

Mutational activation of the c-Ha-ras gene in liver tumors of different rodent strains: Correlation with susceptibility to hepatocarcinogenesis

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Communicated by James A. Miller, October 15, 1990 (received for review July 31, 1990)

ABSTRACT The frequency and pattern of mutations at codon 61 of the c-Ha-ras gene have been analyzed in 195 liver tumors and 132 precancerous liver lesions from various rodent strains with differing susceptibility to hepatocarcinogenesis. By using the polymerase chain reaction and allele-specific oligonucleotide hybridization, C → A transversions at the first base and A → T transversions or A → G transitions at the second base of c-Ha-ras codon 61 were detected in 20–60% of spontaneous or carcinogen-induced liver tumors of the C3H/He, CBA, CF1, and B6C3F1 mouse strains, which are highly susceptible to hepatocarcinogenesis. No such mutations, however, could be found in any of the 31 liver tumors of the insensitive C57BL/6J and BALB/c mouse strains or in any of the 35 liver tumors of the comparatively resistant Wistar rat. Further analyses of c-Ha-ras codon 12 mutations in liver tumors from the three insensitive rodent strains also failed to give any positive results. In early precancerous liver lesions, c-Ha-ras codon 61 mutations were found in 13–14% of lesions of the sensitive C3H/He and B6C3F1 mouse strains but not in any of the 34 lesions of the insensitive C57BL/6J mouse. Taken together, our results indicate a close correlation between the mutational activation of the c-Ha-ras gene in liver tumors of the different rodent strains and their susceptibility to hepatocarcinogenesis, whereby the mutations appear to provide a selective growth advantage, leading to a clonal expansion of the mutated liver cell population, only in livers of sensitive but not of insensitive strains.

Activation of the three different members of the *ras* gene family, *Ha-ras*, *Ki-ras*, and *N-ras*, by point mutations at either codon 12, 13, or 61 has been found to play an important role in the development of a variety of different animal tumors (for a detailed review, see ref. 1). In the liver system, activated *ras* genes have been detected in a high percentage of spontaneously occurring and carcinogen-induced liver tumors of the B6C3F1 mouse, where the predominant types of mutations were found to be specific single-base substitutions at different bases of codon 61 of the c-Ha-ras gene (2–6). In contrast to the B6C3F1 mouse, liver tumors from rats analyzed so far did not contain any mutations within the *Ha-ras* gene (5). There are, however, reports demonstrating mutated *Ki-ras* or *N-ras* genes in some aflatoxin B₁-induced liver tumors of the Fischer 344 rat (7–9). The reason for the differences in the activation of *ras* genes between B6C3F1 mouse and rat liver tumors is not known at present. They might simply be due to strain differences between mice and rats; it appears, however, also feasible that differences in the

susceptibility to hepatic tumor formation between various strains of rodents may play an important role.

Differences in the genetic susceptibility to hepatocarcinogenesis are well documented for a variety of inbred mouse strains. For example, C3H, CBA, CF1, and B6C3F1 mice are characterized by a very high rate of spontaneous liver tumor formation with frequencies, depending on the substrains used, of 20% to >80%, whereas C57BL and BALB/c mice have a comparatively low rate of spontaneous liver tumor formation, with frequencies of only 1–4% (refs. 10 and 11; see also Table 1). Similar to these latter two mouse strains, Wistar rats are characterized by a very low spontaneous liver tumor frequency, which is <3% (12). In parallel to their spontaneous rate of liver tumor formation, the different strains of rodents can be classified either as highly susceptible (sensitive) or as resistant (insensitive) to the action of diverse classes of chemical carcinogens (10, 11).

The genetic basis for the differences in susceptibility to hepatocarcinogenesis is only poorly understood. Recent evidence obtained from segregating crosses between the susceptible C3H/HeJ and the resistant C57BL/6J mouse indicates that allelic differences for at least two gene loci are of importance, whereby ≈85% of the difference in susceptibility could be attributed to a single genetic locus that affects the growth control of both normal and preneoplastic liver cells (13–15). To further address the question of which specific genes are involved in the process of tumor formation, we analyzed liver tumors from various rodent strains with characteristic differences in their susceptibility to hepatocarcinogenesis for the presence of mutations at codon 61 of the c-Ha-ras gene, which have been recognized to play an important role in the development of liver tumors in at least one of the susceptible mouse strains.

