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Genetics

Activation of the c-Ki-*ras* oncogene in aflatoxin B_1-induced hepatocellular carcinoma and adenoma in the rat: Detection by denaturing gradient gel electrophoresis

(polymerase chain reaction/base substitutions/oligonucleotide hybridization)

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ABSTRACT **Sequence alterations in the exon 1 region of the rat c-Ki-*ras* gene were studied in DNA isolated from aflatoxin B_1 (AFB_1)-induced rat liver carcinomas and precursor lesions appearing 56 weeks after administration of the carcinogen. To detect the mutations with high sensitivity, DNA samples were analyzed by using polymerase chain reaction (PCR) amplification in conjunction with allele-specific oligonucleotide (ASO) hybridization together with a modified PCR–G+C clamp–denaturing gradient gel electrophoresis (DGGE) method. Mutations in the Ki-*ras* gene were present in all adenomas and carcinomas examined. The predominant mutation observed was a G·C-to-A·T base transition in codon 12 (GGT to GAT). Also present, but at low frequency, was a G·C-to-T·A base transversion in the same codon (GGT to TGT). In addition, 20% of the samples contained a G·C-to-T·A transversion in the second base position of codon 12 (GGT to GTT), a mutation not previously observed in AFB_1-induced rat liver tumors. These results confirm and extend our previous findings that Ki-*ras* mutation is a prevalent event in hepatocellular carcinogenesis induced in Fischer 344 rats by AFB_1. The modified DGGE method described is applicable to the screening of multiple mutations in neoplastic lesions with high fidelity and sensitivity.**

Aflatoxins have been implicated by epidemiologic studies as a causative factor for hepatocellular carcinoma in humans (1). Metabolic studies of aflatoxin B_1 (AFB_1) have shown that its active form, AFB_1 8,9-epoxide, is highly mutagenic and carcinogenic for the liver in rats and other experimental animals, with mutagenicity correlating with carcinogenicity (2, 3). AFB_1 binds exclusively to guanine residues of DNA, both *in vivo* and *in vitro* (4). The major DNA adduct formed by the binding of AFB_1 at the N^7 position of guanine is highly unstable and depurinates readily or is converted into the more stable, open-ring formamidopyrimidine adduct (1). The presence of DNA adducts in the genome may contribute to genetic alterations in loci involved in neoplastic development. Previous work by our group and others on AFB_1-induced liver carcinogenesis in rodents has demonstrated that activation of cellular *ras* (c-*ras*) genes by specific base substitutions occurs at a high frequency in tumor DNA (5, 6).

In human tumors as well as in animal tumor models, c-*ras* genes have been shown to be mutated, the frequency of mutation varying widely among different tumor types. Accumulating evidence suggests that in many human neoplasms, *RAS* oncogenes may play a role in the early stages of carcinogenesis (7–10). Presently, little information is available regarding the frequency of c-*ras* oncogene activation during the early stages of liver tumorigenesis in humans or in experimental animal models. Previous studies of AFB_1-induced hepatocellular carcinomas in Fischer 344 rats showed activating mutations in codon 12 of the c-Ki-*ras* gene, consisting of single-nucleotide changes (6). To develop a more complete understanding of the role of *ras* oncogenes in liver tumor development, we developed an analytical procedure with improved sensitivity by modifying the PCR–G+C clamp–denaturing gradient gel electrophoresis (DGGE) method (11) for detecting mutant alleles present at low frequency with high fidelity and increased sensitivity.

The DGGE procedure separates DNA molecules based on their melting properties (for a review, see ref. 12) and thus can efficiently distinguish between DNA fragments with minor sequence changes, such as base substitutions, small insertions, or deletions, that alter the melting temperature (t_m) of the fragment (12–18). Addition of a G+C-rich sequence ("G+C clamp") to the region of interest modified the melting properties of the DNA molecule such that the sequence changes occurring at all base pair locations could be detected by DGGE (11, 15). This method proved particularly useful for screening the high-frequency mutant population in any genome. However, the high error frequency of the *Thermus aquaticus* (*Taq*) DNA polymerase (about 0.25% after 30 cycles of amplification) and the high background smear of bands seen during DGGE reduced the sensitivity of detection of low-frequency mutations. We have modified the PCR–G+C clamp–DGGE method (11) to improve the limit of detection of point mutations by using T7 DNA polymerase, taking advantage of the higher fidelity of this enzyme (3.4×10^{-5} vs. 2.1×10^{-4} for *Taq* polymerase after 10^6-fold amplification) (19). We have used this method to analyze the codon 12 region of the rat c-Ki-*ras* gene in AFB_1-induced liver tumors and have found that activating mutations were present not only in carcinomas but also in preneoplastic stages of hepatocellular carcinogenesis.

