The *ras* Oncogenes in Aflatoxin B_1-Induced Rat Liver Carcinomas

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INTRODUCTION

The carcinogenic properties of the aflatoxins have been very extensively studided, and much information has been produced concerning various aspects of their mechanisms of action, occurrence in foods, and their possible involvement as human cancer risk factors (for review, see Busby and Wogan (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). In this model, studies of the metabolism of AFB_1 have revealed activation of this compound 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 a major DNA adduct form, 2,3-dihydro-2-(N^7-guanyl)-3-hydroxy-AFB_1 (4).

Previous work by many other investigators has suggested that the activation of c-*ras* genes by specific single-base mutations may be one of several steps involved in the transformation of a normal cell to malignancy (5,6). In addition, studies of *in vitro* mutagenesis of c-*ras* proto-oncogenes have shown that many different mutations within the 12th, 13th, 59th and 61st codons of the c-Ha-*ras* (7-9) and N-*ras* (10) genes lead to oncoproteins with similar properties with respect to their potential to transform NIH3T3 cells. In contrast, c-*ras* mutations that arise in DNA of tumors of animals after exposure to chemical mutagens often result in a very restricted set of nucleotide substitutions at these positions. This narrower range of mutations could be related to characteristics of binding of chemicals to DNA, together with subsequent DNA repair of specific lesions (11). For instance, it has been suggested that differences in the spectra of mutations in oncogenes from mammary carcinomas induced in rats by methylnitrosourea or dimethylbenz[a]anthracene may be attributed to characteristics of carcinogen-DNA binding and repair of these lesions (12).

Previous studies have indicated that G-C base pairs are sites of AFB_1-DNA adduct formation (4) and are also the primary sites for AFB_1-induced mutagenesis (13). Other studies have indicated that the binding of AFB_1 to guanine residue occurs in a non-random manner suggesting influences of DNA sequence context on the binding, and possibly repair of the primary N^7-guanine DNA lesion in rat liver (14). In addition, G-C-rich regions in the exons and flanking DNA regions of c-*ras* genes have been hypothesized as preferential sites of damage by alkylating agents such as AFB_1 (15). 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* proto-oncogenes. 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 quantitation of c-*ras* oncogene sequences in primary liver tumors induced by AFB_1 would thus provide experimental evidence for the postulated

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DNA binding and mutagenic specificity of this compound within this set of genes that may be genetically altered during the liver cancer process.

Detection and quantitation of c-K-*ras* and N-*ras* oncogene sequences in hepatocellular carcinomas of Fischer rats exposed to AFB_1 are described. The initial phase of the study involved detection of oncogenes through DNA-mediated transfer methods followed by selection of transformed mouse fibroblasts. The second phase utilized polymerase chain reaction (PCR) DNA amplification methodology to analyze DNA from primary liver tumors and control livers for mutated oncogene sequences. Genetic analysis of the tumors indicated that two c-K-*ras* oncogenes contained gene regions indicating a single amino acid change at the site of the 12th codon. In addition, an N-*ras* oncogene contained a gene region indicating a single amino acid difference at the site of the 13th codon. The relationship of these amino acid differences to oncogene activation, proto-oncogene mutagenesis, and models of AFB_1-DNA interaction are discussed.

METHODS

Animals and AFB_1 treatment

To induce liver tumors, male Fischer rats received daily intraperitoneal injections of 25 μg of AFB_1, 5 days/week for 8 weeks. Tumors appeared after 12 to 18 months, after which time animals were killed, liver tumors excised, and separated from surrounding tissue to the extent possible. Portions of the 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% sodium dodecyl sulfate, digested overnight with proteinase K, and DNA was purified by repeated phenol-chloroform extraction, precipitated with ethanol, washed with 70% ethanol, and resuspended in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. For DNA transfections, NIH3T3 mouse fibroblasts were maintained at low cell density in Dulbecco's modified essential medium (DMEM) containing 10% calf serum. Transfection of tumor-derived genomic DNA into NIH3T3 mouse fibroblasts was performed using calcium phosphate coprecipitation, after which the transfection medium was replaced with DMEM containing 5% calf serum. Foci were scored and subcloned between 2 and 3 weeks after transfection. DNA was isolated from clones derived from individual transformants. 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 NIH3T3 cells according to the method of Fasano *et al.* (16). Eight hours after transfection, the medium was replaced with 10 m*l* of DMEM with 10% calf serum. Six hours thereafter, the cells were diluted 1:4 in DMEM with 10% calf serum containing 400 μg/m*l* G418 (Gibco). Medium was replaced twice per week for 18-21 days. At this time, cells from four plates, each containing approximately 200 $G418^R$ clones, were trypsinized, pooled, and centrifuged at $500 \times g$ for 5 min. Cell pellets, containing approximately $2\text{-}4 \times 10^6$ cells, were resuspended in 0.4 m*l* of DMEM with 10% calf serum and injected into 2-4 sites in the scapular and hind flank regions of each athymic nude mouse (Charles River Breeding Laboratories). Animals were marked and monitored for grossly-visible tumor growth over a 10-week period following injection of the cells. Control animals were injected with NIH3T3 cells or normal rat liver DNA.