MATERIALS AND METHODS

Induction of Liver Tumors. C3H/He and C57BL/6J mice (hereafter referred to as C3H and C57BL) were obtained from the Zentralinstitut für Versuchstierkunde (Hanover, F.R.G.) and mated in our laboratory for breeding of C3H, C57BL, and B6C3F1 (C3H × C57BL) mice. Male offspring were given a single i.p. injection of diethylnitrosamine (DEN; 5 or 20 µg per g of body weight) on day 15 after birth. Groups of animals were killed between 11 and 50 weeks after treatment, and liver tumors with diameters >3 mm were dissected out of the liver tissue and frozen in liquid nitrogen. The remaining liver tissue containing small liver foci was frozen on dry ice. All material was stored at –80°C. Wistar rats were obtained from the same source at day 7 of pregnancy. Liver tumors were

induced in female offspring by a single application of DEN (15 μ g per g of body weight) on day 1 after birth followed by continuous administration of phenobarbital (500 ppm in the diet) starting at 4 weeks of age. A second group of rats was treated continuously with *N*-nitrosodiethanolamine (NDEOL; 2000 ppm in the drinking water) starting at 6 weeks of age. In addition, liver tumor material that was available to us from previous experiments was used. Further details on treatment of animals and the respective references are provided in Tables 3 and 4.

Isolation of DNA. DNA from frozen tissue material was extracted by the method of Krieg *et al.* (16). DNA from formalin-fixed, paraffin-embedded tissue blocks was isolated by prolonged treatment with proteinase K as described (17).

Preparation of Tissue Sections. Sections (10 μ m) were prepared from frozen liver tissue with a cryostat microtome, mounted on dialysis bags, and enzyme-histochemically stained for glucose-6-phosphatase (G-6-Pase) activity (18). Thereafter, small tissue samples were taken with punching cannulas both from G-6-Pase-deficient liver lesions and from normal parts of the liver sections and used for polymerase chain reaction (PCR) without isolation of DNA as described (6).

Amplification of DNA by PCR. PCR was performed essentially according to Saiki *et al.* (19) with 1 μ g of DNA from either frozen or formalin-fixed tumor material. In addition, if the quality of the paraffin-embedded tissue blocks was sufficient to give detectable PCR product, single 5- μ m sections were used without prior isolation of DNA. Isolated DNA or tissue sections were suspended in 100 μ l of a reaction mixture containing 67 mM Tris-HCl (pH 8.8), 16.6 mM NH_4SO_4 , 6.7 mM MgCl_2 , 10 mM 2-mercaptoethanol, 6.7 μ M EDTA, bovine serum albumin at 170 μ g/ml, each dNTP at 1.5 mM, and each primer at 1 μ M and subjected to 35 cycles of amplification as described (6). On the basis of the mouse *c-Ha-ras* sequence (20), the primers used for amplification of *Ha-ras* codon 61 sequences were Amp61/1A (5'-CTAAGCCTGTTGTTTGCAGGAC-3') and Amp61/2A (5'-GTGCGCATGTACTGGTCCCGCAT-3') or Amp61/1B (5'-GAGACATGTCTACTGGACATCTT-3') and Amp61/2B (5'-GTGTTGTTGATGGCAAATACACAGAGG-3'), yielding PCR products of 130 base pairs (primer set 1A/2A) and 116 base pairs (primer set 1B/2B), respectively. For additional analyses of *Ha-ras* codon 12 sequences, a DNA fragment of 138 base pairs was amplified using the primers Amp12/1A (5'-CTTGGCTAAGTGTGCTTCTCATT-3') and Amp12/1B (5'-CAGCTGGATGGTCAGGGCACTCT-3'). In all experiments, PCR controls without template DNA consistently gave negative results.

Allele-Specific Oligonucleotide Hybridization. Mutation analysis by allele-specific oligonucleotide hybridization was performed as described (6). In brief, 1–5 μ l of the PCR-amplified reaction mixtures was spotted onto BioTrace RP nylon membranes, hybridized with ^{32}P -labeled oligonucleotide probes (for sequences, see ref. 21), and exposed to Kodak X-AR x-ray film. Final discrimination temperatures were 62°C for the oligonucleotides detecting the wild-type sequence of *Ha-ras* codon 61 (CAA) or the mutated sequences CGA and CTA and 59°C for the oligonucleotide diagnostic for the mutated sequence AAA. The conditions for oligonucleotide probes used to detect additional mutations at *Ha-ras* codon 61 (GAA, CCA, CAC, or CAT) or *Ha-ras* codon 12 were as described by Brown *et al.* (21).

