pH 7.6), 5 mM dithiothreitol, 0.1 mM spermidine hydrochloride, and 0.1 mM EDTA. The radiolabeled oligomer was purified by ion-exchange chromatography using Elutip-d columns (Schleicher & Schuell, Keene, NH). Genomic DNA from NIH 3T3 transformants was digested with *Hin*dIII and electrophoresed in a 0.8% agarose gel. The gel was denatured, neutralized, and dried according to the method of Bos *et al.* (12). The dried gel was prehybridized for 1 hr in a buffer containing 0.5 M sodium phosphate (pH 7.5), 7% $NaDodSO_4$, 1 mg of yeast RNA per ml, and 1 mM EDTA at 50°C. The gel was then hybridized overnight in the same buffer containing 20–40 pmol of ^{32}P-labeled 20-nucleotide oligomers. Hybridized gels were washed three times for 10 min each at 50°C in a buffer containing 5× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate) and 1% $NaDodSO_4$. The gel was then equilibrated for 15 min at 50°C in a solution containing 3.2 M tetramethylammonium chloride with 1% $NaDodSO_4$. The gel was then washed in the same solution for 30 min at 56°C to melt mismatched hybrids. The washed gel was air-dried and exposed to x-ray film with a single Cronex intensifier screen.

Enzymatic Amplification. Enzymatic amplification was performed using a method modified from the procedure of Saiki *et al.* (9). One microgram of genomic DNA or 1 μg of v-Ki-*ras*-containing plasmid pHiHi-3 (13) was precipitated with 2 vol of ethanol and washed twice with 70% ethanol. One microgram of DNA was incubated in a 100-μl reaction mixture with 2.5 μg each of 20-nucleotide primers; 1.5 mM each dGTP, dATP, dTTP, and dCTP; 10 mM Tris·HCl (pH 8.0); 10 mM $MgCl_2$; and 7 mM NaCl. The reaction mixture was initially boiled for 5 min, removed from the water bath at room temperature for 1 min, incubated at 42°C for 1 min, and incubated for 2 min with 1 unit of the large fragment of *Escherichia coli* DNA polymerase I (Klenow) (New England Biolabs). Subsequent cycles were identical except that the reaction mixture was boiled for 1 min. After 24–36 cycles, the reaction mixture was precipitated with ethanol, and the DNA was resolved in a native 10% acrylamide gel. The 90-base-pair (bp) DNA band was excised, eluted from polyacrylamide, and precipitated with ethanol to concentrate. The DNA concentration was estimated by dotting the sample on agarose plates containing ethidium bromide at 1 μg/ml.

Direct DNA Sequencing. Amplified DNA was sequenced according to a modification of the procedures of Sanger *et al.* (14). A 12-μl mixture was prepared containing 1 μl of purified DNA, 2 μl of a 10× buffer containing 100 mM Tris·HCl (pH 8.0), 100 mM $MgCl_2$, and 20–80 pmol of ^{32}P-labeled primer. Three microliters of the mixture was apportioned into reaction mixtures containing 1 μl of 10 mM dideoxy-GTP (ddGTP) and 1 μl of a mixture containing 1.6 mM dATP, dTTP, dCTP and 0.16 mM dGTP; or 1 μl of 10 mM ddATP and 1 μl of a mixture containing 1.6 mM dGTP, dTTP, dCTP and 0.16 mM dATP; or 1 μl of 10 mM ddTTP and 1 μl of a mixture containing 1.6 mM dGTP, dATP, dCTP and 0.16 mM dTTP; or 1 μl of 5 mM ddCTP and 1 μl of a mixture containing 1.6 mM dGTP, dATP, dTTP and 0.16 mM dCTP. The reaction mixtures were boiled for 1 min, allowed to cool for 1 min at room temperature, incubated for 1 min at 42°C, and incubated for 2 min at 42°C with 1 unit of *E. coli* DNA polymerase I. This procedure was repeated twice and stopped by the addition of 2.5 μl of an 80% formamide dye mixture. Four microliters of the reaction mixture was boiled for 4 min and loaded onto a 10% acrylamide/urea gel. The samples were electrophoresed and the gel was exposed to x-ray film at −70°C.

RESULTS

We have previously reported that some AFB_1-induced hepatocellular carcinomas contain activated c-Ki-*ras* genes (6), based on findings that DNA isolated from primary NIH 3T3 mouse transformants contained rat sequences homologous to c-Ki-*ras* DNA probes. Because other c-*ras* genes have been shown to be activated by mutations residing in the 12th or 61st codon (1), we sought to determine whether such mutations were present at these genetic loci in NIH 3T3 transformants containing rat c-Ki-*ras* sequences.