MATERIALS AND METHODS

Materials. To obtain liver lesions at various stages of tumor development, animals were killed at various time points after AFB_1 administration to male Fischer 344 rats (40 daily intraperitoneal injections of 25 μg of AFB_1 each over a period of 8 weeks, beginning when the animals were 36 days old) as described (20). Tissue samples were also examined by histopathology. Adenomas were present 56 weeks after termination of dosing, and at 63 weeks and beyond, hepatocellular carcinomas (HCC) were found (21). The various oligonucleotides used as PCR primers were custom-synthesized by Synthetic Genetics (San Diego). The standard DNA samples

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Abbreviations: AFB_1, aflatoxin B_1; ASO, allele-specific oligonucleotide; DGGE, denaturing gradient gel electrophoresis.

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used in this study are as follows: R8a and R3a were genomic DNAs from AFB_1-induced rat liver tumors containing GAT and TGT substitutions, respectively; R8a.1 and R3a.2 were DNA from the corresponding NIH 3T3 transformant cell lines (22); and SW11.1 (23) and pHiHi3 were plasmid clones (24) carrying GTT and AGT substitutions, respectively.

Isolation of DNA. Genomic DNA was isolated from liver tissues by the sodium dodecyl sulfate (SDS)/proteinase K digestion method (25). An aliquot of the purified DNA was run on a 0.4% agarose gel, stained with ethidium bromide, and visualized under UV light to verify the integrity of DNA used for analysis.

PCR Amplifications. About 5.0 μg of DNA was amplified initially for 30 cycles with Ki-*ras* primers α and β (sequences shown below) and either the high-fidelity modified T7 DNA polymerase enzyme (Sequenase) for the initial DGGE analysis or the thermostable *Taq* DNA polymerase enzyme (in a Perkin–Elmer/Cetus DNA thermal cycler) for all other analyses. The high-fidelity amplification was performed manually under conditions described previously by Keohavong *et al.* (26). Each cycle consisted of successive incubations at 94°C for 1 min, 37°C for 2 min, and again at 37°C for 3 min after addition of fresh enzyme (3–4 units). For *Taq* DNA polymerase, the reaction mixture contained 200 μM of each dNTP, 1 μM each of the two PCR primers, 1× PCR buffer (50 mM KCl/10 mM Tris·HCl, pH 8.3/1.5 mM $MgCl_2$/0.01% gelatin), and 2.5 units of the enzyme. Each cycle consisted of successive incubations at 94°C for 1 min, 37°C for 2 min, and 72°C for 4 min. The 90-base-pair (bp) amplified DNA was purified by electroelution in 1× TBE buffer (50 mM Tris base/50 mM boric acid/1 mM EDTA) and ethanol precipitation. The DNA was never stained by ethidium bromide nor exposed to UV light, since it has been reported that such treatment caused DNA damage that could interfere with DGGE analysis (27). The purified DNA was resuspended in 23 μl of 1 × TBE.

For the addition of a 54-mer G+C-rich sequence to the 5′ end of exon 1 DNA, a second PCR reaction was carried out with the 90-bp amplification product as the template and two PCR primers, G_α (74-mer) and β (for sequence, see below). The amplifications were carried out for 15 cycles as follows: (*i*) with Sequenase, 94°C for 1 min, 37°C for 2 min, and after addition of enzyme, 37°C for 3 min; and (*ii*) with *Taq* DNA polymerase, 94°C for 1 min, 53°C for 2 min, and 72°C for 3 min. The amplified DNA was gel-purified as described earlier and resuspended in 23 μl of low TE buffer. An aliquot of the 144-bp DNA was quantified by densitometric scanning.

The primers used for amplification were α (20-mer), 5′-ATGACTGAGTATAAACTTGT-3′; β (20-mer), 5′-ATCCACAAAGTGATTCTGAA-3′; and G_α (74-mer), 5′-GCCGCCTGCAGCCCGCGCCCCCCGTGCCCCCGCCCCGCCGCCGGCCCGGGCGCCATGACTGAGTAT-3′.

