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Identification of an activated c-Ki-*ras* oncogene in rat liver tumors induced by aflatoxin B_1

(hepatocarcinogen/chemical carcinogen/DNA transfection/mycotoxin)

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ABSTRACT **Weanling male Fischer rats were administered 40 intraperitoneal injections of aflatoxin B_1 (25 μg per animal per day) over a 2-month period. This chronic dosing regimen resulted in the sequential formation of hyperplastic foci, preneoplastic nodules, and hepatocellular carcinomas in all of the animals treated. The presence of transforming DNA sequences was detected by formation of anchorage-independent foci after transfection of tumor-derived DNA in NIH 3T3 mouse fibroblasts. Transfection of genomic DNA isolated from individual tumors from eight animals resulted in specific transforming activities ranging from 0.05 to 0.2 foci per μg of DNA. Primary transfectant DNAs were analyzed by Southern blot hybridization with DNA probes homologous to c-Ha-*ras*, c-Ki-*ras*, and N-*ras* oncogenes. A highly amplified c-Ki-*ras* oncogene of rat origin was detected in transformants derived from tumors in two of the eight animals tested. There was no evidence to suggest the presence of c-Ha-*ras* or N-*ras* sequences in any of the transformants. Analysis of primary liver tumor DNA showed no Ki-*ras* DNA amplification when compared to control liver DNA samples. Increased levels of c-Ki-*ras* p21 proteins were detected in 3T3 transformants containing activated rat c-Ki-*ras* genes. The presence of c-Ki-*ras* sequences of rat origin capable of inducing transformed foci can be taken as evidence that the c-Ki-*ras* gene has been activated in the primary liver tumors.**

Recent studies concerning mechanisms of chemical carcinogenesis have focused on elucidation of relationships between the formation of carcinogen-DNA adducts and genetic changes that take place during tumor formation. Oncogenes have been identified from many human and several animal tumor sources after transfection of primary tumor DNA or DNA derived from established cell lines into appropriate target cells (for review, see refs. 1 and 2). In general, DNA-mediated transfer techniques have been used most frequently with NIH 3T3 mouse fibroblasts, which form anchorage-independent foci when a heterologous transforming gene is transferred. These findings, as well as those relating cellular oncogenes to known RNA tumor viruses, suggest that the activation of cellular protooncogenes may be an important step in the initiation, progression, or maintenance of the malignant cell.

The finding that oncogenes can be activated by single-base mutations provides a rationale for studying oncogenes subsequent to administration of chemical carcinogens (for review, see ref. 3). It has been suggested that a single administration of specific chemical carcinogens is sufficient in certain experimental systems to cause single-base changes that activate known oncogenes (4). As discussed in more detail below, activated oncogenes have been identified in a wide variety of tumor types induced by many different chemical carcinogens in experimental animals. Chemically induced tumors of the liver are of particular interest in the context of studies of mechanisms of carcinogenesis, since this organ is often the primary target organ for carcinogens of very diverse chemical structure. Characterization of oncogenes in liver tumors will also be important in the interpretation of the results of bioassays conducted in the safety evaluation of environmental chemicals, in which liver tumors often constitute the only evidence of carcinogenicity.

In the present study, we describe the detection of an oncogene of the c-Ki-*ras* gene family in primary tumors derived by treatment of rats with the potent hepatocarcinogen aflatoxin B_1 (AFB_1). Studies of AFB_1-DNA binding in rat liver have revealed that the major DNA-bound form of AFB_1 following exposure of rats to AFB_1 is a 2,3-dihydro-2-(N^7-guanyl)-3-hydroxyaflatoxin B_1 adduct (5). The kinetics of formation and removal of this adduct *in vivo* in relation to carcinogenesis have been studied extensively and therefore provide a useful experimental system with which to investigate the process of oncogene activation in relation to carcinogenesis in liver. Since AFB_1 has been shown to be a potent mutagen in both bacterial and mammalian systems (6–8), we sought to determine whether AFB_1 could induce the activation of single-base mutations in an oncogene of the c-*ras* gene family. We describe here our initial characterization of an oncogene in cell lines derived from transfection of DNA isolated from AFB_1-induced liver tumors into NIH 3T3 cells.

