Activation of the Ha-, Ki-, and N-ras Genes in Chemically Induced Liver Tumors from CD-1 Mice

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ABSTRACT

We compared the profile of ras gene mutations in spontaneous CD-1 mouse liver tumors with that found in liver tumors that were induced by a single i.p. injection of either 7,12-dimethylbenz(a)anthracene (DMBA), 4-aminoazobenzene, N-hydroxy-2-acetylaminofluorene, or N-nitrosodiethylamine. By direct sequencing of polymerase chain reaction-amplified tumor DNA, the carcinogen-induced tumors were found to have much higher frequencies of ras gene activation than spontaneous tumors. Furthermore, each carcinogen caused specific types of ras mutations not detected in spontaneous tumors, including several novel mutations not previously associated with either the carcinogen or mouse hepatocarcinogenesis. For example, the model compound DMBA is known to cause predominantly A to T transversions in Ha-ras codon 61 in mouse skin and mammary tumors, consistent with the ability of DMBA to form bulky adducts with adenosine. Our results demonstrate that the predominant mutation caused by DMBA in mouse liver tumors is a G to C transversion in Ki-ras codon 13 (DMBA is also known to form guanosine adducts), illustrating the influence of both chemical- and tissue-specific factors in determining the type of ras gene mutations in a tumor. 4-Aminoazobenzene and N-hydroxy-2-acetylaminofluorene also caused the Ki-ras codon 13 mutation. In addition, we found that N-nitrosodimethylamine, 4-aminoazobenzene, and N-hydroxy-2-acetylaminofluorene all caused G to T transversions in the N-ras gene (codons 12 or 13). This is the first demonstration of N-ras mutations in mouse liver tumors, establishing a role for the N-ras gene in mouse liver carcinogenesis. Finally, comparison of the ras mutations detected in the direct tumor analysis with those detected after NIH3T3 cell transfection indicates that spontaneous ras mutations (in Ha-ras codon 61) are often present in only a small fraction of the tumor cells, raising the possibility that they may sometimes occur as a late event in CD-1 mouse hepatocarcinogenesis.

INTRODUCTION

Characterization of the genetic lesions caused by carcinogens in tumor DNA enhances our understanding of the mechanisms involved in chemical carcinogenesis, while helping to delineate the multiple steps involved in carcinogenesis in different tissues. One of the most highly characterized lesions associated with carcinogenesis is the activation of the ras genes by point mutation (1–7). Several genotoxic carcinogens have been shown to induce particular patterns of ras gene point mutations, which in certain cases can be correlated to the specificity of the carcinogen for interacting with particular bases in DNA (reviewed in Refs. 3 and 4). The specificity of certain carcinogens for inducing particular patterns of ras gene point mutations has been used to help distinguish chemically induced liver tumors from spontaneous liver tumors, which arise at high and variable incidence in several of the mouse strains used for long-term carcinogenicity studies (8, 9). In spontaneous mouse liver tumors, ras gene point mutations are exclusively found in Ha-ras codon 61, although the frequency of these mutations varies among mouse strains (6, 10–13). Previous studies in B6C3F1 mice demonstrated that liver tumors induced by some genotoxic carcinogens (6, 14, 15) and nongenotoxic carcinogens (11) had different frequencies or profiles of ras gene point mutations than were seen in spontaneous tumors. However, B6C3F1, mouse liver tumors induced by benzidine (11), N-OH-AAF (14), and DEN (16) were reported to have a similar pattern of Ha-ras codon 61 mutations to that seen in spontaneous liver tumors.

Determining the general applicability of ras gene analysis for distinguishing chemically induced tumors from spontaneous tumors requires further evaluation, including studies of other carcinogens and other mouse strains. In this study, we examined ras gene activation in liver tumors from Crl:CD-1(ICR)BR mice (referred to as CD-1 mice), a strain used for carcinogenicity studies in several laboratories including our own (8). We compared spontaneous CD-1 mouse liver tumors with liver tumors induced by a single dose of either DMBA, N-OH-AAF, DEN, or AAB.

