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Mutational Analysis of the H-*ras* Oncogene in Spontaneous C57BL/6 × C3H/He Mouse Liver Tumors and Tumors Induced with Genotoxic and Nongenotoxic Hepatocarcinogens[1]

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ABSTRACT

The frequency and mutational profile of H-*ras* gene activation were determined in spontaneous liver tumors of male C57BL/6 × C3H/He mice and in tumors induced with the genotoxic hepatocarcinogen benzidine·2 HCl or the nongenotoxic hepatocarcinogens phenobarbital, chloroform, and ciprofibrate. DNA sequence analysis of the H-*ras* gene from representative tumors revealed that 32 of 50 (64%) spontaneous tumors and 13 of 22 (59%) benzidine·2 HCl-induced tumors contained a point mutation in codon 61. Tumors induced with the nongenotoxic agents had a much lower frequency of codon 61 mutations, *i.e.*, phenobarbital, 1 of 15 (7%); chloroform, 5 of 24 (21%), and ciprofibrate, 8 of 39 (21%). No mutations were observed at codons 12, 13, and 117 in tumors from any of the groups. Only three base pair substitutions within codon 61 were found. The one most frequently detected in all of the groups was a C·G to A·T transversion at the first nucleotide position, occurring at a 59%, 85%, 100%, 80%, and 88% frequency in the spontaneous tumors and in the tumors induced with benzidine 2·HCl, phenobarbital, chloroform, and ciprofibrate, respectively. In these same groups an A·T to G·C transition or an A·T to T·A transversion at the second nucleotide position occurred at a frequency of 34%, 8%, 0%, 0%, and 12%, and 6%, 8%, 0%, 20%, and 0%, respectively. The number of tumors carrying an activated H-*ras* gene in the nongenotoxic treatment groups is within the range that would be expected if those animals had not received any treatment. This indicates that the activation of the H-*ras* gene in those tumors is probably the result of a spontaneous event. The data suggest that these toxicologically and pharmacologically diverse nongenotoxic hepatocarcinogens increase the frequency of liver tumors but do not induce mutations in the H-*ras* gene. Instead these agents appear to interact with a population of cells that do not contain an activated H-*ras* gene. This suggests that the mechanisms of tumor development by these nongenotoxic carcinogens differ at least partially from the mechanisms responsible for the development of spontaneous tumors or those induced by a typical genotoxic agent.

INTRODUCTION

The results of the rodent bioassay play an important role in the evaluation of whether a chemical is considered potentially carcinogenic for humans. However, the use of such test results to predict human risk is often complicated by biological factors peculiar to the strain of animal used. For example, the C57BL/6 × C3H/He (hereafter called B6C3F$_1$) mouse is frequently used to assay various potential carcinogens. Male B6C3F$_1$ mice have an average spontaneous liver tumor incidence of 20 to 30% (1). These mice are apparently hypersensitive to the development of such tumors which calls into question the relevance of a chemically induced increase in tumor frequency as a predictor of human cancer risk. This concern is underscored by the fact that many genotoxic and nongenotoxic agents significantly increase the frequency of liver tumors in this animal but may not be tumorigenic at other sites or in other species (2).

For example, Ashby and Tennant (3) surveyed 222 carcinogen bioassays conducted by the National Cancer Institute/National Toxicology Program. Of the 115 chemicals found to be tumorigenic, nearly 40% were hepatocarcinogenic in the B6C3F$_1$ mouse. For 20% of the 115 carcinogens the only tumorigenic response was an increase in mouse liver tumors. Based on *in vitro* mutagenicity tests many of these tumorigenic chemicals have been shown to have genotoxic activity (*i.e.*, damage DNA). The mutagenic activity of these agents can be initiated from the parent molecule or from electrophilic metabolic intermediates. It is believed that damage to DNA resulting in somatic mutations plays an important role in the tumorigenicity of this class of chemical carcinogens (4).