Dot-Blot Allele-Specific Oligonucleotide (ASO) Hybridization of PCR DNA. Oligonucleotide hybridization was carried out essentially as described (28) with the following modifications. About 10–20 ng of the 90-bp PCR-amplified DNA (exon 1 of Ki-*ras* gene) was spotted onto a GeneScreen nylon membrane (New England Nuclear) and UV-crosslinked. The membrane was prehybridized for 1 hr at 56°C in a sealed bag in 10 ml of buffer, containing 3.0 M tetramethyl ammonium chloride (Me_4NCl), 50 mM Tris·HCl (pH 7.5), 1 mM EDTA, 1% SDS, and 1 mg of transfer RNA (tRNA) per ml and was hybridized for 2 hr at 56°C in 10 ml of fresh buffer containing, in addition, 20 pmol of an end-labeled oligonucleotide primer (20-mer) specific for codon 12 of the Ki-*ras* exon 1 region (GGT, GAT, TGT, GTT, and AGT) (29). The membrane was washed successively in 2× SSC (300 mM sodium chloride/30 mM sodium citrate, pH 7.0) for 5 min at room temperature, in 3.0 M Me_4NCl/1% SDS for 10 min at 56°C, and finally in the same solution for 15–30 min at 58–62°C (depending on the probe used). At the end of the wash, the membrane was briefly washed in 2× SSC, air-dried, and exposed to x-ray film with a single Cronex intensifier screen.

DGGE. DNA with an attached G+C clamp at the 5′ end was analyzed by DGGE as follows. The wild-type allele used as a probe was prepared by using γ-^{32}P-end-labeled primers for the 10–15 cycles of PCR amplification required for addition of the G+C clamp at the 5′ end of the molecule. The probe was purified on a linear gradient of formamide/urea (15%–60%) denaturing gradient gel to remove all nonspecific bands generated during the amplification step. Addition of this modification step considerably reduced the background nonspecific bands initially observed in the DGGE analysis. The probe thus prepared was hybridized to an excess (5×–10×) of the DNA samples by boiling for 5 min and reannealing the mixture at 56°C for 3–4 hr in a reaction buffer containing 300 mM NaCl, 10 mM Tris (pH 7.5), and 1 mM EDTA. After hybridization, the DNA was precipitated with ethanol, dried, and resuspended in 1× loading buffer [1× TAE (40 mM Tris/20 mM sodium acetate/1 mM EDTA, pH 7.5) containing 1% bromophenol blue, 1% xylene cyanol, and 50% (vol/vol) glycerol]. The gel was prerun at 150 V and at 60°C for 30 min. About 5 × 10^3 cpm were loaded per lane. Electrophoresis was carried out for 12–15 hr under the same conditions. The gel was fixed in methanol/acetic acid, vacuum dried, and exposed to Kodak X-ray film with an intensifier screen. Wild type–wild type homoduplex, wild type–mutant heteroduplex, and mutant–mutant homoduplex molecules migrated to different distances in the gel and could be differentiated readily.

Enrichment of the Mutant Fraction. After DGGE, the heteroduplex regions focusing the codon 12 mutant bands (above the wild-type band) were excised from the gel, and DNA was recovered by electroelution followed by ethanol precipitation. The DNA enriched in the mutant fraction was further reamplified by using *Taq* polymerase enzyme and was analyzed on DGGE as described above.

RESULTS

Results of oligonucleotide hybridization analysis of PCR-amplified DNA of liver adenomas or carcinomas from animals killed 56–68 weeks after termination of AFB_1 administration (as described in Table 1) are summarized in Figs. 1 and 2. The activating G·C-to-A·T mutation in codon 12 of the rat c-Ki-*ras* gene (GGT to GAT) was prevalent in the liver DNA samples analyzed (7 of 9). We also detected the base changes GGT to TGT and GGT to GTT in the liver DNA (1 of 9 cases each), while all of the DNA samples were negative for the v-Ki-*ras*-specific GGT-to-AGT transition. Multiple mutations occurring in two of the samples (2a and 7a, respectively,

Table 1. Characterization of base substitutions in the rat Ki-*ras* gene of DNA from livers of rats killed 56–68 weeks after termination of AFB_1 dosing