MATERIALS AND METHODS

Primary Tumors and Cell Lines. To derive liver tumors, weanling male Fischer rats were injected intraperitoneally 5 days per week for 8 weeks with 25 μg of AFB_1 (Makor Chemicals, Jerusalem, Israel) in 50 μl of dimethyl sulfoxide. Animals were killed and liver tumors were excised after 52 weeks. NIH 3T3 mouse fibroblasts were maintained at low cell density in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum. S2-72-1 NIH 3T3 cells were obtained from S. Sukumar (Frederick Cancer Research Facility, Frederick, MD).

DNA Preparation. DNA was isolated from cells of homogenized tumor samples or dispersed cell pellets 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 DNA was purified by phenol/chloroform extraction. The DNA solution was made 2 M in ammonium acetate, reprecipitated with ethanol, and resuspended in 10 mM Tris·HCl, pH 8.0/1 mM EDTA before digestion with restriction endonucleases under conditions specified by the supplier.

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Abbreviations: AFB_1, aflatoxin B_1; kb, kilobase(s).
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Transfection Experiments. Transfection of tumor-derived genomic DNA into NIH 3T3 mouse fibroblasts was performed as described (9). After transfection, the cells were supplemented with DMEM containing 5% calf serum. Anchorage-independent foci were scored and subcloned 2–3 weeks after transfection. Transfection of plasmid pEJ (10) and S2-72-1 3T3 DNA containing an activated rat c-Ha-*ras* oncogene (11) were used as positive controls.

Southern Blot Hybridizations. Southern blot experiments were performed after restriction endonuclease-treated DNA was electrophoresed in agarose gels and transferred to nitrocellulose membranes (12). Alternatively, DNA was denatured in the gels, which then were dried for 2 hr at 60°C using a slab gel dryer (Bio-Rad). Hybridizations were at 65°C for 16 hr in 5× standard saline citrate (1× SSC = 0.15 M NaCl/0.015 M sodium citrate) containing 50 mM sodium phosphate (pH 6.5), 1 mM EDTA, 0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone, 0.1% Ficoll, 0.2% $NaDodSO_4$, 0.2 mg of sonicated salmon sperm DNA per ml, and ^{32}P-labeled DNA probes ($>10^9$ cpm/μg of DNA). Blots and dried gels were washed for 30 min at room temperature in 2× SSC/0.1% $NaDodSO_4$. Then blots were washed at 65°C in 0.5× SSC/0.1% $NaDodSO_4$ and gels were washed at 65°C in 2× SSC/0.1% $NaDodSO_4$. Autoradiograms were obtained after exposure of blots or gels to x-ray film in the presence of a single Cronex intensifier screen (Du Pont).

Preparation of DNA Probes. Restriction fragments were prepared from plasmids pBS9 (v-Ha-*ras*) (13), pHiHi-3 (v-Ki-*ras*) (14), pCDK76 (human c-Ki-*ras* cDNA) (15), and p52C$^-$ (human N-*ras*) (16) after resolution in low-melting-point agarose gels. DNA fragments were radiolabeled with [α-^{32}P]dCTP (3000 Ci/mmol, New England Nuclear; 1 Ci = 37 GBq) by the random-primer method of Feinberg and Vogelstein (17). Plasmid pCDK76 was utilized to identify c-Ki-*ras* genes in rat DNA, since we have observed appreciable cross-hybridization of the pHiHi-3 probe with endogenous viral elements in the rat genome. Plasmid p3B5 (18) was used to identify repetitive rat DNA.

Immunoprecipitation of p21 Protein. Cells were metabolically labeled for 2 hr with 100 μCi of [^{35}S]methionine (1075 Ci/mmol) in methionine-free DMEM plus dialyzed fetal bovine serum. Lysates were prepared in 0.01 M sodium phosphate, pH 7.2/0.15 M NaCl/1% (wt/vol) Triton X-100/0.03 M sodium azide/12 mM sodium deoxycholate/3.0 mM $NaDodSO_4$/1 μM sodium fluoride and incubated with monoclonal antibody Y13-259 (19) and protein A-Sepharose (Bethesda Research Laboratories) at 4°C for 18 hr. Pellets were washed three times with lysate buffer, boiled for 3 min, and electrophoresed in a $NaDodSO_4$/12.5% acrylamide gel at 250 V. Gels were dried, treated for fluorography, and exposed to x-ray film with an intensifier screen at −70°C.