MATERIALS AND METHODS

Induction of CD-1 Mouse Liver Tumors. DEN was purchased from Sigma Chemical Co., St. Louis, MO. DMBA and AAB were purchased from Aldrich Chemical Co., Milwaukee, WI. N-OH-AAF was purchased from CCR, Inc., Chanhassen, MN. Each carcinogen was dissolved in 10% dimethyl sulfoxide in triacetin.

Twelve-day-old male CD-1 mice (Charles River Breeding Laboratories, Wilmington, MA), 41–42 animals/treatment group, were given a single i.p. injection of either DMBA (20 nmol/g body weight), AAB (400 nmol/g), N-OH-AAF (200 nmol/g), DEN (20 nmol/g), or the vehicle alone. Liver tumors were harvested from surviving mice 9 or 12 months after injection of the carcinogens (29 of 36 N-OH-AAF-treated mice had a total of 355 tumors; 23 of 31 DMBA-treated mice had a total of 226 tumors; 26 of 37 AAB-treated mice had a total of 256 tumors; 30 of 35 DEN-treated mice had a total of 256 tumors; 3 of 38 vehicle-treated mice had a total of only 9 tumors). Spontaneous liver tumors were isolated from an aging colony of male CD-1 mice that were approximately 22 months old (94 to 98 weeks) old, when survival was about 54% and approximately 58% of the surviving mice had tumors (282 tumors in 125 mice). Note that number of tumors actually refers to grossly visible nodules. Histological examination was performed on only those tumors that were taken for analysis.

Only those tumors greater than 3 mm in diameter were taken for DNA isolation. A section of each tumor with adjacent normal tissue was examined histologically. A portion of the tumor was carefully trimmed of surrounding normal tissue and rapidly frozen in liquid nitrogen. Tumor samples were stored at −70°C until they were processed for DNA extraction. In addition to the liver tumors, normal liver (or kidney) tissue from each mouse was also frozen for use as a negative control for ras mutation analysis.

DNA Isolation. High molecular weight DNA was purified from pulverized frozen tissue by lysis in 4 M guanidinium isothiocyanate solution followed by CsCl gradient centrifugation, treatment with proteinase K, and phenol-chloroform extraction. DNA was subsequently purified by ethanol precipitation.

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2The abbreviations used are: B6C3F1, mice; C57BL/6 × C3H,F1, mice; N-OH-AAF, N-hydroxy-2-acetylaminofluorene; DEN, N-nitrosodimethylamine; DMBA, 7,12-dimethylbenz(a)anthracene; AAB, 4-aminoazobenzene; CD-1 mice, Crl:CD-1(ICR)BR mice; PCR, polymerase chain reaction.
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Tissue K and RNase A, phenol-chloroform extraction, and ethanol precipitation, essentially as described by others (17).

**ras Mutation Analysis.** Direct preparations of tumor DNA were analyzed for ras mutations by PCR amplification followed by Sanger dideoxy-DNA sequencing using 5′-end-labeled primers. The sequences of the oligonucleotide primers used for amplification and sequencing and the procedures used for amplification, sequencing, and 5′-end labeling are described in detail elsewhere (18). For comparative purposes, tumors were also analyzed by transfection of the tumor DNA into NIH3T3 cells, essentially as described previously (19). After transfection, DNA isolated from the NIH3T3 cell transformants was assayed for ras mutations by the same procedures used for direct tumor DNA analysis. In both the direct tumor DNA analysis and the transfection assay, normal tissue DNA (from the same mouse as each tumor) was used as a negative control.

**RESULTS**

**Induction of CD-1 Mouse Liver Tumors.** Each group of carcinogen-treated mice exhibited a fairly high incidence of liver tumors, ranging from 70 to 86% of the surviving mice, with a total of over 200 tumors in each treatment group (see "Materials and Methods"). In contrast, only 9 liver tumors were observed in vehicle-treated control mice (9 to 12 months old), indicating that the vast majority of tumors in each of the carcinogen-treated groups were induced by the single dose of the chemical. Spontaneous liver tumors were isolated from an aging colony of male CD-1 mice that were approximately 22 months old. At this age, 58% of the surviving mice had liver tumors. Although the spontaneous tumors were taken from older mice, they were similar to the carcinogen-induced tumors in size range and in the proportion of adenomas versus carcinomas (data not shown).