Statistical Analysis. Intergroup differences were analyzed by Fischer's exact test.

RESULTS

Susceptibility of Rodent Strains to Hepatocarcinogenesis. Mutations at codon 61 of the *c-Ha-ras* gene were analyzed in liver tumors from various strains of rodents with differing susceptibility to hepatocarcinogenesis, which were available

to us from either present or previous experiments. To facilitate classification of animals used, a brief summary of literature data on their susceptibility (sensitivity) to hepatocarcinogenesis is provided in Table 1. According to these data, C3H, CBA, CF1, and B6C3F1 mice can be classified as highly susceptible and NMRI mice as intermediately susceptible, whereas C57BL and BALB/c mice and Wistar rats are comparatively resistant to hepatocarcinogenesis. With respect to C3H, B6C3F1, and C57BL mice, these differences were also obvious in our present experiment. As illustrated in Fig. 1, the onset of liver nodule and tumor formation following a single injection of DEN was characteristically different between the three strains. In C3H and B6C3F1 mice, small nodules (diameters <2 mm) started to appear on the surface of the livers at 17–23 weeks after treatment, and liver tumors with diameters >5 mm were observed after 29 weeks. In contrast, the response to DEN treatment was considerably delayed in C57BL mice: small liver nodules were found starting at 36 weeks, and large liver tumors were found starting at 45 weeks after application of the carcinogen.

***Ha-ras* Codon 61 Mutations in Liver Tumors.** For analysis of mutations at codon 61 of the *c-Ha-ras* gene, we used the method of *in vitro* amplification of DNA by PCR followed by allele-specific oligonucleotide hybridization. In addition, some of the results were confirmed for individual tumor samples either by direct sequencing of the amplified DNA fragments or by analysis of restriction fragment length polymorphisms after digestion with the restriction enzyme *Taq* I or *Xba* I. Typical examples of such analyses are shown in Fig. 2. In all tumors analyzed, we were able to detect the wild-type sequence of *Ha-ras* codon 61, which is CAA. In those tumors with a mutation at this codon, the signal intensities obtained for the normal and the mutated sequences were almost identical (for example, see Fig. 2A), suggesting that each cell of the tumors contained both a normal and a mutated *Ha-ras* allele.

A summary of our results with DEN-induced liver tumors from C3H, B6C3F1, and C57BL mice is given in Table 2. Of the seven oligonucleotides used for detection of different single-base substitutions at *Ha-ras* codon 61, positive signals were obtained for the mutated sequences AAA, CGA, and CTA, demonstrating the presence of C $\rightarrow$ A transversions at the first base or A $\rightarrow$ G transitions and A $\rightarrow$ T transversions at the second base of this codon. Such mutations, however, were only detected in liver tumors of the two sensitive mouse strains (i.e., C3H with a frequency of 56% and B6C3F1 with a frequency of 23–33%) but not in any of the 24 liver tumors of the comparatively insensitive C57BL mouse. Interestingly, $\approx$ 60% of spontaneous liver tumors of the C3H mouse were also found to possess mutations at codon 61 of the *Ha-ras* gene (Table 2). Comparison of C57BL with either C3H or B6C3F1 mice revealed significant differences ($P = 10^{-5}$ and $P = 0.01$, respectively). Moreover, the mutation

Table 1. Susceptibility of various rodent strains to liver tumor formation

Rodent strain	% spontaneous liver tumor frequency		Susceptibility	Ref(s).
	Male	Female		
C3H mouse	18–100	5–59	High	10, 11
CBA mouse	41–44	5–27	High	10, 11
CF1 mouse	15–34	13–23	High	10, 22
B6C3F1 mouse	25–40	5–10	High	23
NMRI mouse	6–9	<1	Inter.	24, 25
C57BL mouse	<4	<1	Low	10, 11
BALB/c mouse	<4	<1	Low	10, 11
Wistar rat	<2	<3	Low	12

Inter., intermediate.