Not all carcinogenic chemicals have mutagenic activity as determined by *in vitro* mutagenicity assays. An important class of chemical carcinogens, generally referred to as nongenotoxic, do not cause mutations but still can cause an increase in animal tumors. The mechanism by which these agents increase mouse liver tumors is presently unclear. They may exert their carcinogenic activity by promoting previously initiated cells (5). Alternatively, these agents may induce somatic mutations by indirectly damaging DNA as a consequence to some abnormally induced physiological response. For example, this secondary damage to DNA may occur as a result of the interaction of reactive oxygen radicals with DNA generated by the infiltration of polymorphonuclear leukocytes as a consequence to chemically induced cellular toxicity (6).

Much of the controversy surrounding the interpretation of bioassay results concerns the relevance to human risk from exposure to nongenotoxic agents that cause an increase in mouse liver tumors. In an attempt to gain a better understanding of how liver tumors arise in the B6C3F$_1$ mouse and how this tumor incidence is increased by chemical exposure, several groups have examined the role of cellular oncogenes in the hepatocarcinogenic process of the B6C3F$_1$ mouse. It was demonstrated that the H-*ras* oncogene is activated in a high percentage of both spontaneous tumors and tumors induced with several genotoxic hepatocarcinogens (7–10). In addition Wiseman *et al.* (9) and Reynolds *et al.* (10) showed that certain genotoxic carcinogens cause chemical-specific mutational patterns within the H-*ras* gene.

To investigate the mechanisms by which nongenotoxic hepatocarcinogens enhance liver tumor development in the B6C3F$_1$ mouse, we determined the frequency and mutational profile of H-*ras* gene activation in tumors that arose spontaneously and in tumors induced by exposure to a genotoxic or several nongenotoxic carcinogens. This was carried out by amplifying regions of exons 1, 2, and 3 of the H-*ras* gene which

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include the activating codons 12, 13, 61, and 117 from tumor DNA using the PCR technique.[3] The amplification products were subjected to direct DNA sequence analysis to identify point mutation within these activating codons. The results showed that tumors induced with the nongenotoxic hepatocarcinogens phenobarbital, chloroform, or ciprofibrate have a much lower frequency of H-*ras* gene activation than do those that arose spontaneously or those induced with the genotoxic carcinogen benzidine·2 HCl.

MATERIALS AND METHODS

Tumor Induction. Spontaneous liver tumors were obtained from control male B6C3F$_1$ mice collected at the terminal necropsy of 2-yr bioassays for carcinogenicity conducted at the Environmental and Toxicology Research Laboratory of The Dow Chemical Company (Midland, MI). Chemically induced liver tumors were obtained from independently treated groups of male B6C3F$_1$ mice administered either benzidine·2 HCl (120 ppm, drinking H_2O, 1 yr), phenobarbital (0.05%, drinking H_2O, 1 yr), chloroform (200-mg/kg corn oil gavage, 2 times weekly for 1 yr), or ciprofibrate (0.0125% diet, 2 yr). Animals were put on test at 6 wk of age and necropsied after 18 to 24 mo. At necropsy tumors were excised, and a section of each tumor was fixed in neutral phosphate-buffered 10% formalin. Tissues were processed by conventional techniques, embedded in paraffin, cut at approximately 6 μm, stained with hematoxylin and eosin, and examined using a light microscope. The remainder of the tumor was frozen on dry ice and stored at −70°C until DNA was isolated.

Oligonucleotide Primer Preparation. Oligonucleotide primers used in PCR amplification and DNA sequencing were synthesized using the phosphoramidite method on an Applied Biosystems Model 380A DNA synthesizer. Detritylated primers were purified by HPLC using a 10-μm RP-300 reverse-phase column (Brownlee Laboratories). Elution of primers from the column was achieved with a linear 7 to 17% acetylnitrile gradient (50 min, 1 ml/min) in 0.1 M tetramethylammonium acetate (pH 7.0). HPLC fractions containing the purified primer were pooled, evaporated to dryness, reconstituted with distilled H_2O, and purified by ultrafiltration using a Centricon-3 (Amicon, W. R. Grace, Danvers, MA).