Thus, the 9- to 12-month-old carcinogen-treated mice and the 22-month-old aging mice provided us with essentially pure populations of chemically induced tumors and spontaneous tumors, respectively. For this study, 25 to 40 liver tumors from each treatment group (representing a partial sampling of each tumor group) were examined histologically and assayed for ras gene mutations.

**Frequency and Profile of ras Mutations Detected by Direct Tumor DNA Analysis.** As shown in Table 1, each group of carcinogen-induced liver tumors exhibited a fairly high frequency of ras gene activation, ranging from 32 to 64% as detected by direct tumor DNA analysis. In contrast, only about 8% of spontaneous liver tumors had activated ras oncogenes. Furthermore, the three mutations detected in spontaneous liver tumors were all located in Ha-ras codon 61 (CAA to AAA or CGA), whereas at least one-half of the ras mutations found in each group of carcinogen-induced tumors were located in other ras gene codons. An example of the DNA sequencing results for each type of point mutation detected in the CD-1 mouse liver tumors is shown in Figs. 1, 2, and 3 (for Ha-, Ki-, and N-ras mutations, respectively). In all tumors that had a ras gene mutation, at least 50% of the normal ras allele was also present, suggesting that most or all of these tumors were heterozygous for their mutation.

**DMBA-induced tumors contained predominantly Ki-ras codon 13 (G to C) transversions.** In addition, four DMBA-induced tumors had an A to T transversion in Ha-ras codon 61. Neither of these mutations was detected in the spontaneous CD-1 liver tumors (Table 1). Similarly, AAB-induced tumors had predominantly Ki-ras codon 13 mutations, as well as several N-ras mutations (G to T transversions in codon 12 or 13). Several N-OH-AAF- and DEN-induced tumors had the CAA to AAA Ha-ras codon 61 mutation that was found in spontaneous tumors (Table 1). In addition, DEN caused G to T transversions in codon 12 of N-ras, and AAF caused G to C transversions in Ki-ras codon 13 and G to T transversions in N-ras codons 12 and 13.

**ras Gene Analysis Using the DNA Transfection Assay.** For comparative purposes, in addition to the direct tumor DNA analysis described above, we also examined the liver tumors (from which a sufficient amount of DNA was isolated) using the NIH3T3 cell transfection assay. Note that after transfection, DNA samples from transformed foci of NIH3T3 cells were analyzed for ras mutations by the same procedures used for direct tumor DNA analysis, i.e., sequencing of PCR-amplified genomic DNA. Thus, only the source of DNA differed between the two assays (transfected DNA versus direct tumor DNA). Table 2 shows the percentage of spontaneous and carcinogen-induced tumors that gave positive transformed foci in the transfection assay and also shows the profile of ras mutations that were detected in DNA isolated from the transfected cells. As expected, tumors with ras mutations detected by direct tumor analysis were usually positive in the transfection assay, and the transfected DNA generally had the same mutation as the direct tumor DNA (data not shown on an individual tumor basis). Several Ki-ras and N-ras mutations that were detected by direct tumor DNA analysis were missed by the transfection assay, which is not unexpected given the known limitations of the transfection assay (see "Discussion").

More notably, transfection detected mutations in a number of tumors that were negative in the direct tumor DNA analysis and thereby enhanced the percentage of tumors found to be positive for ras mutation by over 3-fold in spontaneous tumors and AAB-induced tumors, and to a lesser extent in DEN- and N-OH-AAF-induced tumors (compare Table 2 with Table 1). In order to determine the nature of these mutations, we tabulated (Table 2) the ras mutation profile for only those tumors.

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**Table 1 Frequency and profile of activating ras mutations in CD-1 mouse liver tumors: direct tumor DNA analysis**

<table>
<thead>
<tr>
<th>Carcinogen</th>
<th>Fraction of tumors with ras mutations</th>
<th>Ha-ras 61 (CAA)</th>
<th>Ki-ras 13 (GGC)</th>
<th>N-ras 12 (GGT)</th>
<th>N-ras 13 (GGT)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AAA</td>
<td>CGA</td>
<td>CTA</td>
<td>CGC</td>
</tr>
<tr>
<td>Spontaneous</td>
<td></td>
<td>3/36 (8)*</td>
<td>2</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>AAB*</td>
<td></td>
<td>12/37 (32)</td>
<td>9</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>N-OH-AAF</td>
<td></td>
<td>19/34 (56)</td>
<td>4</td>
<td>4</td>
<td>17</td>
</tr>
<tr>
<td>DEN</td>
<td></td>
<td>8/25 (32)</td>
<td>21/33 (64)</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage.