RESULTS

Identification of Transforming DNA. We performed DNA-mediated transfer in order to ascertain whether a transforming DNA sequence was present in DNA derived from tumors induced in rats by AFB_1. Weanling male Fischer rats were administered 25 μg of AFB_1 in 50 μl of dimethyl sulfoxide by intraperitoneal injection 5 days per week for 8 weeks, resulting in a cumulative dose of 1 mg per animal. Subsequent to AFB_1 exposure, the livers showed the sequential appearance of hyperplastic foci, preneoplastic nodules, and malignant tumors, as described (20). After 52 weeks, tumors were excised from livers containing hepatocellular carcinomas as confirmed by microscopic examination of stained tissue sections.

DNA samples from 11 individual AFB_1-induced tumors were tested, and the results (Table 1) indicated that 10 were able to transform NIH 3T3 mouse fibroblasts to produce anchorage-independent foci. In some cases, independent carcinomas were obtained from the same animal. In all cases, care was taken to obtain tissue samples that were most enriched in the neoplastic tissue. However, microscopic examination of the tissue samples revealed a more heterogeneous distribution of cell types containing much smaller regions of neoplastic cells than suggested by gross appearance. Specific transforming activities ranged from 0.05 to 0.2 foci per μg of DNA. Comparison of the specific transforming activities to those obtained with the positive control, a genomic DNA containing a known activated rat c-Ha-*ras* gene (11), indicated that the rat liver DNA preparations contained transforming DNA sequences. Although the specific transforming activities were substantially lower than that of the positive control, they were comparable to those of other transfection experiments utilizing tumor-derived DNA (2). In addition, it was noted that preparations of control rat liver DNA could induce a low, but detectable, number of foci that was comparable to the number of foci obtained after transfection of 3T3 DNA alone. However, most of the tumor-derived DNA exhibited specific transforming activities 4- to 6-fold higher than that obtained from control livers. More importantly, microscopic examination of the tumor-derived foci indicated a transformed cell phenotype, which more closely resembled foci induced by positive-control DNA than those few foci obtained after transfection of control rat liver DNA. In addition, we found that DNA from the majority of tumor-derived foci contained rat-specific

Table 1. Induction of transformed foci by DNA from AFB_1-induced liver tumors

DNA source	Total foci/ no. of plates	Specific transforming activity, no. of foci per μg of DNA
Rat liver DNAs*		
HC R1a	4/12	0.05
HC R1b	4/12	0.05
HC R3a	5/12	0.07
HC R3b	0/12	<0.01
HC R4	8/12	0.12
	13/12	0.17
HC R5	3/12	0.04
	14/12	0.19
HC R6	7/12	0.10
	10/12	0.13
HC R7a	5/12	0.07
	7/12	0.10
HC R7b	9/12	0.12
	10/12	0.13
HC R8a	5/12	0.07
	12/12	0.16
HC R8b	3/12	0.04
	11/12	0.15
CR 1	2/12	0.03
	0/12	<0.01
CR 2	3/12	0 04
	3/12	0.04
Control DNAs		
NIH 3T3 cells	2/6	0.03
	2/6	0.05
	0/6	<0.01
S2-72-1† cells	14/6	0.19
	35/6	0.93
	8/6	0.21

*HC designates hepatocellular carcinoma; lowercase letters denote tumors present in the same liver. CR designates liver from a control (vehicle-treated) rat.

†S2-72-1 is an NIH 3T3 cell line containing an activated rat c-Ha-*ras* gene (11).

DNA sequences as determined by hybridization (data not shown) of transformant DNA to a DNA probe that is specific for rat repetitive DNA (18).