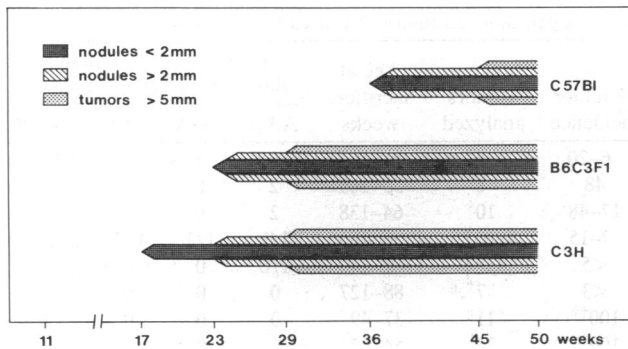


FIG. 1. Time-dependent appearance of liver tumors and nodules in C3H, B6C3F1, and C57BL mice. Mice of either strain were given a single i.p. injection of DEN (20 μ g per g of body weight) on day 15 after birth. Groups of two animals each were killed sequentially over a period of 11–50 weeks after treatment, and livers were inspected for the appearance of macroscopically visible nodules and tumors.

frequency was significantly higher in C3H when compared with B6C3F1 mice ($P = 0.02$), which might reflect the gradual differences in liver tumor susceptibility between these two sensitive strains. Analysis of >30 samples from normal liver tissue of the different mouse strains did not give any indication for Ha-*ras* codon 61 mutations.

To further extend our findings, we analyzed liver tumors from additional strains of mice that were available to us from previous experiments. The material was selectively chosen to allow comparative analyses of Ha-*ras* mutations in a broad spectrum of liver tumors from animals either susceptible or resistant to hepatocarcinogenesis rather than according to the protocol under which these tumors had been induced. In one of the experiments, which has recently been described (17), 13% of spontaneous and 38% of aflatoxin B₁-induced liver tumors of the CF1 mouse contained Ha-*ras* codon 61 mutations (see Table 3). In a second experiment analyzed, spontaneous liver tumors from (C3H $\times$ 101)F₁ mice, which are also highly susceptible to hepatocarcinogenesis, were found to contain such mutations with a frequency of 40% (Table 3). In both experiments, no significant differences in mutation frequency between adenoma and carcinoma were evident. From additional sources, liver tumors from CBA, NMRI, and BALB/c mice that developed either spontaneously or after a single treatment with radionuclides (thorium-227 or radium-224) were used. In these experiments, radionuclide treatment has been employed for induction of leukemia or osteosarcoma. Since liver tumor frequencies and mean latency periods for tumor formation were almost identical in treated animals and untreated controls, except for CBA mice, where a slight increase in liver tumor incidence was observed (A.L., unpublished observation), we believe that the majority of liver tumors from these animals can be regarded as sponta-

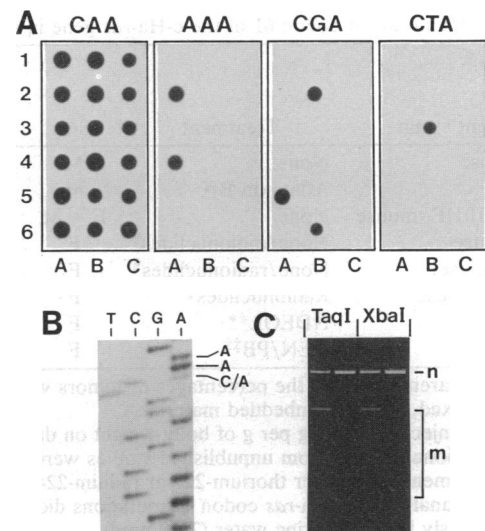


FIG. 2. Representative examples for identification of mutations at codon 61 of the c-Ha-*ras* gene. (A) PCR-amplified DNA samples were dot-blotted onto nylon membranes and hybridized with oligonucleotide probes diagnostic for either the wild-type sequence (CAA) or the mutated sequences AAA, CGA, or CTA, respectively. Liver tumors were from C3H mice (A2–A6), B6C3F1 mice (B2–B6), and C57BL mice (C2–C6). Positions A1, B1, and C1 contain DNA samples from normal liver tissue. (B) Direct sequencing of a PCR-amplified DNA sample from a CF1 mouse liver tumor containing a C $\rightarrow$ A transversion at the first position of Ha-*ras* codon 61. One picomole of PCR product was sequenced as described (17) using 5 pmol of a ³²P-labeled sequencing primer and electrophoresed in a 6% polyacrylamide gel. The gel was exposed to Kodak x-ray film overnight at -70°C . (C) Restriction fragment length polymorphisms after digestion with Taq I or Xba I, which indicate the mutated sequences CGA or CTA, respectively. Ten microliters of PCR-amplified DNA solutions from mouse liver tumors were incubated with 5 units of either restriction enzyme for 3 hr, electrophoresed in a 10% polyacrylamide gel, and stained with ethidium bromide. n, normal allele; m, mutated allele.

