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Genetics

Characterization of c-Ki-*ras* and N-*ras* oncogenes in aflatoxin B_1-induced rat liver tumors

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ABSTRACT **c-Ki-*ras* and N-*ras* oncogenes have been characterized in aflatoxin B_1-induced hepatocellular carcinomas. Detection of different protooncogene and oncogene sequences and estimation of their frequency distribution were accomplished by polymerase chain reaction, cloning, and plaque screening methods. Two c-Ki-*ras* oncogene sequences were identified in DNA from liver tumors that contained nucleotide changes absent in DNA from livers of untreated control rats. Sequence changes involving G·C to T·A or G·C to A·T nucleotide substitutions in codon 12 were scored in three of eight tumor-bearing animals. Distributions of c-Ki-*ras* sequences in tumors and normal liver DNA indicated that the observed nucleotide changes were consistent with those expected to result from direct mutagenesis of the germ-line protooncogene by aflatoxin B_1. N-*ras* oncogene sequences were identified in DNA from two of eight tumors. Three N-*ras* gene regions were identified, one of which was shown to be associated with an oncogene containing a putative activating amino acid residing at codon 13. All three N-*ras* sequences, including the region detected in N-*ras* oncogenes, were present at similar frequencies in DNA samples from control livers as well as liver tumors. The presence of a potential germ-line oncogene may be related to the sensitivity of the Fischer rat strain to liver carcinogenesis by aflatoxin B_1 and other chemical carcinogens.**

The carcinogenic properties of the aflatoxins have been extensively studied, and much information has been produced concerning various aspects of their mechanisms of action, occurrence in foods, and possible involvement as human cancer risk factors (1). Aflatoxin B_1 (AFB_1), in particular, has been shown to be a powerful carcinogen for the liver of many experimental animals, including the rat (2). Studies of the metabolism of AFB_1 have revealed activation to its electrophilic DNA binding form through an epoxidation pathway (3). Furthermore, activation and DNA binding of AFB_1 produces identical DNA-chemical adduct spectra in both man and rat including the major DNA adduct form, 2,3-dihydro-2-(N^7-guanyl)-3-hydroxy-AFB_1 (4).

Previous work by many other investigators has suggested (5) that the activation of c-*ras* genes by specific single-base mutations may be one of several steps involved in the transformation of normal cells to malignancy. In addition, studies of *in vitro* mutagenesis of c-*ras* protooncogenes have shown that many mutations within codons 12, 13, 59, and 61 of the c-Ha-*ras* and N-*ras* genes may lead to oncoproteins with similar properties with respect to their potential to transform NIH 3T3 cells. In contrast, c-*ras* mutations that arise in DNA of tumors of animals after exposure to specific chemicals often result in a more restricted set of nucleotide substitutions at these positions. This narrower range of mutations could be related to characteristics of binding of chemicals to DNA and to subsequent DNA repair of specific lesions. For instance, it has been suggested that differences in the spectra of mutations in oncogenes from mammary carcinomas induced in rats by methylnitrosourea and dimethylbenz[*a*]anthracene may be attributed to specific characteristics of carcinogen–DNA binding and repair of the resulting lesions (6).

Previous studies have indicated that G·C base pairs are the only sites of AFB_1-DNA adduct formation (4) and are also the primary sites for AFB_1-induced mutagenesis (7). Other studies have shown that the binding of AFB_1 to guanine residues takes place in a nonrandom manner, suggesting influences of DNA sequence context on the binding, and possibly repair of the primary N^7-guanine DNA lesion in rat liver (8). In addition, G+C-rich regions in the exons and flanking DNA regions of c-*ras* genes have been hypothesized as preferred sites of damage by alkylating agents such as AFB_1 (9). This study was designed to test the hypothesis that metabolism of AFB_1 to DNA-binding forms could result in somatic mutations in DNA at sites that cause activation of c-*ras* protooncogenes. This conjecture would further predict that activating mutations in oncogenes should take place at G·C base pairs if mechanisms of direct mutagenesis and selection of mutant genes occur during tumor development. Detection and quantification of c-*ras* oncogene sequences in primary liver tumors induced by AFB_1 would thus provide experimental evidence for the postulated DNA binding and mutagenic specificity of this compound within this set of genes that may be genetically altered during the liver cancer process.