Screening of Primary Transformants for c-*ras* Genes. Genomic DNA from transformant cells induced by tumor DNA was analyzed to determine whether a known oncogene could be detected. Foci were subcloned and established as cell lines. DNA was then isolated and screened for the presence of known c-*ras* oncogenes by Southern blot-hybridization techniques. Analysis of 40 cell lines indicated that transformants derived from two of the original tumors (HC 3a and HC 8a) contained DNA homologous to the v-Ki-*ras* oncogene, as revealed by the presence of highly amplified 2.5-kilobase (kb) and 8.5-kb restriction fragments (Fig. 1, lanes c and e). Comparison of these findings to the restriction map of the rat c-Ki-*ras* gene (21) suggested that the activated DNA sequences are of rat origin. In contrast, hybridization analysis failed to reveal sequences homologous to DNA probes specific for c-Ha-*ras* or N-*ras* genes in those transformants that did not contain c-Ki-*ras* DNA sequences.

In addition, we have noted that DNA preparations from two independent tumors from the same animal (e.g., HC 3a and HC 3b; HC 8a and HC 8b) showed comparable increases in specific transforming activity but did both contain c-Ki-*ras* DNA sequences in the transformed clones (see Fig. 1, lanes e and f). Likewise, independently derived foci showed different levels of c-Ki-*ras* gene amplification (see Fig. 1, lanes b and c).

Analysis of c-Ki-*ras* Gene Copy Number in Primary Tumor DNA. Since it has been shown that some primary tumors contain amplified c-Ki-*ras* genes (22), we sought to determine whether DNA was amplified in the original tumor DNA. For reasons described in *Materials and Methods*, we employed a cDNA probe derived from the human c-Ki-*ras-2* (*KRAS2*) allele (15) for this purpose. Hybridization of restriction enzyme-digested tumor DNAs to this probe revealed the presence of an 8.5-kb band (Fig. 2, lanes a–d) that was also present in tumor-derived transformants (lane e), confirming that the transfected gene is of rat origin. However, comparison of the signal intensity of this band in DNA from control livers with that from tumors indicated that there was no detectable increase in copy number of the c-Ki-*ras* gene in the tumor tissue (data not shown). This indicates that the c-Ki-*ras* gene was not amplified in these tumors.

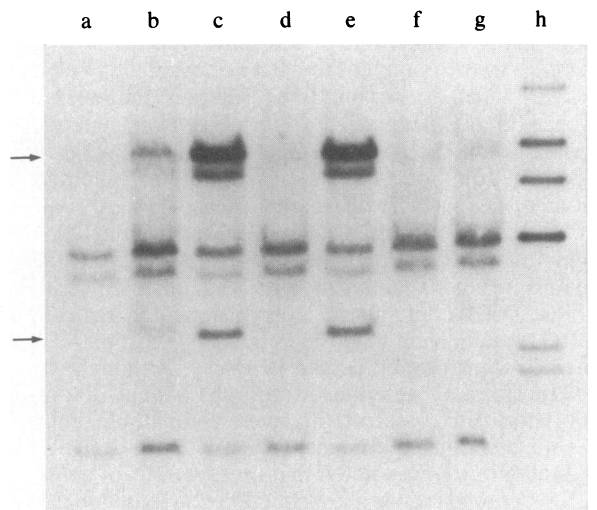

FIG. 1. Identification of a c-Ki-*ras* oncogene in tumor-derived transformants. DNA (20 μg) from tumor-derived 3T3 foci was digested with *Hin*dIII, electrophoresed in 0.8% agarose, blotted to nitrocellulose, and hybridized to a ^{32}P-labeled 1.0-kb *Eco*RI fragment derived from a plasmid containing viral Ki-*ras* DNA (pHiHi-3). The washed blot was exposed to x-ray film for 2 days. DNA samples were derived from tumors HC 6 (lanes a and d), HC 3a (lanes b and c), HC 8a (lane e), and HC 8b (lane f) and from NIH 3T3 cells (lane g). *Hin*dIII-digested λ DNA provided size markers (lane h). Arrows designate restriction fragments derived from the rat c-Ki-*ras* gene.