Detection of oncogenes

DNA (20 μg) isolated as described above from NIH3T3 foci or nude mouse tumors was restricted with *Hind*III or *Eco*RI, electrophoresed in a 0.8% agarose gel, and bloted to Genescreen Plus membrane (New England Nuclear) as described (17). The membrane was hybridized to a random-primer

radiolabeled (18) 380 bp Sst2-*Xba*I restriction fragment derived from a v-K-*ras*-containing plasmid pHiHi-3 (19) or 1.0 kb *Eco*RI restriction fragment derived from a human N-*ras*-containing plasmid p52C$^-$ (20). The hybridization was performed overnight at 65°C in 1 M NaCl, 10% dextran sulfate, 1.0% BSA, 1.0% SDS, and 0.2 mg/m*l* denatured salmon sperm DNA. The membrane was washed to final stringency in a buffer containing 0.1 X SSC (1X = 0.15 M NaCl and 0.015 M sodium citrate) at 65°C and exposed to X-ray film with an intensifier screen.

PCR-cloning and DNA sequencing

DNA amplification was performed by PCR using 2.5 units of *Taq* polymerase (Perkin-Elmer Cetus) in a 100 μl reaction containing 1 μg of rat liver or NIH3T3 transformant DNA, 1.0 μM dGTP, 1.0 μM dATP, 1.0 μM TTP, 1.0 μM dCTP, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), and 0.1 pmoles of primers (21). The primers used for the amplification of both c-K-*ras* and N-*ras* genes delineated codons 8-23 and 9-30, respectively. The primer sets used for PCR of c-K-*ras* were:

BK12: 5'CGGATCCGATGACTGAGTATAAACTTGT3'
EK12: 5'GGAATTCCATCCACAAAGTGATTCTGAA3'
Those used for N-*ras* were:
BN12: 5'CGGATCCGGACTGAGTACAAACTGGTGG3'
EN12: 5'GGAATTCCCTCTATGGTGGGATCATATT3'

Both primer sets were derived from published DNA sequence information pertaining to the rat c-K-*ras* (22) and mouse N-*ras* genes (23). Primer sets contained nucleotides specifying either an *Eco*RI or *Bam*HI linker for unidirectional cloning The reaction was overlain with 100 μl of mineral oil (Sigma) and subjected to a cycle consisting of a 1 min denaturation step at 94°C, followed by a 2 min annealing step at 37°C, and followed by a polymerization step for 4 min 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 bp (c-K-*ras*) or 125 bp (N-*ras*) 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) and 1 mM EDTA. Yields ranged 100 – 500 ng of PCR product from each amplification. Alquots containing 100 – 200 ng of the PCR product were restricted with *Eco*RI and *Bam*HI and ligated into the multiple cloning site of M13mp18. Single-strand phage DNA was prepared from individual white plaques after transformation of *E. coli* JM101. DNA was sequenced using modified T7 polymerase (Sequence), α -[^{35}S] dCTP (500 Ci/mmole) (New England Nuclear) and the nucleotide-specific termination buffers supplied in the Sequenase kit (US Biochemicals). The reaction was stopped by the addition of 4.0 μl of formamide-dye mix and then heated to 100°C for 2 min. The samples were electrophoresed in an 8% urea sequencing gel, fixed, dried and exposed to X-ray film without an intensifier screen.