METHODS

Animals and AFB_1 Treatment. To induce liver tumors, weanling male Fischer rats received daily intraperitoneal injections of 25 μg of AFB_1 (Makor Chemicals, Jerusalem) 5 days/week for 8 weeks. When tumors appeared after 12–18 months, animals were killed, and liver tumors were excised and separated from surrounding tissue to the extent possible. Portions of tissues were also fixed in buffered formalin for histopathologic examination.

Isolation of DNA and Transfections. Liver tumors or cell pellets were disrupted by manual homogenization 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% SDS and digested overnight with proteinase K, and DNA was purified by repeated phenol/chloroform extraction. DNA was precipitated with ethanol, washed with 70% ethanol, and resuspended in 10 mM Tris·HCl (pH 8.0) plus 1 mM EDTA. DNA transfections and the focus formation assay were performed as described (10). For nude mouse tumorigenicity assays, 30 μg of rat tumor DNA and 300 ng of the plasmid pSV2neo were transfected into a single dish containing 7.5×10^5 NIH 3T3 cells and the tumor formation assay in athymic mice was performed by the method of Fasano *et al.* (11).

Detection of Oncogenes. DNA (20 μg) isolated as described above from NIH 3T3 foci or nude mouse tumors was digested

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Abbreviations: AFB_1, aflatoxin B_1; PCR, polymerase chain reaction.
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with *Hin*dIII or *Eco*RI, electrophoresed in a 0.8% agarose gel, and blotted to GeneScreen*Plus* membrane (New England Nuclear) as described (10). The membrane was hybridized to a random-primer radiolabeled (12) 380-base-pair Sst II–Xba I restriction fragment derived from a v-Ki-*ras*-containing plasmid pHiHi-3 (13) or a 1.0-kilobase *Eco*RI restriction fragment derived from a human N-*ras*-containing plasmid p52C$^-$ (14).

Polymerase Chain Reaction (PCR) Cloning and DNA Sequencing. DNA amplification was performed by PCR using 2.5 units of *Thermus aquaticus* polymerase (Perkin–Elmer/Cetus) in a 100-μl reaction mixture containing 1 μg of rat liver or NIH 3T3 transformant DNA, 0.2 mM dGTP, 0.2 mM dATP, 0.2 mM TTP, 0.2 mM dCTP, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris·HCl (pH 8.3), and 0.5 μg each of primers (15). The primers used for the amplification of c-Ki-*ras* and N-*ras* genes delineated codons 8–23 and 9–30, respectively. The primer sets used for PCR of c-Ki-*ras* were BK12 (5′-CG*GATCCG*ATGACTGAGTATAAACTTGT-3′) and EK12 (5′-GG*AATTCC*ATCCACAAAGTGATTCTGAA-3′). Those used for N-*ras* were BN12 (5′-CG*GATCCG*GACTGAGTACAAACTGGTGG-3′) and EN12 (5′-GG*AATTCC*TCTATGGTGGGATCATATT-3′). Both primer sets were derived from published DNA sequence information pertaining to the rat c-Ki-*ras* (16) and mouse N-*ras* genes (17). Primer sets contained nucleotides specifying either an *Eco*RI or *Bam*HI linker for unidirectional cloning (italic). The reaction was overlain with 100 μl of mineral oil (Sigma) and subjected to a cycle consisting of a 1-min step at 94°C, followed by a 2-min annealing step at 37°C, and followed by a 4-min polymerization step at 72°C. Amplification reactions were performed for 40 cycles using a Perkin–Elmer/Cetus thermal cycler. The reaction was extracted once with chloroform and the aqueous phase was precipitated with 70% ethanol. The precipitate was electrophoresed in a 10% native polyacrylamide gel, stained with ethidium bromide, and photographed. DNA bands of 106 (c-Ki-*ras*) or 125 (N-*ras*) base pairs were cut from gels and eluted overnight by diffusion in a buffer containing 0.5 M ammonium acetate and 1 mM EDTA. Eluted DNA was precipitated with ethanol and resuspended in 10 mM Tris·HCl (pH 8.0) plus 1 mM EDTA. Yields ranged from 100 to 500 ng of DNA from each PCR amplification. Aliquots containing 100–200 ng of DNA were digested with *Eco*RI and *Bam*HI and ligated into the multiple cloning region of M13mp18. Single-strand phage DNA was prepared from individual white plaques after transformation of *Escherichia coli* JM101. DNA was sequenced using modified T7 polymerase (Sequenase), deoxycytidine 5′-[α-[^{35}S]-thio]triphosphate (500 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear), and the nucleotide-specific termination buffers supplied in the Sequenase kit (United States Biochemicals). The reaction was stopped by the addition of 4.0 μl of formamide/dye mixture and then heated to 100°C for 2 min. The samples were electrophoresed in an 8% polyacrylamide gel with urea, fixed, dried, and exposed to x-ray film without an intensifier screen.