To accomplish this, we performed hybridization experiments using synthetic 20-nucleotide oligomers that were specific for the 12th and 61st codon regions. Using the method of Bos *et al.* (12), we found that hybridization of a wild-type 61st codon oligonucleotide with transformant DNA digested with *Hin*dIII revealed the presence of a rat-specific 8.5-kilobase (kb) restriction fragment, indicating that no mutation had taken place at the 61st codon region. Hybridization of the same DNA to a wild-type 12th codon oligonucleotide probe failed to reveal a rat-specific 2.5-kb restriction fragment, suggesting the presence of mutations at that site. In both cases, the oligonucleotides were shown to hybridize to restriction fragments characteristic of mouse-specific proto-oncogenes derived from the NIH 3T3 DNA, thus verifying the ability of the probes to recognize homologous sequences. Taken together, these findings suggested that the transformants contained mutations in the 12th codon region of the rat c-Ki-*ras* genes.

To establish the nature of these mutations, we hybridized transformant DNA to oligonucleotides containing various nucleotide substitutions in the first and second position of the 12th codon. As shown in Fig. 1, hybridization of specific anti-sense oligomers containing thymidine substitutions at these positions revealed the presence of a 2.5-kb rat-specific restriction fragment. These results indicated that a G·C to A·T base transition had occurred at either of two positions in the rat c-Ki-*ras* 12th codon (GGT). Such single-base changes would result in the amino acid substitution of aspartate (AGT) and serine (GAT) for the glycine present in wild-type polypeptide. Hybridization of the same transformant DNA to adenine-substituted oligonucleotides did not reveal the presence of G·C to T·A base transversions.

To confirm the results obtained through the oligonucleotide probe technique, an approach was developed to provide unambiguous evidence of sequence changes at appropriate positions in this region of the gene. The procedure of Saiki *et al.* (9) for enzymatic DNA amplification was adapted for this purpose. Two 20-nucleotide oligomers were synthesized and used as primers to amplify a gene region that spans the 12th codon. As shown in Fig. 2, a region spanning 90 bp (codons 1–30) was bracketed by the primers. By using sequential cycles of denaturation, primer annealing, and polymerase extension, multiple copies of the c-Ki-*ras* gene region were generated. As shown in Fig. 3, template DNA from NIH 3T3 cells and a v-Ki-*ras*-containing plasmid was subjected to 36 cycles of polymerase extension and electrophoresed on a

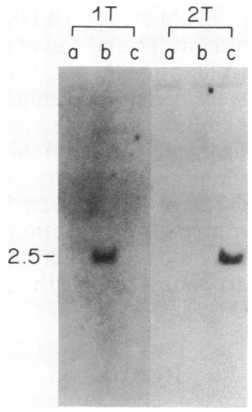

FIG. 1. Determination of mutations in c-Ki-*ras*-containing NIH 3T3 transformants. Genomic DNA was prepared from NIH 3T3 cells (lanes a), NIH 3T3 cell line R3a.2 (lanes b), and NIH 3T3 cell line R8a.1 (lanes c). Twenty micrograms of DNA was digested with *Hin*dIII, electrophoresed in a 0.8% agarose gel, and dried according to the procedure of Bos *et al.* (12). Dried gels were hybridized to primers 1T: 5′ CCTACGCCA*CTA*GCTCCAAC 3′, or 2T: 5′ CCTACGCCA*TCA*GCTCCAAC 3′ as described. The hybridized gels were washed in tetramethylammonium chloride at 56°C and exposed to x-ray film. The resultant autoradiogram is shown. The 2.5-kb band is indicated.

native polyacrylamide gel. Analysis of amplified mouse DNA indicated a predominant 90-bp DNA band corresponding to the size predicted from reported sequence analysis of the c-Ki-*ras* gene (13), together with additional unidentified bands. Amplification of a plasmid containing a portion of the v-Ki-*ras* gene produced only the 90-bp band. We conclude, therefore, that the additional DNA sequences produced from genomic DNA templates may be related to alternative primer sites, which occur during the enzymatic amplification procedure. For this reason, the 90-bp DNA band had to be purified prior to sequence analysis. It is noteworthy that amplification of NIH 3T3 control DNA revealed a 90-bp band, the intensity of which was comparable to the amplified sequences derived from transformant DNA. This was unanticipated, since our previous study showed that the rat c-Ki-*ras* gene in the transformant DNA was highly amplified (6) and therefore suggested that amplification would result in disproportionate production of the c-Ki-*ras* sequences. This would indicate that the amount of DNA produced by the amplification system under these conditions is not directly dependent on the initial quantity of template DNA.