neous tumors. In CBA mice, mutations at codon 61 of the Ha-*ras* gene were found in 28% of liver tumors, in which no obvious differences in mutation frequencies were seen between radionuclide treated and untreated mice (Table 3). In contrast to this sensitive strain, only 1 out of 15 liver tumors (7%) of the intermediately susceptible NMRI mouse and 0 out of 7 liver tumors of the insensitive BALB/c mouse contained mutations at Ha-*ras* codon 61. In addition, we were unable to detect such mutations in any of the 35 carcinogen-induced liver tumors of the Wistar rat (Table 3). Further analyses with liver tumors from the insensitive rodent strains (Wistar rats and BALB/c and C57BL mice) aimed to detect mutations at codon 12 of the Ha-*ras* gene also failed to give any positive results (see footnotes to Tables 2 and 3).

Table 2. Mutations at codon 61 of the c-Ha-*ras* gene in liver tumors from male C3H, B6C3F1, and C57BL mice

Mouse strain	Treatment	Tumors analyzed*	Age at sacrifice, weeks	Tumors with the mutated sequence at Ha- <i>ras</i> codon 61			
				AAA	CGA	CTA	Total†
C3H/He	DEN‡	18	29–45	5	5	0	10 (56%)
	None	15	52	7	1	1	9 (60%)
B6C3F1	DEN‡	22	29–50	2	2	1	5 (23%)
	DEN§	15	68	1	3	1	5 (33%)
C57BL/6J	DEN‡	24¶	45–48	0	0	0	0

*Mean tumor diameters were 5.8 mm (2–15 mm) for C3H mice, 5 mm (2–10 mm) for B6C3F1 mice, and 7.5 mm (2–20 mm) for C57BL mice.

†Values in parentheses are the percentages of tumors with Ha-*ras* mutations.

‡Single injection (20 μ g per g of body weight) on day 15 after birth.

§Single injection (1.2 or 2.5 μ g per g of body weight) on day 15 after birth (data from ref. 6).

¶Additional analyses for Ha-*ras* codon 12 mutations did not give any positive results.

Table 3. Mutations at codon 61 of the *c-Ha-ras* gene in spontaneous and carcinogen-induced tumors from various sources

Rodent strain	Treatment	Sex	Ref. for tumor source	% tumor incidence	Tumors analyzed	Age at sacrifice, weeks	Tumors with the mutated sequence at <i>Ha-ras</i> codon 61			
							AAA	CGA	CTA	Total*
CF1 mouse	None	M	17	6–20	8 [†]	91–104	0	1	0	1 (13%)
	Aflatoxin B ₁ [‡]	M	17	48	8 [†]	55–103	2	0	1	3 (38%)
(C3H × 101)F ₁ mouse	None	F + M	26 [§]	17–48	10 [†]	64–138	2	0	2	4 (40%)
CBA mouse	None/radionuclides [¶]	F	27 [§]	7–15	8/10 [†]	56–165	1/1	1/1	0/1	5 (28%)
NMRI mouse	None/radionuclides [¶]	F	28	<5	8/7 [†]	87–140	1/0	0	0	1 (7%)
BALB/c mouse	Radionuclides [¶]	F	27 [§]	<3	7 ^{†,}	88–127	0	0	0	0
Wistar rat	NDEOL**	F	This work	100 ^{††}	11	37–49	0	0	0	0
	DEN/PB ^{‡‡}	F	This work	100 ^{††}	24	54–93	0	0	0	0

*Values in parentheses are the percentages of tumors with *Ha-ras* mutations.

[†]Formalin-fixed, paraffin-embedded material.

[‡]Single i.p. injection of 6 µg per g of body weight on day 7 after birth (data from ref. 17).

[§]Some additional tumors from unpublished studies were included.

[¶]Single treatment with either thorium-227 or radium-224.

^{||}Additional analyses for *Ha-ras* codon 12 mutations did not give any positive results.

**Continuously in the drinking water (2000 ppm).

^{††}No tumors were observed in untreated rats, whereas all carcinogen-treated rats developed multiple liver tumors.