* Two AAB-induced tumors had coincident mutations detected in two ras gene codons.

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that were negative in the direct tumor DNA assay but positive in the transfection assay. Remarkably, all of the ras mutations detected in these transfectants were Ha-ras codon 61 mutations characteristic of spontaneous tumors, regardless of whether they were from spontaneous or induced tumors.

Silent ras Gene Mutations in Carcinogen-induced Liver Tumors. Using direct tumor DNA analysis, we detected silent ras gene mutations (mutations that do not change the amino acid sequence of the encoded ras protein) in 4 DEN-induced tumors and in 2 N-OH-AAF-induced tumors (data not shown). In 3 of the 4 DEN-induced tumors and in both N-OH-AAF-induced tumors, the silent mutation was an A to C transition in the third base of Ha-ras codon 12. The fourth DEN-induced tumor had a silent T to C transition in the third base of Ki-ras codon 12. Four of the 6 silent mutations were accompanied by a coincident activating mutation within the same tumor. The silent mutations were not detected in the transfection assay (not shown) and thus appear to be nontransforming, as expected. These results suggest that the third base of codon 12 in Ha- and Ki-ras may be hypermutable in CD-1 mouse liver cells.

Histopathology: Correlation of Specific Carcinogen-induced Mutations with Tumor Malignancy. Comparison of the mutational patterns observed between adenomas and carcinomas induced by each carcinogen revealed an interesting correlation in the case of DMBA-induced tumors (data not shown). The Ki-ras codon 13 (G to C) mutation was found in 7 adenomas and 10 carcinomas induced by DMBA. However, the Ha-ras codon 61 (A to T) mutation was in 4 adenomas but no carcinomas (data not shown). This mutation was not detected in any of the other carcinogen-induced or spontaneous CD-1 mouse liver tumors (Tables 1 and 2). These results suggest that CD-1 mouse liver tumors with the A to T transversion may have a low probability of progressing into carcinomas relative to liver tumors carrying the Ki-ras codon 13 mutation.

DISCUSSION

Consistent with earlier studies in mice, our results demonstrate that populations of chemically induced CD-1 mouse liver tumors can be distinguished from spontaneous liver tumors by ras gene analysis. In contrast to B6C3F1 mice, spontaneous liver tumors in CD-1 mice have a relatively low frequency of ras mutations, and chemically induced CD-1 mouse liver tumors can be distinguished by quantitative as well as qualitative differences in ras gene mutations.

Both chemical- and tissue-specific factors can influence the type of ras mutation found in a tumor, as exemplified by the DMBA-induced liver tumors. DMBA is thought to have a strong preference for chemically interacting with adenine (20-22), and the predominant mutation detected in mouse skin and mammary tumors induced by DMBA is an A to T transversion in the second base of Ha-ras codon 61 (3, 4). A small fraction of the DMBA-induced liver tumors in our study had the A to T transversion in Ha-ras codon 61, which was specific for DMBA since it was not found in the other groups of chemically induced or spontaneous tumors. However, the majority of DMBA-induced liver tumors had a G to C transversion in Ki-ras codon 13, which has not been seen in DMBA-induced skin or mammary tumors.