Week	Liver sample	Histology	Ki-*ras* codon 12 sequence
56	1a	A	GGT, *GAT*
	2a	A	GGT, GAT, *TGT*, *GTT*
	3a	A	GGT, *GAT*
63	4a	A	GGT, GAT, *TGT*
	5a	A	GGT, *GAT*
	6a	A	GGT, *GAT*
68	7a	HCC	GGT, GAT, *TGT*
	8a	HCC	GGT, GAT, *TGT*
	8b	HCC	GGT, GAT, *TGT*, *GTT*
	9a	A	GGT, *GAT*

The nucleotide change is italicized; A, adenoma; HCC, hepatocellular carcinoma.

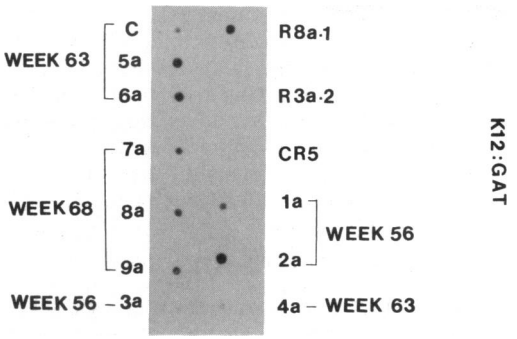

FIG. 1. Dot blot–ASO hybridization analysis. PCR-amplified DNA from adenomas and carcinomas were screened by using ^{32}P-labeled oligonucleotide probes for Ki-*ras* codon 12 substitutions. R8a.1, R3a.2, SW11.1, and pHiHi3 DNA (described in text) were used as positive controls. CR5 and C are DNA from normal rat liver; 1a to 9a represented DNA from the liver samples analyzed. The oligonucleotide probe used detected GAT mutations in codon 12.

carrying GAT, GTT and GAT, TGT) may be explained by the heterogeneity of the cell population within each sample.

For DGGE analysis of the liver lesions for Ki-*ras* gene mutations, the melting behavior of the PCR-amplified DNA with or without the addition of the G+C clamp was computer simulated (see Fig. 3*B*) by using an algorithm developed by Lerman and co-workers (13, 14). Fig. 3*A* outlines the amplification scheme employed. Positive control genomic DNA from which the 144-bp exon 1 region of Ki-*ras* gene was amplified was initially analyzed on a linear gradient of formamide/urea denaturing gel (20%–60% denaturant). These DNA samples carried known base substitutions in the codon 12 region as described in *Materials and Methods*. Mutant alleles were not distinguishable from the wild-type allele under nondenaturing conditions (data not shown). On the other hand, in the DGGE analysis (Fig. 4), all mutant bands formed heteroduplexes with the wild-type allele and focused above the wild-type band in a sequence-specific manner. Since only the wild-type DNA was radiolabeled, the mutant–mutant homoduplex fraction was not visible in the gel, facilitating detection of base substitutions occurring in the region of interest. Densitometric scanning of the respective bands in the autoradiogram was used to identify the mutant population in each DNA sample. These positive control DNA samples were employed in further studies to locate the position of each mutant band in the gel. For unknown reasons, mutant bands from the R8a.1 transformant displayed mobility slightly different from those from the primary tumor DNA, R8a.

Genomic DNA isolated from AFB_1-induced adenomas and carcinomas from livers of rats killed 56–68 weeks after AFB_1 treatment was analyzed for Ki-*ras* gene codon 12 mutations by using modified T7 DNA polymerase (Sequenase) for high-fidelity amplification, with results shown in Fig. 5. DGGE analysis was found to be more sensitive in detecting base substitutions compared with ASO hybridization, when one takes into consideration the quantity of DNA required and the number of mutations visible per analysis. DNA from samples 2a, 7a, 8a, 8b, and 9a showed multiple mutations, with 2a and 8b DNA carrying three different substitutions in