Plaque Screening Assay. Plates (150 mm) containing ≈100 recombinant plaques were transferred to membrane filters (New England Nuclear) that had been soaked in a logarithmic-phase culture of *E. coli* strain JM101. The membrane was air-dried and incubated overnight at 37°C in a 150-mm plate containing bacterial agar to amplify plaques prior to immobilization of DNA. The membranes were then treated with a solution containing 0.2 M NaOH and 1.0% SDS for 20 min and then with 1 M Tris·HCl (pH 7.5) for 20 min. The membrane filters were air-dried at room temperature and then prehybridized in a solution containing 3 M tetramethylammonium chloride, 50 mM Tris·HCl (pH 8.0), 2.0 mM EDTA, salmon sperm DNA (100 μg/ml), 0.1% SDS, and 5× Denhardt's solution (1× Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone) for 18 hr at 56°C. The prehybridized membranes were hybridized in the same buffer containing 20 pmol of ^{32}P-radiolabeled oligomers (>10^9 dpm/μg) at 56°C for 2 hr. The membrane filters were successively washed in 2× SSPE (1× SSPE = 0.15 M NaCl/0.01 M sodium phosphate, pH 7.0/1 mM EDTA)/0.1% SDS at room temperature for 20 min; 5× SSPE/0.1% SDS at 63°C for 5 min; prehybridization solution (without Denhardt's solution or salmon sperm DNA) for 20 min at room temperature; and finally in the same modified prehybridization solution at 60°C for 1 hr. The membranes were air-dried, exposed to x-ray film with an intensifier screen, and the resultant autoradiograms were developed. Filters were used for further hybridizations after treatment of the membranes at 42°C for 1 hr in 0.5 M NaOH and then treatment at 42°C for 30 min in a solution containing 0.2 M Tris·HCl (pH 7.5), 0.1× SSC, and 0.1% SDS (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0). The frequencies were estimated by enumerating the fraction of positive plaques hybridizing to a given oligomer in relation to the total number of recombinant plaques containing cloned PCR product. The 20-mer oligonucleotide probes for detection of recombinant plaques containing cloned PCR product were GGT(K12) (5′-CAACCTCG*ACCA*CCGCATCC-3′), TGT(K12) (5′-CAACCTCG*AACA*CCGCATCC-3′), GAT(K12) (5′-CAACCTCG*ACTA*CCGCATCC-3′), GTT(N14) (5′-TCGTCCACCG*CAA*CCCTTTT-3′), ATT(N14) (5′-TCGTCCACCG*TAA*CCCTTTT-3′), and GTT(N13) (5′-TCGTCC*ACAA*CAACCCTTTT-3′).

RESULTS

The induction of liver tumors in rats by AFB_1 is a useful experimental model for the study of several aspects of chemical carcinogenesis in the liver, including its metabolism (1), formation of AFB_1-DNA adducts (18), and changes in hepatocellular cytology after AFB_1 exposure (2). In this present study, induction of liver tumors was accomplished in Fischer rats by repeated intraperitoneal injection of AFB_1 during a 2-month period, starting at 3 weeks of age. As anticipated by results of our earlier studies, all nine AFB_1-treated animals developed overt liver tumors between 1 and 2 years after AFB_1 exposure. Also consistent with earlier findings, no control animals developed liver tumors or other histopathologic lesions. Histological examination of the liver tumors indicated that all were carcinomas containing liver parenchymal cells and exhibiting various degrees of trabecular cord formation, glandular character, cystic degeneration, or liver necrosis. Livers of animals treated with the vehicle alone showed no abnormalities in morphology or hepatocellular histology.