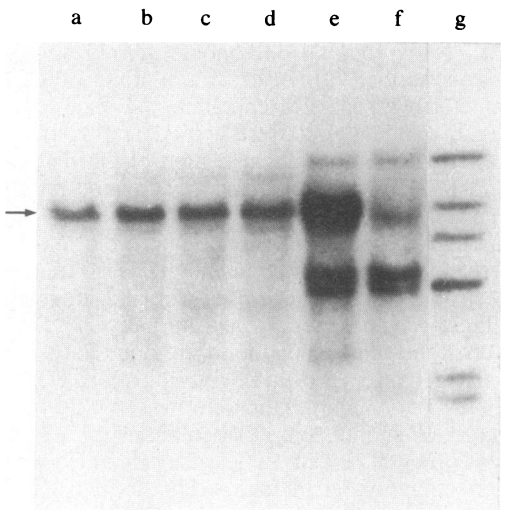

FIG. 2. Analysis of c-Ki-*ras* gene in primary rat liver tumors. Rat liver tumor DNA was digested with *Hin*dIII and electrophoresed in a 0.8% agarose gel. The DNA was denatured, and the gel was dried and probed with a ^{32}P-labeled *Pst* I–*Bam*HI fragment of plasmid pCDK76 (15). The gel was washed in 2× SSC/0.1% $NaDodSO_4$ and exposed to x-ray film for 2 days. DNA samples were from tumors HC 5, HC 6, HC 7a, and HC 8a (lanes a–d), an NIH 3T3 HC 8a transformant (lane e), and NIH 3T3 cells (lane f). *Hin*dIII-digested λ DNA was used as size markers (lane g). Arrow designates the position of restriction fragments containing the rat c-Ki-*ras* gene.

Expression of the p21 Protein. Levels of c-Ki-*ras* p21 proteins were measured in cell lysates derived from transformants containing rat c-Ki-*ras* genes. This was done by immunoprecipitation of cell lysate with a monoclonal antibody specific for c-*ras* polypeptides (19) (Fig. 3, lanes d and e). In all cases, the transformants contained more p21 protein than did nontransformed 3T3 cells. Electrophoretic mobility of the p21 protein in the transformants was not different from that of the mobility of the wild-type p21 protein (data not shown). In addition, we noted increased levels of c-Ki-*ras*-specific mRNA when cellular RNA derived from positive transformants was analyzed by electrophoresis followed by blot hybridization (data not shown). Both of these observations indicate that the transformed cell lines containing an activated rat c-Ki-*ras* gene showed elevated levels of c-Ki-*ras*-specific mRNA and protein.

DISCUSSION

Our objective was to determine whether AFB_1-induced hepatocellular carcinomas contain DNA sequences capable of inducing anchorage-independent foci after transfection of tumor-derived DNA into NIH 3T3 cells. Tumors were induced by treating rats with multiple administrations of the carcinogen, known by extensive previous experience to induce hepatocellular carcinomas in all treated animals (20). No liver tumors were found in control rats given the vehicle, dimethyl sulfoxide.

We found that DNA derived from 10 of 11 hepatocellular carcinomas contained transforming sequences, as detected after transfection into NIH 3T3 cells. In addition, 2 of 10 of these transforming DNAs were shown to contain an activated c-Ki-*ras* oncogene of rat origin, but the nature of the transforming sequences in the remaining tumor DNA samples was not established. In addition, the c-Ki-*ras*-containing transformants exhibit elevated levels of p21 protein. Taken