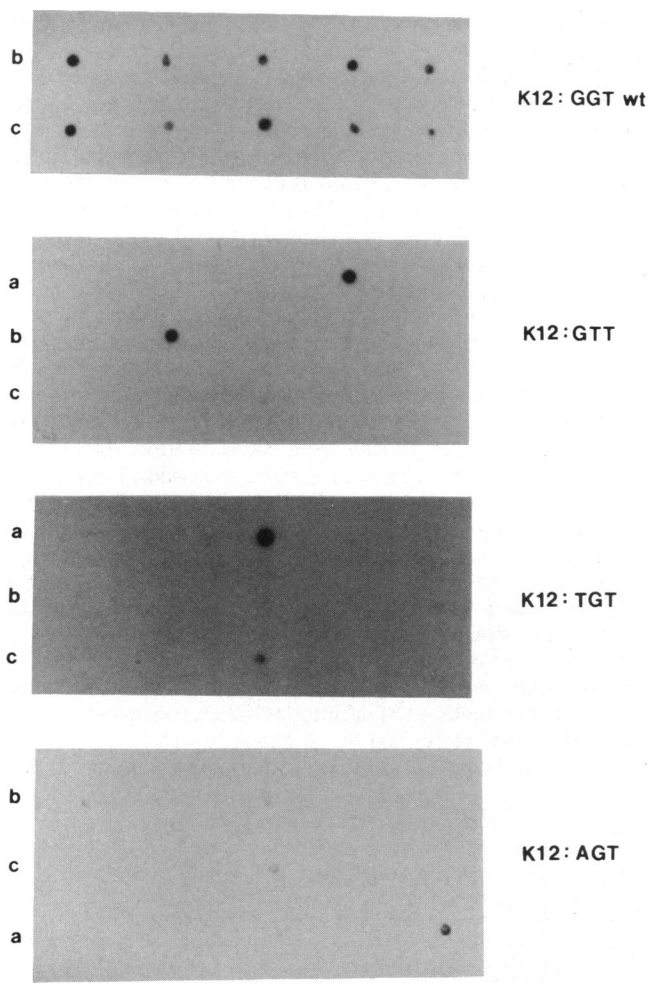

FIG. 2. Wild-type (wt) (GGT) and mutant alleles (GTT, TGT, and AGT, respectively) were screened by dot blot–ASO hybridization with appropriate oligonucleotide probes as indicated. The various DNA sources were as follows: in rows a, CR5, R8a.1, R3a.2, SW11.1, and pHiHi3; in rows b, 1a, 2a, 3a, and 4c; and in rows c: 5a, 6a, 7a, 8a, and 9a.

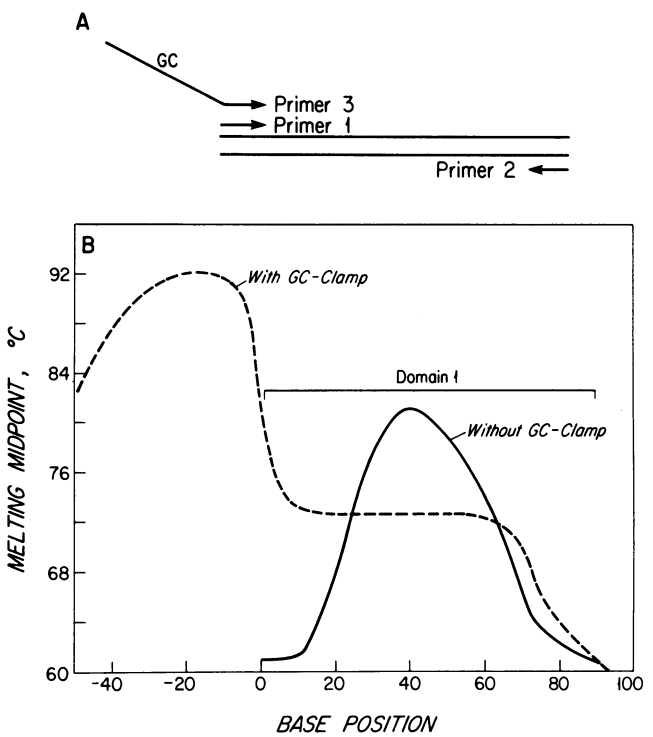

FIG. 3. Melting profile of the exon 1 region of Ki-*ras* gene. (*A*) Schematic representation of the amplification steps used for the addition of a G+C clamp at the 5′ end of the DNA molecule. Primers 1 and 2 amplified a 90-bp region, while primers 2 and 3 amplified a 144-bp DNA. (*B*) Melting behavior of the exon 1 region of rat Ki-*ras* gene with and without the addition of a G+C clamp at the 5′-end.