In the initial phase of the study, DNA was isolated from excised hepatocellular carcinomas, whenever possible from single gross tumors that could be dissected free of surrounding tissue. Most samples, however, had to be obtained from gross lesions that lacked clear definition or encapsulation and, therefore, may have included nontumor as well as tumor cells. For detection of oncogenes, DNA was transfected into NIH 3T3 mouse fibroblasts and assayed for its potential to induce transformed foci and the formation of subcutaneous tumors after injection of transfected cells into athymic nude mice. DNA was prepared from morphologically transformed foci or subcutaneous nude mouse tumors and analyzed by Southern hybridization for the presence of c-*ras* genes of rat origin. In a total of nine liver tumors analyzed, two samples (R3 and R8) indicated the presence of c-Ki-*ras* oncogenes and an additional two samples (R4 and R10) showed the presence of N-*ras* oncogenes by these assays (Fig. 1). The detection of these two classes of genes confirms and extends our own previous work (10) as well as that of Sinha *et al.* (19).

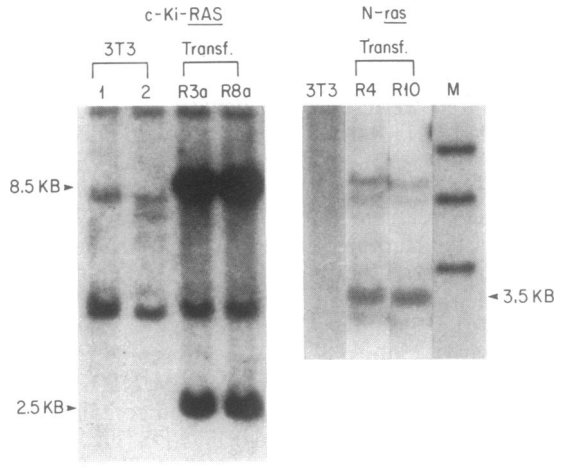

FIG. 1. Detection of oncogenes in NIH 3T3 transformants derived from AFB_1-induced hepatocellular carcinomas. DNA was prepared from nude mouse tumors derived by transfection of AFB_1-induced liver tumor DNA. Control NIH 3T3 and transformant DNA samples (20 μg) were digested with either *Hin*dIII (*Left*) or *Eco*RI (*Right*), electrophoresed, blotted, and hybridized to ^{32}P-radiolabeled DNA probes specific for c-Ki-*ras* or N-*ras* genes. After hybridization, the membrane was washed to a final stringency in 0.1× SSC/1.0% SDS at 65°C and exposed to x-ray film with an intensifier screen. Arrows indicate the presence of rat c-Ki-*ras* (*Left*) and rat N-*ras* (*Right*) oncogenes in the NIH 3T3 transformant DNA. Lane M contains radiolabeled *Hin*dIII-digested λ DNA molecular size markers. 3T3 lanes 1 and 2 show restriction fragments containing a mouse c-Ki-*ras* protooncogene.

The apparent absence of oncogenes identifiable by these assays in the remaining liver tumors prompted us to undertake a more detailed analysis of DNA sequence changes in the c-Ki-*ras* and N-*ras* genetic loci of tumors and control rat livers by using PCR DNA amplification. Genetic analysis of DNA from primary tumors would circumvent negative findings attributable to lack of productive recombinations during the DNA transfection procedure with apparent lack of mutated oncogenes in some tumor samples. PCR DNA amplification procedures were, therefore, used to survey genetic changes in the vicinity of codon 12 of the c-Ki-*ras* gene because previous studies in our own laboratory (20) and another (19) indicated that region as a potential site of AFB_1-induced mutagenesis in the tumor DNA. To the best of our knowledge, no prior information was available concerning N-*ras* sequences in the rat. Using primer sets delineating regions of the first exon, PCR amplification of DNA was performed (15). Sequence information for construction of the primer sets was obtained from the rat c-Ki-*ras* gene (16) or mouse N-*ras* gene (17). The design of primer sets included a restriction enzyme linker region for unidirectional cloning of the PCR-generated DNA fragment into M13 phage. DNA from individual recombinant phage were isolated and sequenced using dideoxynucleotide chain-termination methods. In addition, plaques were transferred to membranes and hybridized with radiolabeled probes specific for individual alleles by using a plaque screening assay.