PCR Amplification. Total genomic DNA was isolated from liver tumor tissue as previously described (5). Prior to PCR amplification approximately 2 μg of tumor DNA were digested with 2 units of *Bam*HI restriction endonuclease (37°C, 30 min) as recommended by the manufacturer (Bethesda Research Laboratories). Restriction endonuclease digestion of the genomic DNA resulted in an increased amplification yield of the H-*ras* gene sequences presumably by increasing the template accessibility to the PCR-polymerizing enzyme.[4]

The regions of the H-*ras* gene illustrated in Fig. 1 were PCR amplified as described by Mullis *et al.* (11) with the following modifications. Amplification was conducted in a total volume of 50 μl which was initiated with the addition of *Taq* enzyme followed by 30 cycles of successive incubations for 1 min each at 65°C (annealing), 70°C (polymerization), and 94°C (denaturing). Confirmation of amplification was assessed by agarose gel electrophoresis. Ten μl of the PCR reaction mixture were loaded onto a 3% Nusieve (FMC Bioproducts, Rockland, ME)-1% agarose gel, and DNA bands were visualized by ethidium bromide staining.

DNA Sequencing. The protocol for DNA sequence analysis was adopted from that of Yang *et al.* (12). Following the termination of amplification the PCR reaction mixture was purified by centrifugation through a Centricon-30 to remove unincorporated nucleotides and excess oligonucleotide PCR primers. The Centricon-30 retentate was sequenced directly using a modification of the Sanger dideoxy chain termination protocol (13). The deoxynucleotide:dideoxynucleotide ratio used in the sequencing reactions was 10:1. Deoxynucleotide concentrations were 33 μmol, and dideoxynucleotides were 3.3 μmol. Sequencing primers were end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase as described by the supplier (BRL, Gaithersburg, MD). Specific activities of the sequencing primers routinely ranged from 1 to 5×10^8 cpm/μg. At the termination of the sequencing reaction, 2.5 ul of the reaction sample were loaded onto an 8% polyacrylamide·8 M urea gel and electrophoresed at 40 mA. The gel was transferred to Whatman No. 3MM paper, dried under vacuum, and exposed to Kodak XAR-5 film at −70°C for 24 to 48 h.

[3] The abbreviations used are: PCR, polymerase chain reaction; HPLC, high-pressure liquid chromatography.

[4] T. Fox, unpublished observations.

RESULTS

Tumor Induction. Tissue used for analysis in this study was derived from male B6C3F$_1$ murine liver tumors occurring spontaneously or induced by treatment with the genotoxic hepatocarcinogen benzidine·2 HCl or the nongenotoxic hepatocarcinogens phenobarbital, chloroform, or ciprofibrate. Mice bearing spontaneous tumors, obtained from the control groups of 6 different bioassays, had an average tumor incidence of 42% with 22% of the tumor-bearing animals having multiple liver tumors (Table 1). A treatment-related effect was observed for all compounds within the treatment groups as indicated by the increase in percentage of tumor incidence and tumor multiplicity compared with the control animals (Table 1). In many of the tumor-bearing animals exposed to benzidine·2 HCl, the livers were so tumorous that it was difficult to distinguish individual tumors. Consequently, multiple tumors could not be counted in these animals, resulting in an artificially lower frequency of tumor multiplicity. Tumors ranged in size from approximately 1 mm to 2.5 cm in diameter.