^{‡‡}Single i.p. injection of DEN (15 µg per g of body weight) on day 1 after birth followed by continuous phenobarbital (PB) treatment (500 ppm in diet).

Taken together, the data from Tables 2 and 3 demonstrate that 37% (i.e., 42 out of a total of 114) spontaneously occurring or carcinogen-induced tumors from the highly susceptible C3H, (C3H × 101)F₁, CBA, CF1, and B6C3F1 mouse strains possess *Ha-ras* codon 61 mutations, whereas only 7% of tumors from the intermediate NMRI mouse and 0 out of a total of 66 tumors from the insensitive C57BL and BALB/c mouse and Wistar rat contain such mutations. Irrespective of the gradual differences in mutation frequencies within the group of sensitive strains, these differences were highly significant when comparing sensitive versus insensitive strains ($P = 10^{-8}$) and sensitive versus intermediate strains ($P = 0.02$).

***Ha-ras* Codon 61 Mutations in Precancerous Liver Lesions.** Mutations at *Ha-ras* codon 61 were also analyzed in small precancerous (G-6-Pase-deficient) liver lesions with diameters of <2 mm by using punched tissue samples from enzyme-histochemically stained liver sections. *Ha-ras* codon 61 mutations were found within 13–14% of DEN- or ethylnitrosourea (ENU)-induced liver lesions of B6C3F1 and C3H mice, respectively (Table 4). Again, the signals obtained for the normal and the mutated sequences were roughly equal within individual lesions, and the types of mutations were identical to those already detected in liver tumors. In contrast, no *Ha-ras* codon 61 mutations could be found in any of the 34 ENU-induced precancerous liver lesions of the insensitive C57BL mouse (Table 4), and this difference was statistically significant ($P = 0.04$). Samples taken from normal parts of the liver of either strain did not contain any mutations at *Ha-ras* codon 61.

DISCUSSION

In this study we have analyzed mutations at codon 61 of the *c-Ha-ras* gene in liver tumors from different rodent strains, which were selected from present and previous experiments in order to allow comparisons with a broad spectrum of animals either susceptible or resistant to hepatocarcinogenesis. Although in some experiments the number of tumors available to us was comparatively small, taken together the data from all experiments clearly demonstrate that *Ha-ras* mutations are only present in liver tumors and precancerous liver lesions of sensitive but not of insensitive rodent strains.

Our findings considerably extend previous observations on the role of *ras* mutations during hepatocarcinogenesis reported in the literature so far. These studies have primarily been focused on liver tumors of the B6C3F1 mouse, which, due to its sensitivity, is widely used as a test animal for the assessment of carcinogenic risks associated with exposure to chemical carcinogens and other xenobiotics. In this strain, *Ha-ras* mutations have been detected in a high percentage of spontaneous and carcinogen-induced liver tumors with frequencies ranging from ≈30% to 100% (2–6). The predominant types of mutations observed were single-base substitutions at either the first or second base of codon 61 of the *Ha-ras* gene, with only a minor fraction of liver tumors carrying additional mutations at either codon 13 or 117 (4).

Studies with the Fischer 344 rat failed to detect *Ha-ras* mutations in DEN-induced liver tumors (5). In accordance with this finding, we were unable to observe any mutations at either codon 12 or codon 61 of the *Ha-ras* gene in DEN- or NDEOL-induced liver tumors of an additional rat strain (i.e.,

Table 4. Mutations at codon 61 of the *c-Ha-ras* gene in small precancerous liver lesions of male C3H, C57BL, and B6C3F1 mice

Mouse strain	Treatment	Lesions analyzed*	Age at sacrifice, weeks	Lesions with the mutated sequence at <i>Ha-ras</i> codon 61			
				AAA	CGA	CTA	Total [†]
B6C3F1	DEN [‡]	69	23–28	4	4	1	9 (13%)
C3H/HeJ	ENU [§]	29	26	2	2	0	4 (14%)
C57BL/6J	ENU [§]	34	26	0	0	0	0

*All lesions were G-6-Pase-negative and had sphere diameters of <2 mm, with the majority being around 1 mm as estimated by three-dimensional reconstructions with serial sections.

[†]Values in parentheses are the percentages of lesions with *Ha-ras* mutations.

[‡]Single i.p. injection (5 or 20 µg per g of body weight) on day 15 after birth (data from ref. 6 are included).