A compilation of the types of c-Ki-*ras* and N-*ras* gene sequences detected in the Fischer rat liver is shown in Fig. 2. Analyses of the c-Ki-*ras* gene region from control rat liver revealed a DNA sequence identical to that previously published for the rat gene. Analysis of >200 independent phages derived from control rat livers produced identical DNA sequences and validated the PCR cloning methodology as a means of detecting DNA sequence changes within this gene region. Additional control experiments using cloned PCR-amplified DNA derived from plasmids containing genes of known sequence indicated no DNA regions within the c-Ki-*ras* or N-*ras* first exons that contained sequence artifacts resulting from the PCR DNA amplification or subsequent cloning procedures. In addition, multiple and independent PCR reactions were performed by several investigators using separate reagents, and multiple phages were isolated and sequenced from a given PCR DNA preparation to arrive at an invariate consensus sequence. Two c-Ki-*ras* oncogene sequences were detected in DNA derived from three of eight AFB_1-induced liver tumors (R3, R8, and R9). In PCR DNA from primary liver tumors as well as NIH 3T3 transformant cells derived from them, identical gene regions were found to contain single nucleotide changes (G·C to T·A or A·T base pairs) in codon 12. These mutations resulted in single amino acid substitutions at this site (cysteine or aspartate for

SAMPLE	CODON 8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Human K-ras2	GTA	GTT	GGA	GCT	GGT	GGC	GTA	GGC	AAG	AGT	GCC	TTG	ACG	ATA	CAG
Mouse K-ras	GTG	GTT	GGA	GCT	GGT	GGC	GTA	GGC	AAG	AGC	GCC	TTG	ACG	ATA	CAG
Rat K-rasA	GTA	GTT	GGA	GCT	GGT	GGC	GTA	GGC	AAG	AGT	GCC	TTG	ACG	ATA	CAG
	Val	Val	Gly	Ala	Gly	Gly	Val	Gly	Lys	Ser	Ala	Leu	Thr	Ile	Gln
Rat K-rasB ONC					TGT										
					Cys										
Rat K-rasC ONC					GAT										
					Asp										
Human N-ras	GTG	GTT	GGA	GCA	GGT	GGT	GTT	GGG	AAA	AGC	GCA	CTG	ACA	ATC	CAG
Mouse N-ras	GTG	GTT	GGA	GCA	GGT	GGT	GTT	GGG	AAA	AGC	GCC	CTG	ACG	ATC	CAG
Rat N-rasA	GTG	GTT	GGA	GCA	GGT	GGC	GTT	GGG	AAA	AGT	GCT	TTG	ACA	ATC	CAG
	Val	Val	Gly	Ala	Gly	Gly	Val	Gly	Lys	Ser	Ala	Leu	Thr	Ile	Gln
Rat N-rasB							ATT								
							Ile								
Rat N-rasC ONC	GTA					GTT					GTT				
	Val					Val					Val				

FIG. 2. Sequence and amino acid comparison of c-K-*ras* and N-*ras* gene regions from exon 1. A comparison of c-Ki-*ras* and N-*ras* sequences is illustrated and compared to sequences for human c-Ki-*ras*, mouse c-Ki-*ras*, rat c-Ki-*ras*, human N-*ras*, and mouse N-*ras* genes. ONC, presence of this gene in liver tumor-derived NIH 3T3 cells and detection of the gene region by PCR analysis. Codons containing putative activating mutations resulting in amino acid substitutions are indicated by boldface type.

Table 1. Frequency of various c-Ki-*ras* and N-*ras* gene regions as determined by a plaque screening assay of cloned PCR-amplified DNA derived from rat livers

Sample	Frequency, no. positive plaques/no. total plaques			Sample	Frequency, no. positive plaques/no. total plaques		
	Ki-*rasA**	Ki-*rasB*	Ki-*rasC*		N-*rasA*	N-*rasB*	N-*rasC*
CR5	148/148	0/17	0/131	CR5	4/16	4/16	8/16
R3	348/377	29/377		R4	10/51	28/51	13/51
R8	74/123		49/123	R5	11/33	13/33	9/33
R9	615/632		17/632	R10	26/60	25/60	9/60

*See Fig. 2.

glycine). The absence of such mutations in DNA of livers from control rats suggested that the c-Ki-*ras* sequences detected in the primary tumors reflected changes resulting from treatment of the rats with AFB_1. Quantitative estimates of the frequencies at which these sequences were present in different DNA samples, calculated with respect to the total number of phage analyzed, ranged from 8% (R9; 3 of 36 clones) to 24% (R8; 24 of 100 clones). Results of the plaque screening assay confirmed and extended data on the presence and distribution of mutated c-Ki-*ras* sequences in the same three tumors and also indicated frequency distributions comparable to those detected by the PCR-cloning procedures (Table 1). Using the latter assay, a larger number of recombinant plaques could be surveyed with relative ease when compared to the DNA sequencing procedure and substantially improved the accuracy with which the frequency of different sequences in DNA preparations could be estimated. In addition, mutated gene regions could be distinguished from germ-line regions using the same DNA-containing membrane. The presence of either of these two sequences as determined by either methodology was confirmed by sequence analysis of PCR product derived from tumor-derived NIH 3T3 transformants containing c-Ki-*ras* oncogenes.