Plaque screening assay

Plates (150 mm) containing approximately 100 recombinant plaques were transferred to membrane filters that had been previously soaked in a log phase culture of *E. coli* JM101. The membrane was air-dried and incubated overnight at 37°C on 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 followed by a 20 min treatment in 1 M Tris-HCl (pH 7.5). The membrane filters were air-dried at room temperature and then prehybridized in a solution containing 3 M tetramethylammonium choloride, 50 mM Tris-HCl (pH 8.0), 2.0 mM EDTA, 100 g/m*l* salmon sperm DNA, 0.1% SDS, and 5X Denhardts solution (1X Denhardts = 0.02% bovine serum albumin, 0.02% ficoll and 0.02% polyvinylpyrrolidone) for 18 hrs at 56°C. The prehybridized membranes were

incubated in the same buffer containing 20 pmoles of [^{32}P]-radiolabeled oligomers (>10^9 dpm/μg) at 56 °C for 2 hrs. The membrane filters were successively washed in 2XSSPE (1XSSPE = 0.15 M NaCl, 0.01 M sodium phosphate, and 1 mM EDTA) and 0.1% SDS at room temperature for 20 min; 5XSSPE and 0.1% SDS at 63°C for 5 min prehybridization solution (without Denhardts or salmon sperm DNA) for 20 min at room temperature; and finally in the modified prehybridization solution 60°C for 1 hr. The membranes were air-dried, exposed to X-ray film with an intensifier screen, and the resultant autoradiograms developed. Filters were used for further hybridizations after treatment of the membranes at 42°C for 1 hr in 0.5 M NaOH followed by treatment at 42°C for 30 min. in a solution containing 0.2 M Tris-HCl (pH 7.5), 0.1XSSC, and 0.1% SDS. 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'CAACCTCGACCACCGCATCC3'
TGT(K12): 5'CAACCTCGAACACCGCATCC3'
GAT(K12): 5'CAACCTCGACTACCGCATCC3'
GTT(N14): 5'TCGTCCACCGCAACCCTTTT3'
ATT(N14): 5'TCGTCCACCGTAACCCTTTT3'
GTT(N13): 5'TCGTCCACAACAACCCTTTT3'

RESULTS

The induction of liver tumors in rats by AFB_1 has proven to be a useful experimental model for the study of several aspects of chemical carcinogenesis in the liver, which include its metabolism (1), formation of AFB_1-DNA adduct (24), and changes in hepatocellular cytology following AFB_1 exposure (2). In this study, induction of liver tumors was accomplished in Fischer rats by repeated intraperitoneal injection with AFB_1 during a two 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 1-2 years after AFB_1 exposure (see Table 1). Also consistent with earlier findings, no control animals developed liver tumors or other lesions. Histological examination of the liver tumor indicates that all were carcinomas containing liver parenchymal cells exhibiting varying degrees of trabecular cord formation, gladular character, cystic degeneration, or liver necrosis. Livers of animals treated with the vehicle alone showed no abnormalities in morphology or hepatocellular histology.

Table 1. Identification of oncogenes in AFB_1-induced rat liver carcinomas.

Parameter	Incidence	Animal
Rats Bearing Hepatocellular Carcinomas		
AFB_1-treated	9/9	R3 to R10
DMSO-treated	0/5	
Tumors Positive in Focus Formation Assay		
AFB_1-treated	2/9	R3, R8
DMSO-treated	0/5	
Tumors Positive in Nude Mouse Tumorigenicity Assay		
AFB_1-treated	4/9	R3, R8, R4, R10
DMSO-treated	0/5	
Tumors Containing		
c-k-*ras* oncogene	2/9	R3, R8
N-*ras* oncogene	2/9	R4, R10

In the initial phase of the study, DNA was isolated from excised hepatocellular carcinomas, whenever possible from single gross tumors that could 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 non-tumor as well as tumor cells. For detection of oncogenes, DNA was transfected into NIH3T3 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 novel c-*ras* genes of rat origin. In a total of nine liver tumors analyzed, two samples (R3 and R8) indicated the presence of c-K-*ras* oncongenes and an additional two samples (R4 and R10) showed the presence of N-*ras* oncogenes by these assays (see Table 1). The detection of these two classes of genes confirms and extends our own previous work (17) as well as that of Sinha *et al.* (25).

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-K-*ras*-genetic loci of tumors and control rat livers using polymerase chain reaction (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 the 12th codon of both c-K-*ras* genes because previous studies in our own laboratory (26) and another (25) indicated that the 12th codon of the c-K-*ras* gene was a potential site of AFB_1-induced mutagenesis in the tumor DNA. No prior information was available concerning N-*ras* sequences in rat liver. Using primer sets delineating regions of the first exon, PCR amplification of DNA was performed using *Taq* polymerase (21). Sequence information for construction of the primer sets was obtained from published rat c-K-*ras* gene (22) or mouse N-*ras* gene (23). The design of primer sets included a restriction enzyme linker for unidirectional cloning of the PCR-generated DNA fragment into M13 phage. DNA from individual recombinant phage was isolated and sequenced using dideoxy chain termination methods. In addition, plaques were transferred to membranes and hybridized with radiolabeled probes specific for individual alleles using a plaque screening assay (see Materials and Methods).