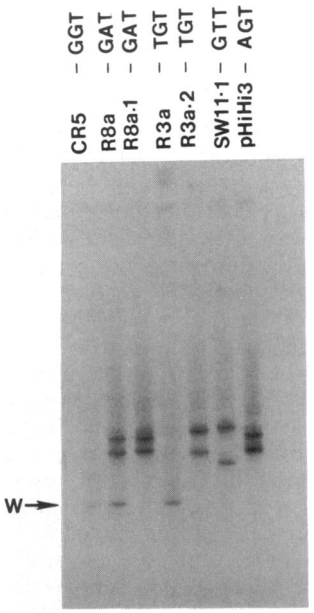

FIG. 4. DGGE analysis of control DNA. Mutant DNA from primary tumors (R8a and R3a) and from 3T3 transfectants (R8a.1 and R3a.2) as well as normal DNA were analyzed on DGGE as described. The mutant alleles formed heteroduplexes with the wild-type allele and were separated from the wild-type homoduplex molecule, denoted "W."

the codon 12 region. As described earlier, the ASO hybridization method also detected multiple mutations, but only in two samples. In Fig. 5, lane C, the region above the wild-type homoduplex signal showed the background generated probably by the errors inherent in the primers as well as in the amplification process. The background was noticeably reduced by the procedures described here compared with that produced in a similar study using previously reported PCR–G+C clamp–DGGE techniques (data not shown). The mutant bands in the liver DNA samples were detected against the background by simultaneous analysis of the wild-type control DNA (codon 12: GGT) and DNA carrying known codon 12 mutations. Since mutant alleles could be physically separated from the wild-type allele, mutations occurring at very low frequency could be enriched in the sample population by reamplification of the mutant fraction previously separated on a denaturing gradient gel. Fig. 6 shows the PCR–DGGE analysis of mutant DNA from samples 2a, 8a, and 8b after reamplification. The corresponding region from the control sample, C, was also simultaneously analyzed for comparison. As shown in the figure, although the low-frequency mutant population became enriched after reamplification, the process concurrently produced and increased background signal. However, the background bands were consistent and reproducible. Analysis of the remaining DNA samples after reamplification of the mutant fraction showed additional mutations, and the results are summarized in Table 1. Results from both PCR–ASO hybridization and PCR–DGGE analyses were in good agreement, with the latter providing increased sensitivity by allowing enrichment of mutant fractions. All (10 of 10) rat liver adenomas/carcinomas analyzed showed point mutations in codon 12 of the Ki-*ras* gene. Multiple mutations were observed in many instances, presumably indicating cellular heterogeneity in the tumors.

DISCUSSION

Many tumors induced by chemical carcinogens in experimental animals have been shown to contain oncogenes activated by base-substitution mutations. In experimental liver tumorigenesis models with rats or mice, activation of both Ha-*ras* and Ki-*ras* oncogenes has been associated with different carcinogens (30–32). Previous studies by our group and others on AFB_1-induced rat liver tumorigenesis have identified two activating mutations in the codon 12 region of Ki-*ras* genes (GGT to GAT and TGT, respectively) in DNA of end-stage hepatocellular carcinomas (5, 33). The present study was designed to confirm our earlier findings by analysis of DNA obtained from a new group of animals bearing AFB_1-induced tumors and to extend the analysis to earlier stages of tumor development for a more complete elucidation of the role of Ki-*ras* gene activation in the tumorigenesis process. The treatment protocol used to induce tumors was essentially the same as in the previous experiment, except that carcinogen dosing was initiated at 36 rather than 21 days of age. As expected, this change in protocol resulted in a longer latency period for tumor development and, consequently, reduced the incidence of end-stage hepatocellular carcinomas at the time the experiment was terminated (68 weeks after termination of dosing), compared with similar studies involving treatment of younger animals (22). All liver tissues obtained from animals killed 63 weeks after termination of dosing and beyond showed morphological changes characteristic of malignant neoplasms (21). Consistent histopathologic abnormalities associated with earlier stages of tumorigenesis (preneoplastic lesions), including increased mitotic activity, basophilia, and hyperplasia were present in the livers of animals killed 20 weeks after termination of dosing or later. The results of our analysis of carcinomas or precursor lesions appearing from week 56 after dosing and beyond showed the presence of the Ki-*ras* codon 12 mutation GGT → GAT in the liver DNA, indicating that Ki-*ras* mutation is a prevalent event in liver carcinogenesis induced by AFB_1.