Analysis of the corresponding N-*ras* gene regions in AFB_1-induced liver tumors and control livers revealed several important differences from the findings on c-Ki-*ras*. First, three N-*ras* gene regions were detected that contained different nucleotides in codons 8, 13, 14, and 18 (Fig. 3). The N-*rasA* sequence was found to be homologous to mouse and human N-*ras* genes and no divergence in amino acid sequence was found for this gene region (Fig. 2). The N-*rasB* sequence differed from N-*rasA* only in a single nucleotide change (G·C to A·T) in codon 14 that resulted in an amino acid substitution (isoleucine for valine). The N-*rasC* sequence, when compared to N-*rasA*, contained four nucleotide differences (G·C to A·T in codon 8; G·C to T·A and C·G to T·A in codon 13; and C·G to T·A in codon 18). These differences resulted in two amino acid substitutions, at codon 13 (valine for glycine) and codon 18 (valine for alanine).

It is important to note that the N-*rasC* sequence was detected in DNA from two primary tumors (R4 and R10) as well as in DNA of nude mouse tumors derived from cells transformed by them. Thus, this gene region is associated with properties ascribed to oncogenes (Table 2). An unanticipated finding was the presence of this same sequence in DNA from all control livers as well as tumors, even though no control liver DNA gave positive results in the NIH 3T3 transformation or nude mouse assays. In addition, the frequency distribution of N-*rasA*, N-*rasB*, and N-*rasC* gene regions in both AFB_1-induced liver tumors and normal rat livers was strikingly similar. The presence of a valine for glycine substitution at codon 13 is consistent with oncogenic-activating mutations of the N-*ras* gene observed in other systems (21, 22). This unexpected finding, confirmed by DNA sequencing of many independently derived representative clones, the plaque screening assay, and many successive experiments, suggests that the N-*rasC* gene region is of germ-line origin in the Fischer rat.

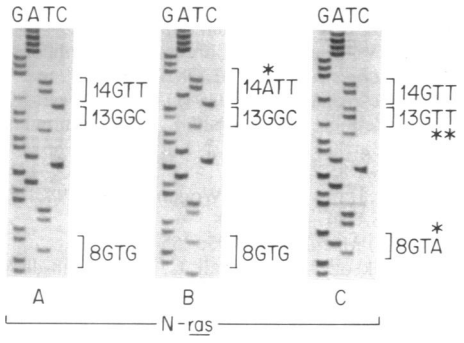

FIG. 3. DNA sequence analysis of rat N-*ras* gene regions. Three M13 phage containing rat N-*rasA* (lanes A), N-*rasB* (lanes B), and N-*rasC* (lanes C) genes were sequenced by dideoxynucleotide chain-termination methods. Arrows denote nucleotide differences within codons 8, 13, and 14 for each of the respective alleles.

DISCUSSION

Our previous studies identified c-Ki-*ras* oncogenes in NIH 3T3 fibroblasts derived from AFB_1-induced liver tumors and characterized mutations in codon 12 as the putative mechanism of activation (20). Sinha *et al.* (19) also identified c-Ki-*ras* and N-*ras* oncogenes in transformants derived from liver tumors of AFB_1-fed rats but did not report sites of activation. Findings reported here confirm and substantially extend these earlier results. Cloning of PCR-amplified DNA followed by sequencing or oligonucleotide hybridization revealed sequence differences in codon 12 of c-Ki-*ras* in DNA from three of nine primary liver tumors. These same gene regions were detected in PCR-amplified DNA from NIH 3T3 transformants derived from two of these tumors. Analyses failed to detect any differences in comparison to the protooncogene sequence with respect to codon 12 of c-Ki-*ras* in

Table 2. Activating mutations in exon 1 of c-Ki-*ras* and N-*ras* oncogenes from AFB_1-induced hepatocellular carcinomas and normal livers of Fischer rats