By using a modification of the Sanger DNA sequencing method (14), we sequenced directly both strands of the 90-bp amplified DNA through the use of radiolabeled forms of either of the two 20-nucleotide primers used initially to amplify the DNA. Sequences were determined for amplified regions from control and transformant DNA, and the results are shown in Fig. 4. Sequence analysis at nucleotide positions corresponding to the 12th codon (positions 34 and 35) indicated that single-base mutations had occurred at these two sites in c-Ki-*ras*-containing transformant DNA. Comparison of sequences derived from guanine- and adenine-specific sequence reactions indicated the simultaneous presence of both guanine and adenine at either nucleotide position 34 or 35. Analysis of the sequence data revealed the same G·C to A·T base transitions which had previously been identified by oligonucleotide hybridization, thus providing independent confirmation of the nature of the mutations present in the transformants. These observations are in sharp contrast to

FIG. 2. Scheme of enzymatic amplification. This diagram illustrates the experimental scheme used to amplify a portion of the rat c-Ki-*ras* gene. Primer I and primer II were annealed to genomic DNA to initiate the synthesis of a portion of the gene from codon 7–23 after the addition of Klenow enzyme. Subsequent cycles of denaturation, primer annealing, and polymerase extension lead to the amplification of DNA corresponding to nucleotide positions 1–90.

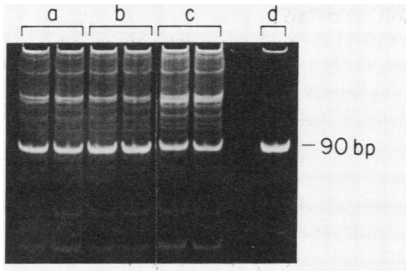

FIG. 3. Analysis of amplified DNA. Genomic DNA prepared from NIH 3T3 cell line R3a.2 (lanes a), NIH 3T3 cell line R8a.1 (lanes b), NIH 3T3 cells (lanes c), and plasmid pHiHi-3 containing v-Ki-*ras* (lane d) and amplified through 36 cycles. The DNA was resolved by electrophoresis in a native 10% acrylamide gel, stained with ethidium bromide, and photographed as shown.

the sequences derived from either v-Ki-*ras* or NIH 3T3 cells, which were unique in that a single sequence was found in each case. Analysis of cytosine- and thymidine-specific reactions did not reveal G·C to T·A mutations at the 12th codon (data not shown). In addition, no evidence was found of simultaneous amplification of the c-Ha-*ras* or N-*ras* genes derived from mouse genomic DNA, indicating that these genes did not act as alternative sites for primer annealing during amplification.

The fidelity of the procedure for amplifying this gene region was evaluated by comparison of sequences of plasmid DNA before and after amplification. Results indicated no detectable misreplication, within limits of sensitivity of the methodology used. As mentioned previously, the amount of 90-bp amplified DNA seemed to be relatively independent of the c-Ki-*ras* gene copy number present in the original template. However, mouse transformants containing amplified rat c-Ki-*ras* genes showed a distribution of rat-specific nucleotide changes (i.e., nucleotide position 24), which reflected the amount of rat gene in the transformant. This indicated that the original distribution of species-specific c-Ki-*ras* alleles in the template DNA is preserved during amplification and analysis by DNA sequencing. Therefore, the relative distribution of mutant c-Ki-*ras* alleles at positions 34 and 35 can reasonably be assumed to reflect the distribution of nucleotide changes present in the original template DNA.