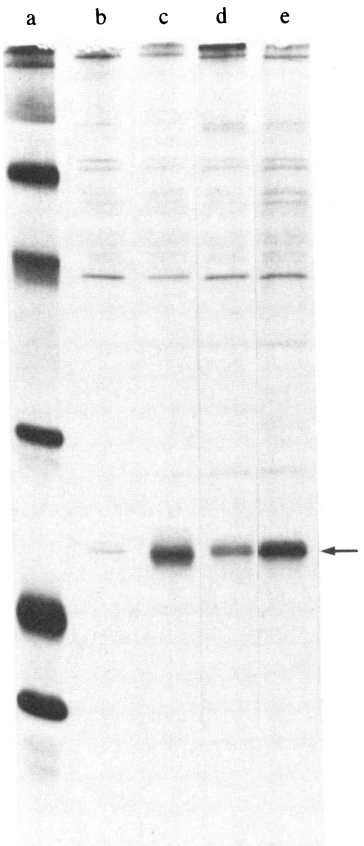

FIG. 3. Detection of p21 in cell lysates derived from transformed NIH 3T3 cells. Cell lysates, derived from NIH 3T3 cells transfected with various c-*ras* genes and labeled with [^{35}S]methionine, were precipitated with Y13-259 antibody (19) and resolved in a NaDodSO$_4$/12.5% polyacrylamide gel. The dried gel was fluorographed and shows precipitated proteins derived from lysates of NIH 3T3 cells (lane b), NIH 3T3 cells transformed with pEJ (10) (lane c), and NIH 3T3 transformants R3a.2 (lane d) and R8a.1 (lane e). Lane a: ^{14}C-labeled molecular weight standards (Bethesda Research Laboratories) lysozyme, β-lactoglobulin, chymotrypsinogen, ovalbumin, phosphorylase *b*, and myosin heavy chain. Arrow indicates the position of the p21 protein.

together, this evidence suggests that an oncogene of the c-Ki-*ras* gene family has been activated in some of the primary liver tumors. There has been a previous report of oncogene activation in a chemically induced rat liver tumor: Ochiai *et al.* (23) reported the identification of an activated c-*raf* gene in a rat hepatocellular carcinoma induced by the carcinogen 2-amino-3-methylimidazo[4,5-*f*]quinoline. However, Ishikawa *et al.* (24) subsequently showed that the c-*raf* activation took place by recombination during transfection, and it remains unclear whether these sequences were present during the process of tumor development *in vivo*.

Other studies on liver tumors have employed the B6C3F1 hybrid mouse strain. Fox and Watanabe (25) originally reported that DNA isolated from spontaneous liver tumors in this strain was capable of inducing foci in NIH 3T3 cells, but the identity of the oncogene was not determined. Reynolds *et al.* (26) subsequently showed that 30% (3/10) of hepatocellular adenomas and 77% (10/13) of hepatocellular carcinomas contained activated oncogenes, as detected by the transfection technique. Transformed cells derived from all of the adenomas and from 8 of 10 carcinomas were shown to contain an activated c-Ha-*ras* gene. Recently, Wiseman *et al.* (27) showed that 100% of hepatomas induced by *N*-hydroxy-2-acetylaminofluorene, vinyl carbamate, and 1′-hydroxy-2′,3′-dehydroestragole in the same mouse strain contain transforming DNA sequences, which were identified as c-Ha-*ras* oncogenes.

With respect to chemically induced tumors of other tissues in rats, activated oncogenes have been found in mammary carcinomas induced by *N*-methyl-*N*-nitrosourea and 7,12,-dimethylbenz[*a*]anthracene (c-Ha-*ras*) (4), mesenchymal kidney tumors induced by *N*-methyl-*N*-methoxymethylnitrosamine (c-Ki-*ras* and N-*ras*) (28), neuroblastomas induced by *N*-ethyl-*N*-nitrosourea (*neu*) (29), fibrosarcomas induced by 1,8-dinitropyrene (c-Ki-*ras*) (21), and nasal carcinomas induced by methyl methanesulfonate (unidentified) (30). In chemically induced tumors of mice, Balmain and coworkers (31, 32) identified activated c-Ha-*ras* genes in 7,12-dimethylbenz[*a*]anthracene-initiated, phorbol 12-myristate 13-acetate-promoted skin carcinomas. Eva and Aaronson (33) showed activation of c-*kis* in 3-methylcholanthrene-induced fibrosarcomas. Murine thymic lymphomas induced by methylnitrosourea were found to contain an activated N-*ras* gene (34), whereas the same tumor type induced by ionizing radiation contained activated c-Ki-*ras* DNA sequences (35). In general, it appears that the particular oncogene which is activated in tumor cells induced by chemical carcinogens may be dependent on the target organ and species, as well as the specific chemical used to induce the tumor. It is, therefore, not surprising that an N-*ras* oncogene has been detected in cells derived from primary hepatocellular carcinomas in humans (36). Thus, different c-*ras* oncogenes have been detected in liver tumors of mice, rats, and humans.