Activated Ki-*ras* oncogene was detected in all of the late-stage adenomas and carcinomas analyzed, with the G·C-to-A·T transition mutation in the second position of codon 12 being the predominant substitution. In addition, G·C-to-T·A transversions in the first position of codon 12 were also found in 5 of 10 adenomas and carcinomas. These observations are consistent with the spectrum of mutations reported by previous investigators for aflatoxin-induced liver tumors in rats

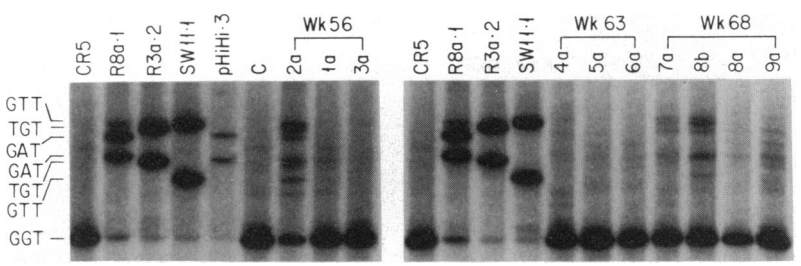

FIG. 5. DGGE analysis of DNA from adenomas and carcinomas. The mutant heteroduplexes were separated from the wild-type homoduplex molecule and are denoted at the left-hand side of the figure. Liver DNA was isolated from animals killed at weeks (Wk) 56, 63, and 68 after termination of AFB_1 dosage. The region of the gel focusing the mutant heteroduplexes was excised, and DNA was recovered for reamplification.

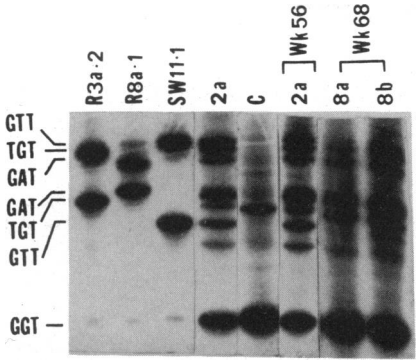

FIG. 6. DGGE analysis of mutant-enriched DNA. The mutant heteroduplexes are denoted against the background bands. The mutant bands of R8a.1 migrated slightly differently; therefore, DNA bands corresponding to GAT alleles from 2a were used as markers.

(5, 33). However, in the previous studies, Ki-*ras* activation was observed in AFB_1-induced hepatocellular carcinomas at a much lower frequency, and no information was available concerning the possible presence of the activated gene in earlier stages of tumor development. In the present experiment, some of the adenomas and carcinomas analyzed showed more than one type of base substitution in the Ki-*ras* gene. The variability in Ki-*ras* gene sequences within the same tumor or precancerous lesion is thought to reflect heterogeneity of cell types present in the tissue from which DNA was extracted. Another significant finding was the presence of a G·C-to-T·A transversion in the second position of codon 12 (GGT to GTT) in 2 of 10 adenomas and carcinomas examined. This mutation was not previously found in AFB_1-induced liver tumors but has been shown to occur frequently in the human c-Ki-*ras2* gene as an activating mutation in various neoplasms.

The results presented in this study clearly demonstrate the greater sensitivity of the DGGE method over the oligonucleotide hybridization technique for detection of base substitutions. The DGGE method further allowed the physical separation of the mutant population from the wild-type DNA. Earlier attempts to use the PCR–G+C clamp–DGGE method could be successfully applied only to the analysis of end-stage tumors or adenomas carrying a high mutant fraction because of the high level of background bands present during the first round of DGGE separation. The modification used in this study has extended the applicability of the method for the analysis of lesions containing a low mutant population. However, the method in its present state is not suitable for analysis of base changes occurring very early during the liver tumorigenesis process because the reamplification step employed produces significant background noise that could interfere with the interpretation of data, especially when the mutant population is very low. The sensitivity with which mutations can be detected may be enhanced by application of the method to the analysis of DNA from isolated preneoplastic foci or from cells showing morphological changes characteristic of neoplastic development enriched by separation from normal hepatocytes and other cells present in liver.

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