[§]Single i.p. injection (0.5 µmol per g of body weight) on day 12 after birth (see ref. 14).

the Wistar rat). In contrast to the *Ha-ras* gene, *Ki-ras* mutations have been described in some aflatoxin B₁-induced liver tumors of the Fischer rat (7–9). However, since these mutations were only present in a relatively small percentage of tumor cells (9), they may represent a late rather than an early event during hepatocarcinogenesis in the rat. Additional mutations observed in the *N-ras* gene of aflatoxin B₁-induced liver tumors of the Fischer rat (8, 9) were found to be present in normal liver tissue and were therefore suggested to result from a germ-line mutation, which might predispose the Fischer rats to be somewhat more sensitive to liver tumor formation than other rat strains (9).

The reason for the observed differences in the mutational activation of the *Ha-ras* gene in strains with low and high liver tumor susceptibility is not known so far. With respect to chemically induced liver tumors, it would appear possible that metabolic activation of procarcinogens to their ultimate carcinogenic metabolites, which form DNA adducts and thus induce mutations at critical gene loci, occurs more efficiently in livers of sensitive than in livers of insensitive mouse strains. This assumption, however, is contradicted by almost identical overall DNA alkylation intensities and DNA repair capacities in sensitive and insensitive mouse strains following application of different carcinogens (13, 29). Moreover, our observation that ethylnitrosourea, a carcinogen that does not require metabolic activation, induces *Ha-ras* codon 61 mutations in liver lesions of the sensitive C3H but not in those of the insensitive C57BL mouse strongly argues against this possibility.

If differences in the susceptibility to hepatocarcinogenesis do not result from alterations in metabolic activation of carcinogens or initiation of tumorigenesis, an alternative explanation could be that liver cells carrying a *ras* mutation undergo a different biological fate in the various rodent strains—i.e., that the mutations lead to a selection and clonal expansion of the mutated hepatocyte population in livers of sensitive but not in livers of insensitive strains. This assumption is substantiated by the observation of *Ha-ras* codon 61 mutations in 13–14% of early liver lesions of the sensitive C3H and B6C3F1 mouse strains, whereas no such mutations could be detected in any of the 34 early liver lesions of the insensitive C57BL mouse. Such differential behavior could be explained by the assumption that additional genes that suppress the action of the mutated *ras* gene are present in the insensitive mouse strains but are not expressed in livers of the sensitive strains. Alternatively, the sensitive mouse strains may possess gene functions that cooperate with the mutated *ras* gene and thereby stimulate the outgrowth of the mutated liver cell population. In this context, it is noteworthy that preneoplastic liver lesions have been found to possess a higher growth rate in livers of sensitive as compared to insensitive mouse strains (14, 30). From this observation, it has been concluded that the increased susceptibility to liver tumor formation is related to the promotional phase rather than to initiation of hepatocarcinogenesis, whereby a specific genetic locus, denoted as hepatocarcinogen sensitivity locus, appears to be of major importance (13–15).

In summary, our results indicate that mutations in the *c-Ha-ras* gene significantly contribute to liver tumor formation in those rodent strains characterized by a high susceptibility to hepatocarcinogenesis but not in strains with low susceptibility. Besides the fact that mutations at codon 61 of the *Ha-ras* gene have been shown to exert oncogenic potential in *in vitro* systems, there are two additional reasons that, taken together, make it highly unlikely that these mutations are random events unrelated to the carcinogenic process.

First, we have demonstrated in this and a previous report that *Ha-ras* codon 61 mutations occur very early during hepatocarcinogenesis in the mouse, probably close to the level of initiation (6). Second, our finding that up to 60% of liver tumors of the sensitive rodent strains harbor *Ha-ras* codon 61 mutations, whereas no such mutations could be detected in the normal liver tissue, clearly indicates that the mutations provide a strong selection advantage, which leads to a clonal expansion of the mutated liver cell population. However, the observation that even a certain percentage of liver tumors from the sensitive strains does not possess mutations in the *Ha-ras* gene suggests that additional, yet unknown pathways of tumorigenesis must exist. Here, activation of other dominantly transforming genes, which can substitute for the *ras* mutation, or loss of suppressor genes and inactivation of other genes responsible for maintenance of normal growth and differentiation may come into play.

We thank Drs. A. Balmain and K. Brown for helpful advice during the initial phase of this study; Dr. S. Moolgavkar for critically reading the manuscript; and R. Schmitt, G. Robinson, and U. Britzelmaier for excellent technical assistance. This study was partly supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 302).

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