Gene region	Incidence		Oncogene	Codon	Putative mutation
	Control livers	Liver tumors			
Ki-*rasA*	3/3	8/8	−	—	—
Ki-*rasB*	0/3	1/8	+	12	GGT → TGT
Ki-*rasC*	0/3	2/8	+	12	GGT → GAT
N-*rasA*	3/3	5/5	−	—	—
N-*rasB*	3/3	5/5	−	—	—
N-*rasC*	3/3	5/5	+	13	GGC → GTT

Oncogene was detected by focus formation or nude mouse tumorigenicity assays. +, Oncogene present; −, oncogene absent. Incidence is the number of positive plaques divided by the total number of plaques.

the remaining six tumors. The observed sequence differences consisted of single-nucleotide changes at either of two G·C base pairs in codon 12, both resulting in amino acid changes at this site. This finding plus extensive evidence indicating the preferential binding of AFB_1 to guanine residues (3, 4) strongly suggest that both c-Ki-*ras* oncogenes could have resulted from direct mutagenesis of the germ-line protooncogene.

The distribution of various c-Ki-*ras* sequences containing changes in codon 12 was highly variable in different liver tumors. Two samples containing the highest frequency of c-Ki-*ras* gene regions (R3 and R8) also scored positive in the DNA transfection assays. Another sample contained a low, but detectable, frequency of the same oncogene-containing region (R9) and scored negative in these assays. These data represent estimates of the frequency of particular c-Ki-*ras* or N-*ras* gene regions in PCR-amplified DNA derived from individual liver DNA preparations. However, these estimates would not necessarily reflect accurately the distribution of these gene regions within a clonal population of hepatocytes. Several important sources of heterogeneity can be identified that could contribute to the observed variability in the frequencies of c-Ki-*ras* gene regions, such as the simultaneous presence of multiple liver cell types, heteroploidy of hepatocytes, and the presence of transformed hepatocytes arising through multiple independent genetic events. In the latter regard, histological examination of the hepatocellular carcinomas did not reveal obvious enrichment of any particular cell types. In addition, the absence of detectable c-Ki-*ras* oncogenes in most of the samples as well as the substantiation of this finding by extensive DNA sequence analysis of the remaining tumors suggest that either the genetically altered cells were absent or were greatly underrepresented in the liver cells from which the DNA was prepared.

N-*ras* oncogenes were identified in two of nine tumors after transfection of liver tumor DNA and production of subcutaneous tumors in nude mice. PCR DNA amplification and sequence analysis of cloned PCR-amplified DNA from the subcutaneous tumors confirmed the presence of a rat N-*ras* gene. Genetic analysis of a gene region corresponding to the oncogene revealed sequence changes resulting in amino acid differences at codons 13 and 18, compared to mouse and human DNA sequences. Comparison of these changes to amino acid substitutions previously associated with oncogene activation implicates the glycine to valine substitution at codon 13 as the putative site of activation. Unexpectedly, however, DNA sequence analysis of this same gene region in PCR-amplified DNA derived from livers of control as well as tumor-bearing animals revealed the presence of three N-*ras* gene regions including that detected in the oncogenes of subcutaneous mouse tumors.

The consistent and unequivocal detection of all three N-*ras* gene regions in livers of all Fischer rats suggests that these sequences are present in the germ line. The finding of a single rat-derived N-*ras* oncogene in NIH 3T3 cells transformed by liver tumor DNA further suggests that such a gene may be potentially oncogenic in this rat strain. However, it is unclear whether the limited gene region detected in livers of control and tumor-bearing rats would necessarily signify the presence of oncogene sequences fully capable of expressing the oncoprotein detected in NIH 3T3 transformants. The lack of induction of nude mouse tumors in transfection assays of control rat liver DNA may reflect this inability. Genetic alterations at sites other than the region we have analyzed could be required for activation of the germ-line N-*ras* gene harboring the potential to express an oncogenic protein. Alternatively, the activated oncogene sequence could be unexpressed in normal animals but could be expressed as a result of carcinogen treatment. In addition, AFB_1-induced cytotoxicity and resultant hepatocyte regeneration could result in the selective growth of cells containing transcriptionally active germ-line oncogenes, ensuring further genetic events sufficient for malignant transformation. The possible mechanistic significance of these factors, as well as others not yet identified, in tumor induction by AFB_1 cannot be assessed on the basis of present evidence. Fischer rats are particularly sensitive, compared to other rat strains, with respect to induction of liver tumors by AFB_1 as well as other chemical carcinogens (1). Findings reported here suggest that the presence of a potential N-*ras* oncogene in the germ line of animals of this strain could contribute to this sensitivity.

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