H-*ras* Gene Activation. The activation of the H-*ras* gene in a variety of human and rodent tumors has been shown to involve point mutations at codons 12, 13, 61, or 117 (10, 14). The extent of H-*ras* gene activation in the B6C3F$_1$ mouse liver tumors of the various treatment groups was assessed by determining the presence or absence of point mutations within these activating codons. Codons 12 and 13, 61 and 117 reside in exons 1, 2, and 3, respectively, of the H-*ras* gene (15). Point mutations in these codons were identified by direct sequence analysis of amplified regions within the correponding exons from genomic tumor DNA. These exon regions were amplified using the PCR technique. The amplified regions of the H-*ras* gene with the corresponding PCR and sequencing primers are illustrated in Fig. 1.

Sequence analysis of the H-*ras* gene was carried out on a total of 151 tumors from the combined groups. Mutations were detected only in codon 61 and not at codons 12, 13, and 117 in any of the tumors. As a result, the percentage of tumors with a mutation in codon 61 is equivalent to the percentage of tumors with an activated H-*ras* gene.

As can be seen in Table 2, the percentage of tumors with an activated H-*ras* gene in the spontaneous and benzidine·2 HCl-induced tumors is considerably higher than in the tumors

Table 1 *Incidence of spontaneous and chemically induced liver tumors in male B6C3F$_1$ mice*

Treatment used to induce tumors	No. of animals	Animals with tumors[a]	% of animals with tumors	% of tumor-bearing animals with multiple tumors
None (spontaneous)	293	124	42	22
Benzidine·2 HCl	27	23	85	39
Phenobarbital	25	17	68	65
Chloroform	17	15	80	80
Ciprofibrate	31	29	94	79

[a] All animals were necropsied after 18 to 24 mo of age, and liver tumors were removed for further analysis.

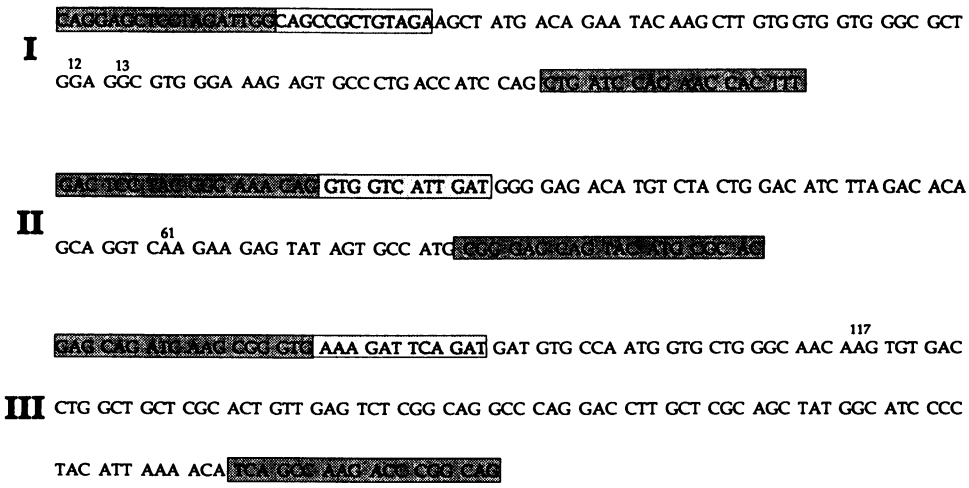

Fig. 1. Nucleotide sequence of the coding strand for the H-*ras* gene which was PCR amplified and subsequently sequenced. *Fragment I* represents the region of exon 1 containing codons 12 and 13. *Fragment II* is the region of exon 2 containing codon 61. *Fragment III* is the entire sequence of exon 3 containing codon 117. The *darkly shaded areas* indicate the position of the 5′ and 3′ PCR primers. *Lightly shaded areas* represent the sequencing primers as derived from BALB/c murine sarcoma virus (16).