The tissue specificity in the type of ras mutations detected in DMBA-induced tumors could be due to DMBA actually causing different types of mutations in liver versus skin or mammary cells or to the different ras mutations induced by DMBA having different transforming potencies in each tissue-type. In support of the latter possibility, Nakazawa et al. (23) demonstrated that DMBA efficiently induced Ha-ras (A to T) transversions in BALB/c 3T3 cells, but transformed foci that selectively grew out of the DMBA-treated cultures did not carry this mutation. The Ha-ras codon 61 (A to T) transversion may be weakly
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Fig. 3. Representative DNA sequences of the codon 12–13 region of the N-ras gene. The sequences shown are of the antisense strand and were obtained by direct sequencing of PCR-amplified liver tumor (or normal liver tissue) DNA. A, wild-type sequence derived from normal liver tissue DNA; B, C→A transversion in antisense strand of codon 12 (a GGT→GTT transversion in the sense strand) detected in a N-OH-AAF-induced tumor; C, C→A transversion in antisense strand of codon 13 (a GGT→TGT transversion in the sense strand) detected in a N-OH-AAF-induced tumor; D, two coincident N-ras mutations detected in an AAB-induced tumor, C→A transversions in the antisense strand of both codons 12 and 13 (corresponding to GGT→TGT transversions in the sense strands of both codons 12 and 13).

Table 2 Analysis of ras mutations in NIH3T3 cell transfectants

<table>
<thead>
<tr>
<th>Carcinogen</th>
<th>Ha-ras 61 (CAA)</th>
<th>Ki-ras 13 (GGC)</th>
<th>N-ras 12 (GTT)</th>
<th>N-ras 13 (GTT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneous</td>
<td>9/32 (28)</td>
<td>8/1</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>AAB</td>
<td>31/36 (86)</td>
<td>22</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>N-OH-AAF</td>
<td>21/33 (64)</td>
<td>15</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>DEN</td>
<td>11/23 (48)</td>
<td>7</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>DMBA</td>
<td>19/30 (63)</td>
<td>3</td>
<td>12</td>
<td>1</td>
</tr>
</tbody>
</table>

Profile of mutations detected in transfectant DNA but missed in the direct tumor analysis

<table>
<thead>
<tr>
<th>Carcinogen</th>
<th>Spontaneous</th>
<th>AAB</th>
<th>N-OH-AAF</th>
<th>DEN</th>
<th>DMBA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6</td>
<td>22</td>
<td>6</td>
<td>3</td>
<td>1</td>
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<td></td>
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</tr>
</tbody>
</table>

* These foci did not contain point mutations in codons 12, 13, or 61 of Ha-, Ki-, or N-ras.
* Numbers in parentheses, percentage.
* Two AAB-induced tumors had coincident mutations detected in two ras gene codons.

Transforming in CD-1 mouse liver tumors, since all 4 of the DMBA-induced liver tumors carrying this transversion were adenomas, whereas 10 of 17 DMBA-induced liver tumors carrying the Ki-ras codon 13 (G to C) transversion were carcinomas. Interestingly, Brown et al. (24) noted that even in skin, tumors with the A to T transversion have a low probability of progressing into carcinomas.

Mutations at guanine residues by DMBA are not unprecedented, since ras mutations detected in primary bladder epithelial cells treated with DMBA in vitro were invariably G to A transitions in Ki-ras codon 12 (24). In addition, DMBA is known to cause guanosine adducts as well as adenosine adducts (3, 4, 22). Also, other polycyclic aromatic hydrocarbons are known to interact extensively with guanine residues (20, 24). Similarly, in our study, we found that a high percentage of the ras mutations detected in AAB and N-OH-AAF-induced CD-1 mouse liver tumors were the same Ki-ras codon 13 (G to C) transversion found in DMBA-induced liver tumors.

Previously, N-OH-AAF- (14) and DEN-induced (16) liver tumors in B6C3F1 mice were shown to primarily contain C to A transversions in Ha-ras codon 61, which is the major mutation found in spontaneous liver tumors, and thus tumors induced by these carcinogens could not clearly be distinguished from spontaneous tumors. In this study, we found that in addition to the Ha-ras codon 61 (C to A) transversions, N-OH-AAF- and DEN-induced CD-1 mouse liver tumors also contain G to T transversions in the N-ras gene (codons 12 or 13). These results show that N-OH-AAF- and DEN-induced mouse liver tumors can be distinguished from spontaneous liver tumors and represent the first demonstration of N-ras mutations in mouse liver tumors (AAB also caused N-ras mutations), illuminating a role for the N-ras gene activation in mouse liver carcinogenesis.