A compilation of the types of c-K-*ras* and N-*ras* gene sequences in the Fischer rat liver is shown in Fig. 1. Analyses of the c-K-*ras* gene region from

Sample	Codon														
	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Human K-*ras* 2	GTA	GTT	GGA	GCT	GGT	GGC	GTA	GGC	AAG	AGT	GCC	TTG	ACG	ATA	CAG
Mouse K-*ras*	GTG	GTT	GGT	GCT	GGT	GGC	GTA	GGC	AAG	AGC	GCC	TTG	ACG	ATA	CAG
Rat K-*ras* A	GTA	GTT	GGA	GCT	**GGT**	GGC	GTA	GGC	AAG	AGT	GCC	TTG	ACG	ATA	CGA
	Val	Val	Gly	Ala	**Gly**	Gly	Val	Gly	Lys	Ser	Ala	Leu	Thr	Ile	Gln
Rat K-*ras* B ONC					**TGT**										
					Cys										
Rat K-*ras* C 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-*ras* A	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-*ras* B							ATT								
							Ile								
Rat N-*ras* C ONC	GTA					**GTT**					GTT				
	Val					**Val**					Val				

Fig. 1. Sequence and amino acid comparison of c-K-*ras* and N-*ras* gene regions from exon I. A comparison of c-K-*ras* and N-*ras* sequences is illustrated and compared to published information for human c-K-*ras* (41), mouse c-K-*ras* (42), rat c-K-*ras* (22), human N-*ras* (31), and mouse N-*ras* (23) genes. ONC signifies the presence of this gene in liver tumor-derived NIH3T3 cells and the detection of the gene region by PCR analysis.

control rat liver revealed a DNA sequence identical to that previously published for the rat gene (22). Analysis of more than 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 (19, 20) indicated no DNA regions within the c-K-*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 phage were isolated and sequenced from a given PCR DNA preparation to arrive at an invariable consensus sequence. A summary of these results is listed in Table 2 and 3. Two different c-K-*ras* oncogene sequences were detected in DNA derived from 3 of 8 AFB_1-induced liver tumors (R3, R8, and R9). In PCR DNA from primary liver tumors as well as transformant cells derived from them, identical gene regions were found to contain single nucleotide changes (G-C to T-A or A-T) in the 12th codon. These mutations resulted in single amino acid substitutaions at this site (cysteine or aspartate for glycine). The absence of such mutations in DNA of livers from control rats suggested that the c-K-*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 to 24% (see Table 3). Results of the plaque screening assay confirmed and extended data on the presence and distribution of mutated c-K-*ras* sequences in the same three tumors and also indicated frequency distributions comparable to those detected by the PCR-cloning procedures (see Table 4). 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 germline regions using the same DNA-containing membrane.

Table 2. Putative activation mutations in exon I of c-K-*ras* and N-*ras* oncogenes in AFB_1-induced hepatocellular carcinomas and nomal livers of Fischer rats.

Gene Region	Incidence: Control Livers	Incidence: Liver Tumors	Oncogene[a]	Codon	Putative Mutation
K-*ras* A	3/3	8/8	–	– –	–
K-*ras* B	0/3	1/8	+	12	GGT ↓ T
K-*ras* C	0/3	2/8	+	12	GGT A
N-*ras* A	3/3	5/5	–	– –	–
N-*ras* B	3/3	5/5	–	– –	–
N-*ras* C	3/3	5/5	+	13	GGC ↓↓ TT

[a]Detected by focus formation or nude mouse tumorigenicity assays

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-K-*ras*. First, three N-*ras* gene regions were detected that contained different nucleotides in codons 8, 13, 14, and 18. The N-*ras* A sequence was found to be homologous to both mouse and human N-*ras* proto-oncogenes and no divergene in amino acid sequence was found for this gene region (Fig. 1). The N-*ras*B sequence differed from N-*ras*A 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-*ras*C sequence, when compared to N-*ras*A, contained 4 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-*ras*C se-

Table 3. Frequency of various rat c-K-*ras* and N-*ras* gene region as determined by sequencing of cloned PCR DNA derived from rat liver tumors and tumor-derived NIH3T3 transformants.