Table 2 *Frequency of activation of the H-ras gene at codon 61 in male B6C3F$_1$ mouse liver tumors*

Treatment used to induce tumors	No. of tumors analyzed[a]	Tumors with an activated H-*ras*[a]
None (spontaneous)	50	32 (64)[b]
Benzidine·2 HCl	22	13 (59)
Phenobarbital	15	1 (7)
Chloroform	24	5 (21)
Ciprofibrate	39	8 (21)

[a] Total genomic DNA was isolated from liver tumor tissue. Defined regions of exons 1, 2, and 3 of the H-*ras* gene incorporating codons 12–13, 61, and 117, respectively, were PCR amplified and sequenced directly. The sequence was determined for 97 of the total 190 codons of the H-*ras* gene.
[b] Numbers in parentheses, percentage.

Table 3 *Frequency of H-ras gene activation in hepatocellular adenomas and carcinomas in male B6C3F$_1$ mice*

Treatment used to induce tumors	% of adenomas with activated H-*ras*[a]	% of carcinomas with activated H-*ras*[a]
None (spontaneous)	83 (15/18)	71 (10/14)
Benzidine·2 HCl	53 (8/15)	67 (4/6)
Phenobarbital	11 (1/9)	0 (0/5)
Chloroform	15 (2/13)	33 (3/9)
Ciprofibrate	21 (6/29)	22 (2/9)

[a] A portion of each tumor was excised, fixed at necropsy in neutral phosphate-buffered 10% formalin, processed by standard techniques, and embedded in paraffin. Sections (6 μm) were cut, stained, and evaluated by light microscopy.

induced by treatment with phenobarbital, chloroform, or ciprofibrate. Analysis of spontaneous tumors revealed that 64% contained a point mutation in codon 61. A similar response was observed in the benzidine·2 HCl-induced tumors, where 59% of tumors had codon 61 mutations. This is in contrast to the tumors induced by the nongenotoxic agents, where activating H-*ras* gene mutations were detected at a much lower frequency, *i.e.*, phenobarbital, 7%, chloroform, 21%, and ciprofibrate, 21%.

Histopathological examinations were conducted on all tumors except those that were too small to forfeit tissue for analysis. A correlation between the incidence of H-*ras* gene activation and the development of either a hepatocellular adenoma or hepatocellular carcinoma was made, and the data are summarized in Table 3. There was no statistically significant difference between the frequency of H-*ras* gene activation in the hepatocellular adenomas and carcinomas (statistical analysis determined using a χ^2 contingency table test). Histopathological examination of the spontaneous tumors and the tumors induced with benzidine·2 HCl, phenobarbital, and chloroform did not reveal any significant differences in morphology or staining characteristics. The ciprofibrate-induced tumors were more eosinophilic as were the surrounding normal hepatocytes. This observation would be expected from a treatment-related increase in cytoplasmic peroxisomes.

The mutational spectrum of activating codon 61 mutations within the H-*ras* gene was determined, and the data are summarized in Table 4. The normal nucleotide sequence for codon 61 in the mouse H-*ras* gene is CAA. The activating mutations observed in all tumor groups involved a single base pair substitution, changing this sequence to AAA, CGA, or CTA. Representative examples of sequence gels exhibiting these three mutations are shown in Fig. 2.

The most frequently detected mutation was a C·G to A·T transversion at the first nucleotide position. The frequency of this base pair substitution in the spontaneous tumors and in the tumors induced with benzidine·2 HCl, phenobarbital, chloroform, and ciprofibrate was 59%, 85%, 100%, 80%, and 88%, respectively. An A·T to G·C transition or a A·T to T·A transversion at the second nucleotide position occurred in these same groups at a frequency of 34%, 8%, 0%, 0%, and 12%, and 6%, 8%, 0%, 20%, and 0%, respectively.

Many of the animals in the chemically induced tumor groups had multiple tumors as is indicated in Table 1. A comparison of the H-*ras* codon 61 nucleotide sequence of the individual tumors from animals with multiple tumors revealed that, in approximately one-half of those animals examined, a different codon 61 sequence was observed.