A wide diversity of chemically induced tumors has been shown to contain activated oncogenes. However, the frequency with which DNA isolated from primary tumors has given positive results in the transfection assay has been highly variable. For example, DNA from 86% of methylnitrosourea-induced mammary carcinomas (4) and 89% of 7,12-dimethylbenz[*a*]anthracene-initiated skin carcinomas (31) were able to transform NIH 3T3 cells. In contrast, only 6 of 29 dimethylbenz[a]anthracene-induced mammary carcinomas (4) and 11 of 25 methyl(methoxymethyl)nitrosamine-induced renal tumors (28) contained transforming DNA sequences. Our present findings that 10 of 11 liver tumor-derived DNA preparations gave positive results in the transfection assay are noteworthy in view of the report by Reynolds *et al.* (26) that spontaneous liver tumors in the Fischer rat strain showed no evidence of transforming activity. Therefore, our findings suggest that the activation of transforming sequences was related to AFB_1 treatment.

The occurrence of identifiable oncogenes in the DNA of transformant 3T3 cells induced by transfection of primary tumor DNA is also variable and appears to be unrelated to the specific transforming capability of the original tumor DNA. For example, 100% of transformants from dimethylbenz[a]-anthracene-induced mammary carcinomas showed the presence of an activated c-Ha-*ras* gene even though the initial transforming frequency was only 21% (4). Only 26% of 3T3 transformants derived from methylnitrosourea-induced mammary carcinomas showed the presence of an c-Ha-*ras* gene, whereas the occurrence of transforming genes in the primary tumor DNA was 86% (4). In the present study, 10 of 11 individual liver tumors gave positive results in the transfection assay, whereas only 20% of the DNA samples derived from 3T3 transformants showed the presence of a c-Ki-*ras* oncogene. Our findings are therefore more comparable to those for mammary carcinomas induced by methylnitrosourea rather than for the same tumors induced by dimethylbenz[*a*]anthracene. These seemingly anomalous observations may be related to differences in the sets of oncogenes present in DNA derived from individual, independently transformed primary tumors, possibly due to differential probability for activation resulting from multiple dosing. In addition, the level of an activated oncogene present in a given

DNA preparation may be variable. Therefore, the low incidence of c-Ki-*ras* genes in tumor-derived transformants in the experiments reported here may be attributable to a relatively low level of the activated allele in the original tumor DNA preparation.

With regard to the mechanism of c-*ras* activation, there is extensive evidence from studies in rodent and human tumors to indicate that activation results from point mutations in either the 12th or the 61st codon (3). Since AFB_1 is known to be a potent mutagen in bacterial and mammalian cells (6–8), it is logical to propose a mutational mechanism of c-Ki-*ras* gene activation to account for the findings presented here, and, indeed, preliminary work indicates that a mutation is present in the 12th codon of the rat c-Ki-*ras* gene in transformants derived from AFB_1-induced tumors (unpublished data).

In addition, it has been shown by Pulciani *et al.* (22) that a small number of human solid tumors contain amplified normal alleles in the c-Ki-*ras* genetic locus. As shown in this report, we have not detected appreciable DNA amplification of this gene in AFB_1-induced liver tumor DNA. This would indicate that the absence or presence of an activated c-Ki-*ras* gene in the tumors does not correlate with gene amplification of a normal allele. However, the inability to detect the activated gene in some of the primary tumors may still be due, in part, to the dilution of the mutant allele by DNA derived from non-tumor cells. In any case, the lack of c-*ras* oncogenes in the majority of the tumor-derived transformants indicates the possibility that a non-*ras* oncogene(s) may be activated in the primary tumors.

Plasmids pBS9 and pHiHi-3 were obtained from E. Scolnick (Merck, Sharp, and Dohme Research Laboratories, West Point, PA). Plasmids p52C$^-$, pCDK76, and pEJ were obtained from R. Weinberg (Whitehead Institute for Biomedical Research, Cambridge, MA). Plasmid p3B5 was obtained from A. Furano (National Institutes of Health, Bethesda, MD). Research support for these studies was obtained from National Institutes of Health Grant 2PO1-ES00597. G.M. was supported by National Institutes of Health Training Grant 5T32-ESOT020.

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