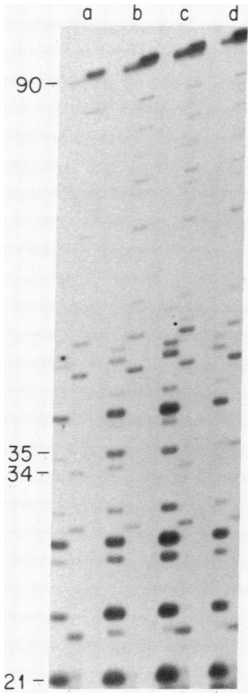

FIG. 4. Direct DNA sequencing of amplified DNA. DNA was enzymatically amplified from plasmid pHiHi-3 (lane a), NIH 3T3 cells (lane b), NIH 3T3 cell line R3a.2 (lane c), and NIH 3T3 cell line R8a.1 (lane d), annealed to a ^{32}P-labeled 5′ ATGACTGAGTATAA-AACTTGT 3′ OH primer, then incubated to reveal nucleotide-specific DNA breaks by the method of Sanger *et al.* (14). The left lane corresponds to a guanine-specific reaction and the right lane corresponds to an adenine-specific reaction. The DNA was analyzed by electrophoresis in a 10% acrylamide/urea gel and exposed to x-ray film. The resultant autoradiogram is shown. Nucleotide analysis ranged from position 21 to 90. Mutations in the 12th codon are shown at nucleotide positions 34 and 35.

DISCUSSION

These studies have demonstrated that mutations in the c-Ki-*ras* gene are associated with activation of the oncogene. The evidence for this derives from the presence of G·C to A·T base transitions in the first and second position of the 12th codon of the gene in DNA from transformants derived from aflatoxin-induced liver tumors. This is taken to indicate that the liver tumors themselves contained activated c-Ki-*ras* oncogenes. It is of interest to compare these results with those of other investigations that have addressed oncogene activation in chemically induced tumors. For instance, mutations have been shown to be present in activated c-Ha-*ras* oncogenes in MNU-induced rat mammary carcinomas (2), c-Ha-*ras* oncogenes of 7,12-dimethylbenz[*a*]anthracene-initiated mouse skin carcinomas (15), and in N-*ras* oncogenes of MNU-induced mouse thymic lymphomas (16). In general, studies such as these typically included a dosing schedule in which the tumors were induced by single dose or limited number of exposures. Analysis of NIH 3T3 transformants derived from tumor DNA indicated the presence of a single activated oncogene in a given tumor. For example, in MNU-induced mammary carcinomas (2), many separate transformants were shown to contain a single G·C to A·T base transition in the second position of the 12th codon. In contrast, the present results have demonstrated the presence of two alleles in the activated c-Ki-*ras* genes of two transformants derived from aflatoxin-induced liver tumors, suggesting that multiple c-Ki-*ras* oncogene alleles may have been present in a single liver tumor. We have, in fact, obtained preliminary evidence that both alleles were present in DNA isolated from a single tumor (unpublished data). However, because of the limited number of transformants that have been studied to date, we cannot make a general statement concerning this point. By application of the methodology described here, we are investigating the relative amounts of multiple alleles present in genomic DNA of individual tumors as well as in the tissues from which they are derived.

The presence of multiple alleles in the AFB_1-induced tumors raises important questions concerning the mechanisms of their induction. AFB_1 forms DNA adducts exclusively at the N^7 position of guanine after metabolic activation (3). The presence of the two mutations identified in these studies could result from multiple mutagenic effects occurring simultaneously from a single exposure to the carcinogen. On the other hand, because of the repeated dosing regimen used to induce the tumors, it is possible that they were produced as a consequence of multiple exposures. Both possibilities assume that the mutations were direct results of DNA modification by the carcinogen or its repair. It is also possible that the multiple genetic changes arose through as yet uncharacterized mechanisms related only indirectly to DNA damage in the course of tumorigenesis. We interpret our data as supporting the former hypothesis, since the only mutations identified thus far involve G·C to A·T base tran-

sitions, which would arise from adducts formed with guanine residues.

The combined amplification–direct sequencing methodology described here provides a rapid means of detecting and characterizing mutations in c-*ras* oncogenes. The method in its present form has been applied only to transformant DNA containing multiple copies of the mutant c-Ki-*ras* gene. The sensitivity of the approach for detection of mutant alleles in nonamplified genes has not yet been established, but preliminary experiments indicate that the sensitivity of the method is adequate to detect mutant alleles in DNA of primary liver tumors. In principle, the method should be applicable to any other genes or regions of DNA for which sequence information is available that permits the implementation of primer-directed enzymatic amplification.

Note Added in Proof. In addition to mutations in transformed NIH 3T3 cells described in this report, we have also detected both G·C to A·T base transitions and G·C to T·A base transversions in DNA isolated directly from tumors R3a and R8a, which had induced the transformants.

We thank Richard Cate (Biogen) for advice concerning oligonucleotide hybridization and Neal Cariello (Massachusetts Institute of Technology) for helpful discussions of the amplification procedure. Financial support for these studies was provided by National Institutes of Health Grant 2P01ES00597-16.

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