The primary assay we used to detect ras mutations involved a direct analysis of tumor DNA, which is the most straightforward and consistent method for detecting ras mutations. For comparative purposes, we also used the NIH3T3 cell transfection assay as a means of detecting ras mutations. We found that transfection missed several Ki-ras and N-ras mutations that were readily detected in direct tumor DNA samples. Transfection has several well-known inherent limitations that can cause it to miss ras mutations. Transfection is sensitive to
variability in the quality of DNA, since an intact ras gene is needed to transform NIH3T3 cells. In addition, the large size of the Ki-ras gene causes it to be more sensitive than Ha- or N-ras to factors that reduce transfection efficiency (26). Furthermore, some ras mutations do not cause a pronounced morphological transformation of NIH3T3 cells and can be missed in the transfection assay (27). Thus, in addition to the additional steps, transfection adds significant inconsistencies to ras gene analysis and therefore direct tumor DNA analysis is generally the method of choice for analyzing ras gene mutations.

Our results do illustrate a case when transfection is advantageous. Transfection was able to detect ras mutations in many tumors that were negative in the direct tumor DNA analysis. Since sequencing of the direct tumor DNA preparations from these tumors showed only the normal ras allele, the mutant ras alleles must have been present in only a small fraction of the cells in the tumor sample. The transfectant DNA must be enriched (relative to the direct tumor DNA) for the mutated ras allele, since the same PCR and sequencing methods were used in both assays. The ability to select for activated ras alleles (capable of transforming NIH3T3 cells) is central to the transfection assay, and others have demonstrated that transfection can detect mutations that were present in too few of the cells of a tumor to be detected in the direct tumor DNA (28, 29).

Most significantly, all of the mutations that were detected in transfectant DNA but not in direct tumor DNA, whether they were found in chemically induced or spontaneous tumors, are Ha-ras codon 61 mutations (CAA to AAA or CGA). Since these mutations are identical to the mutations characteristic of spontaneous tumors and since they are present in only a small fraction of tumor cells, it is likely that these mutations are spontaneous events that occurred later during tumor development, independently of any initiating dose of carcinogen. This interpretation is supported by the fact that all of the ras mutations that are unique to the carcinogen-induced tumors were readily detected by the direct tumor analysis, consistent with them being caused by the single initiating dose of the carcinogen.

Alternative hypotheses (to a late event) could account for the presence of a mutation being in only a small fraction of cells in the tumor sample. The simplest alternative is contamination of the tumor sample with normal tissue. However, extreme care was taken to trim tumors away from the surrounding normal liver tissue, and histological examination of the tumor specimens indicated that gross contamination of the tumors with normal tissue was unlikely. Also, it is unlikely that only the Ha-ras codon 61 mutations characteristic of spontaneous tumors would be afflicted with excess normal tissue contamination. A second alternative is that the mutation is an early event but that cells carrying this mutation grow more slowly than other cells within the tumor. We believe that this slower growth is unlikely since in many N-OH-AAF-induced tumors, Ha-ras codon 61 (C to A) mutations are found in a high percentage of cells and are readily detected by direct tumor analysis. Thus, we favor the notion that the Ha-ras codon 61 mutations, when present in only a small percentage of the tumor cells, represent spontaneous mutations that occurred later during tumor development and independently of the initiating carcinogen.

Paradoxically, AAB enhances the frequency of these putatively late occurring Ha-ras codon 61 mutations relative to their frequency in spontaneous tumors (Table 2), which would not be expected if these mutations were spontaneous and not influenced by AAB. It is possible that the codon 61-activated Ha-ras oncogene may be a particularly effective complement (for promoting growth of the tumor) to an initiating lesion caused directly by AAB. Alternatively, AAB-initiated tumors may be hypermutable or more prone to spontaneous Ha-ras codon 61 mutations later during tumor development.

In conclusion, our results demonstrate that in CD-1 mouse liver tumors, genotoxic carcinogens cause an increased frequency and different profile of ras mutations than that found in spontaneous tumors. All of the ras mutations that are unique to chemically induced tumors are present in the majority of cells within the tumors, consistent with a putative role in the initiation of the tumors by the carcinogen. However, the Ha-ras codon 61 mutations characteristic of spontaneous tumors are often present in only a small fraction of cells, suggesting that they can occur later in tumor development, independent of any initiating carcinogen.

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