DISCUSSION

Mutational analysis of tumor DNA from spontaneous tumors and tumors induced with benzidine·2 HCl, phenobarbital, chloroform, or ciprofibrate revealed activating mutations exclusively in codon 61. Only 3 activating point mutations within this codon were detected: a C·G to A·T transversion at the first nucleotide position; an A·T to G·C transition; or an A·T

Table 4 *Mutation spectrum in codon 61 (CAA) of the H-ras gene in male B6C3F$_1$ mouse liver tumors*

Treatment used to induce tumors	Tumors with an activated H-*ras* gene	No. of tumors with specific codon 61 mutations[a]		
		AAA	CGA	CTA
None (spontaneous)	32	19 (59)[b]	11 (34)	2 (6)
Benzidine·2 HCl	13	11 (85)	1 (8)	1 (8)
Phenobarbital	1	1 (100)	0	0
Chloroform	5	4 (80)	0	1 (20)
Ciprofibrate	8	7 (88)	1 (12)	0

[a] Codon 61 sequence was determined as described in Table 2.
[b] Numbers in parentheses, percentage.

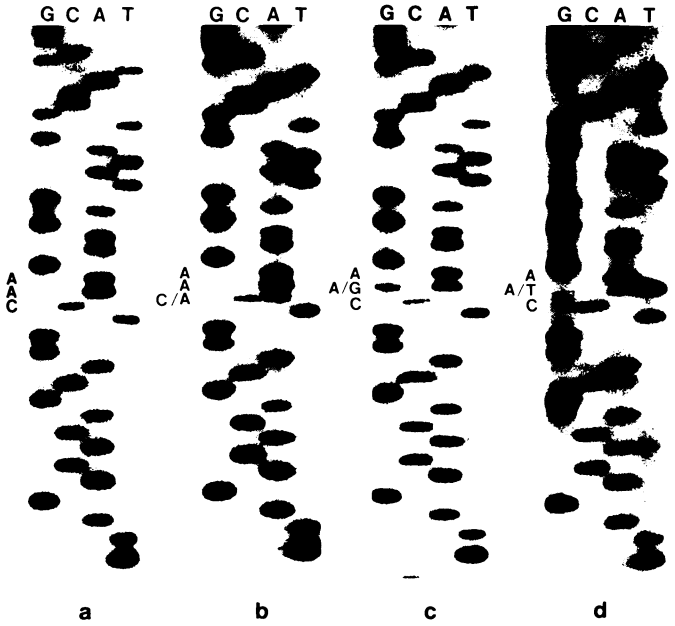

Fig. 2. Representative DNA sequence of the H-*ras* gene codon 61 region derived from PCR-amplified liver tumor DNA. *a*, normal codon 61 sequence (CAA) derived from a ciprofibrate-induced tumor; *b*, C·G → A·T transversion mutation at codon 61 (AAA) from ciprofibrate-induced liver tumor; *c*, A·T → G·C transition (CGA) from benzidine·2 HCl-induced liver tumor; and *d*, A·T → T·A transversion (CTA) from spontaneous liver tumor.

to T·A transversion at the second nucleotide position. These point mutations resulted in the amino acid substitution of glycine in the H-*ras* p21 with lysine, arginine, or leucine, respectively.

The predominant mutation in all treatment groups was the point mutation converting the normal codon 61 sequence of CAA to AAA. It has been demonstrated by Strauss *et al.* (17) that, during DNA synthesis on a noninstructional template, the most frequent polymerase error involves the preferential insertion of an adenine, the "A rule." In the spontaneous tumors the high frequency of C·G to A·T transversions observed at this nucleotide position may be attributed to this type of polymerase error.