Sample	Frequency No. positive/Total No. Plaques						
	K-*ras*A[a]	*K-ras*B TGT(12)[b]	K-*ras*C GAT(12)	N-*ras*A	N-*ras*B	N-*ras* GTT(13)	
Transformants:							
R3a.2		20/20[c]					
R8a.1			17/17				
R4N						2/2	
R10N						5/5	
Control rat livers:							
CR2	45/45			5/14	8/14	1/14	
CR3	52/52			n.d.[d]	n.d.	n.d.	
CR4	n.d.			6/18	9/18	3/18	
CR5	105/105			12/28	13/28	3/28	
AFB_1-induced liver tumors:							
R3	38/47	9/47					
R4	58/58			9/24	12/24	3/24	
R5	18/18			6/20	10/20	4/20	
R6	12/12			4/11	5/11	2/11	
R7	36/36			n.d.	n.d.	n.d.	
R8	76/100		24/100	5/14	7/14	2/14	
R9	33/36		3/36	n.d.	n.d.	n.d.	
R10	25/25			7/25	14/25	4/25	

[a]See Fig. 1
[b]Codon DNA sequence (codon number)
[c]Excluding phages containing mouse genes
[d]Not determined

quence 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 of a genetic element ascribed to oncogenes (Table 3). 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 NIH3T3 transformation or nude mouse assays (Table 1). In addition, the frequency distribution of N-*ras*A, N-*ras*B, and N-*ras*C gene regions in both AFB_1-induced liver tumors and normal rat liver was strikingly similar. The presence of valine for glycine substitution at codon 13 is consistent with oncogenic activating mutations of the N-*ras* gene previously observed in other systems (28-30). This unexpected finding, confirmed by DNA sequencing of many independently-derived representative clones, the plaque screening assay, and in many successive experiments suggests that the N-*ras*C gene region is of germline origin in the Fischer rat.

Table 4. Frequency of various c-K-*ras* and N-*ras* gene regions as determined by a M13 plaque screening of cloned PCR DNA derived from rat livers.

	Frequency No. Positive/Total No. Plaques		
Sample	K-*ras* A	K-*ras* B	K-*ras* C
CR5	148/148	0/17	0/131
R3	348/377	29/377	
R8	74/123		49/123
R9	615/632		17/632
	N-*ras* A	N-*ras* B	N-*ras* C
CR5	4/16	4/16	8/16
R4	10/51	28/51	13/51
R5	11/33	13/33	9/33
R10	26/60	25/60	9/60

In summary, the nature of the amino acid changes that impart oncogenicity in either the focus formation or tumorigenicity assay can be inferred by analysis of PCR-amplified DNA from tumor-derived NIH3T3 transformants and confirmed by analysis of primary liver tumors. Putative activating mutations in the c-K-*ras* genetic locus have been shown to involve a single-base modification of either G-C base pair at codon 12 leading to aspartate or cysteine substitutions for glycine. The oncogenicity of an N-*ras* oncogene containing the N-*ras* C gene region may be related to an amino acid substitution of valine for glycine at codon 13.

DISCUSSION

Our previous studies identified c-K-*ras* oncogenes in NIH3T3 fibroblasts derived from AFB_1-induced liver tumors, and characterized mutations in the 12th codon as the mechanism of activation (17). Sinha *et al.* (25) also identified c-K-*ras* and N-*ras* oncogenes in transformants derived from liver tumors of AFB_1-fed rats, but did not report sites of activation. Our present findings confirm and substantially extend these earlier results. Cloning of PCR-DNA followed by sequencing or oligonucleotide hybridization revealed sequence differences in the 12th codon of c-K-*ras* in DNA from 3 of 9 primary liver tumors. These same gene regions were detected in PCR-DNA from NIH3T3 transformants derived from 2 of these tumors. Analyses failed to detect any differences in comparison to the proto-oncogene with respect to the 12th codon of c-K-*ras* in the remaining 6 tumors. The observed sequence differences consisted of single nucleotide changes at either of two G-C base pairs in the 12th codon, both resulting in amino acid changes at this site. This finding, together with extensive evidence indicating the preferential binding of AFB_1 to guanine residues (3,4), strongly suggests that both c-K-*ras* oncogenes could have resulted from direct mutagenesis of the germline proto-oncogene.