The frequency of AAA codon 61 mutations in DNA from tumors taken from benzidine·2 HCl-treated animals was slightly higher than that observed in the DNA from spontaneous tumors. χ^2 analysis showed that this increase was not statistically significant. Some of the tumors carrying this specific mutation could be of spontaneous origin considering the high frequency of this mutation observed in spontaneous tumors. However, this increase could be due to the mutagenic activity of benzidine·2 HCl. Benzidine·2 HCl is a potent genotoxic agent which has been shown to cause primarily C-8 adducts on guanine (18). The formation of such an adduct on the guanine opposite cytosine at the first position in codon 61 could be expected to cause a C·G to A·T transversion. This transversion could occur either by the preferential insertion of an adenine across from a noninstructive base or as a result of the adduct allowing stable base pairing between the modified guanine with an adenine as was reported recently for guanine carrying an Aminofluorine residue at the C-8 position (19).

Several investigators have also determined the mutational spectra of codon 61 within the H-*ras* gene from B6C3F$_1$ mouse liver tumors induced with various genotoxic carcinogens. Wiseman *et al.* (9) induced B6C3F$_1$ liver tumors with *N*-hydroxy-2-acetylaminofluorene, vinyl carbamate, or 1′-hydroxy-2′,3′-dehydroestragole. These agents produce codon 61 mutational spectra that are distinct from the mutational spectra observed in spontaneous tumors. A similar analysis by Reynolds *et al.* (8) of tumors induced with furan and furfural observed mutations not only in codon 61 but at codons 13 and 117, two activating codons that have not been detected previously in tumor DNA. The carcinogenic agents used in these studies therefore produce unique chemical mutation spectra within the H-*ras* gene which are consistent with their apparent genotoxic activity. It has been suggested that the evaluation of the mutational spectra within the H-*ras* gene of B6C3F$_1$ mouse liver tumors may be useful in determining the *in vivo* genotoxic potential of an agent which is positive for tumorigenicity in the rodent bioassay (10). This type of analysis may prove valuable in selected circumstances, but there are limitations that must be realized. This is illustrated by the analysis of liver tumors generated in the B6C3F$_1$ mouse with the potent mutagenic carcinogen *N*-diethylnitrosamine. The codon 61 mutation spectrum observed in these tumors was similar to the mutation spectrum observed in spontaneous tumors (20). Consequently, the presence or absence of genotoxic activity for this particular chemical cannot be concluded using this procedure. This type of analysis is useful only if the mutational spectra of the chemically induced tumors deviate significantly from the spontaneous tumor spectrum. Because the spontaneous tumor spectrum is used as a benchmark for this analysis, a high degree of confidence in this spectrum is an obvious prerequisite.

One of the objectives of the present study was to examine a large number of spontaneous tumors to increase the statistical confidence in this spectrum. Our results have indicated that the mutational spectrum observed in the spontaneous liver tumors is similar to the mutational spectrum previously reported for 15 spontaneous tumors (10). The inclusion of these data in the preexisting data base should increase the degree of confidence in this spectrum.

Analysis of the codon 61 mutational spectra of the tumors induced with the nongenotoxic agents used in this study was not meaningful, because so few of the tumors contained codon 61 mutations. However, it was noted that the most frequently observed mutation in these tumors, a C·G to A·T base transition at the first nucleotide position, was the most common mutation detected in the spontaneous tumors.

Analysis in the frequency of H-*ras* gene activation did reveal significant differences between the tumors induced with the nongenotoxic carcinogens compared with the spontaneous tumors or tumors induced with the genotoxic agent benzidine·2 HCl. The H-*ras* gene was activated to a similar frequency in spontaneous and benzidine·2 HCl-induced tumors (64% and 59%, respectively). The frequency of H-*ras* gene activation was significantly lower in tumors induced with phenobarbital, chloroform, and ciprofibrate (7%, 21%, and 21%, respectively). If one determines the number of tumors within the nongenotoxic induced groups that would be of spontaneous origin (42%), and then calculates the subgroup of these that can be expected to exhibit an H-*ras* activation, *i.e.*, 27% (64% of 42%), this value approximates the number of tumors observed within these groups having an activated H-*ras* gene. In other words, the number of tumors in these groups that contained an activated H-*ras* gene is the number that would be expected if those animals had received no treatment. This suggests that the tumors induced with the nongenotoxic agents having an activated H-*ras* gene are probably of spontaneous origin and, therefore, the increase in tumor frequency above background caused by exposure to these nongenotoxic agents results from their interaction with a population of non-H-*ras*-activated cells.

We concluded that these nongenotoxic agents do not cause mutations within the H-*ras* gene and that they increase the frequency of mouse liver tumor development by a mechanism that differs from the process occurring spontaneously or after treatment with a genotoxic carcinogen.

Activation of this gene was detected in hepatocellular adenomas as small as 1 mm in diameter. Balmain *et al.* (21) have detected an activated H-*ras* gene in chemically induced benign mouse skin papillomas. The detection of an activated H-*ras* gene in very small hepatocellular adenomas as well as in benign skin papillomas suggests that activation of this gene may be an early initiating event in tumorigenesis. The high frequency of H-*ras* gene activation in spontaneous tumors also indicates that this gene plays an important role in B6C3F$_1$ mouse liver tumor development. However, approximately 35% to 40% of the spontaneous and benzidine·2 HCl-induced tumors and approximately 80% to 90% of the tumors induced with the nongenotoxic agents did not have a detectable activated H-*ras* gene. This indicates that alternative pathways independent of H-*ras* gene activation can also lead to hepatic tumor development. These data suggest that there are at least two populations of cells which can give rise to tumors within the liver, one consisting of cells having an activated H-*ras* oncogene and another which does not. The data do not preclude the possibility that cells giving rise to tumors that do not have an activated H-*ras* gene may have other activated *ras* genes (*i.e.*, K-*ras*, N-*ras*). Reynolds *et al.* (10) did detect an activated K-*ras* gene in 7% and 15% of liver tumors in the B6C3F$_1$ mouse induced with furfural and furan, respectively.

The three nongenotoxic agents used in this study have diverse pharmacological and toxicological activities. Phenobarbital has general hepatostimulatory properties resulting in increased gene expression with an associated induction of cellular proliferation (22). Animal tumors induced with chloroform are accompanied by cell necrosis, death, and subsequent regeneration (23). Although cellular proliferation alone is probably not sufficient to cause tumors, it has been shown to be a necessary prerequisite for the fixation of the initiating event in cell culture, and it is believed to be important for the clonal expansion of the initiated cell population *in vivo* (24, 25). Peroxisome proliferators, such as ciprofibrate, have been hypothesized to be responsible for tumor induction by various mechanisms. One of these suggests that they induce indirect secondary damage to DNA as a consequence of an increased production of oxygen-free radicals. However, ciprofibrate has not been shown to cause mutations *in vitro* (26). Alternatively it has been proposed that such agents stimulate the growth of preneoplastic foci, suggesting that they are acting as tumor promoters (27). Even though the biological activity of these nongenotoxic animal tumorigens is diverse, their tumorigenic activity is only observed at doses that cause perturbations in the cells' normal biochemistry and/or physiology. This implies that, at lower doses, they will not cause tumors.

In summary, results from the present study indicate that the nongenotoxic agents phenobarbital, chloroform, and ciprofibrate induce liver tumors in the B6C3F$_1$ mouse by a process that does not require the activation of the H-*ras* gene. These data suggest that at least two different populations of cells, one requiring H-*ras* activation and the other independent of this event, can give rise to liver tumors in these animals. The nongenotoxic agents used in this study apparently increase B6C3F$_1$ mouse liver tumors by interacting with a population of cells that do not contain an activated H-*ras* gene. Additional studies comparing tumors with and without an H-*ras* gene mutation will be required to elucidate the molecular and cellular differences between these cell populations. Understanding these differences should provide insight into how various classes of carcinogens interact with cells to give rise to liver tumors in the B6C3F$_1$ mouse. Such results will be useful in extrapolating bioassay results using this strain of mouse to evaluate potential chemical carcinogenic risk for humans.

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