The distribution of various c-K-*ras* sequences containing changes in the 12th codon was highly variable in different liver tumors. Two samples containing the highest frequency of c-K-*ras* gene region (R3, R8) also scored positive in the DNA transfection assays. Another sample contained a low, but detectable, frequency of the same oncogene-containing region (R9) but scored negative in the transfection assays. The data in Tables 3 and 4 represent statistical estimates of the frequency of particular c-K-*ras* or N-*ras* gene regions in PCR-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 and would contribute to the observed variability in the frequencies of c-K-*ras* gene regions. These might include the simulataneous presence of multiple liver cell types, the heteroploidy of hepatocytes, and the presence of transformed hepatocytes which may have arisen by multiple independent genetic events. In this latter regard, histological examination of the liver samples revealed hepatocellular carcinomas which did not contain any obvious

enrichment of predominant liver cell types. In addition, the complete lack of c-K-*ras* oncogenes in most of the samples, as well as the substantiation of this finding by extensive DNA sequence analysis of the remaining tumors, suggests that either the genetically-altered cells were absent or they were greatly underrepresented in the liver cells from which the DNA was prepared.

N-*ras* oncogenes were identified in 2 of 9 tumors after transfection of liver tumor DNA followed by production of subcutaneous tumors in nude mice. PCR-DNA amplification and sequence analysis of cloned PCR-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 when compared to published mouse (23) and human (31) 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. The possible existence of other functionally important amino acid differences outside the restricted DNA region we have analyzed remains unknown. Unexpectedly, however, DNA sequence analysis of this same gene region in PCR-DNA derived from livers of control as well as tumor-bearing animals revealed the presence of three different N-*ras* gene regions including that detected in the oncogenes of subcutaneous tumor-bearing mice.

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 germline. The finding of a single rat-derived N-*ras* oncogene in NIH3T3 cells transformed by liver tumor DNA 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 an oncogene capable of expressing the oncoprotein detected in NIH3T3 tumors. The lack of induction of nude mouse tumors in transfection assays of control rat liver DNA may reflect this inactivity. Genetic alterations at sites other than the region we have analyzed may activate germline N-*ras* genes harboring the potential to express an oncogenic protein. In addition, AFB_1-induced cytotoxicity and resultant hepatocellular regeneration may promote the selection of transcriptionally-active germline oncogenes, ensuring further genetic events sufficient for malignant transformation. In this latter scenario, rare spontaneous mutations or the presence of potential germline oncogenes might predispose the livers of Fischer rat to tumor development. In the case of AFB_1, this is especially relevant in the liver, where DNA-reactive or cytotoxic intermediates represent products of significant metabolic pathways.

In other studies of chemical carcinogenesis in the Fischer rat, *ras* oncogenes have been detected in NIH3T3 transformants derived from methyl (methoxymethyl) nitrosamine-induced kindney mesenchymal tumors (c-K-*ras* and N-*ras*) (32); a 1,8-dinitropyrene-induced fibrosarcoma (c-K-*ras*) (33); radiation-induced skin tumors (c-K-*ras* and N-*ras*) (32); a dibutylnitrosamine-induced hepatocellular carcinoma (N-*ras*) (34); a 1,6-dinitropyrene-induced fibrosarcoma (N-*ras*) (35); a 2-aminodipyrido [1,2-a;3',2'-d]imidazole-induced intestinal adenocarcinoma (N-*ras*) (36); and methyl (acetoxymethyl) nitrosamine-induced liver neoplasms (c-K-*ras*) (37). It is noteworthy that N-*ras* oncogenes have also been detected in NIH3T3 cells derived from primary human hepatocellular carcinomas (38). The concurrent presence of c-K-*ras* and N-*ras* oncogenes have been detected in several systems including chemically-induced rat kidney tumors (34), a chemically-induced murine lymphoma (39), and a spontaneous human leukemia (40).

AFB_1 is a particularly potent liver carcinogen in Fischer rats compared to other rat strains (1). Findings reported here suggest that the pressence of a potential N-*ras* oncogene in the Fischer rat might potentiate the transformation of liver cells after chemical treatment. AFB_1-induced mutagenesis of c-K-*ras* proto-oncogenes may con-

tribute to this protential as well. In a more general sense, the role of chemical carcinogens in the induction of liver tumors may involve the mutation of genes, cellular events that increase the frequency, selection or expression of spontaneous mutation (s), or the potentiation of germline genes by epigenetic mechanisms. Experiments designed to identify the relative contributions of chemicals in the direct mutagenesis of genes and in the selection of potentially oncogenic germline genes would be of value in the assessment of environmental chemicals as agents that increase cancer risk.

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