Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 33–37, January 1986
Biochemistry

Detection and identification of activated oncogenes in spontaneously occurring benign and malignant hepatocellular tumors of the B6C3F1 mouse

(Ha-*ras*/non-*ras*/hepatocellular adenoma/hepatocellular carcinoma)

STEVEN H. REYNOLDS*†, SHARI J. STOWERS*, ROBERT R. MARONPOT‡, MARSHALL W. ANDERSON*, AND STUART A. AARONSON§

*Laboratory of Biochemical Risk Assessment and ‡National Toxicology Program, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709; and §Laboratory of Cellular and Molecular Biology, National Institutes of Health, Bethesda, MD 20205

Communicated by George H. Hitchings, August 28, 1985

ABSTRACT **Species- and strain-specific spontaneously occurring tumors have been observed in rodents maintained under normal laboratory conditions. Elucidation of the molecular mechanisms associated with the development of these spontaneous tumors may provide a better understanding of tumor development associated with exposure to chemical carcinogens. In view of the high frequencies of oncogene activation shown in rodent tumors induced by known chemical carcinogens, we have investigated oncogene activation in spontaneous tumors of the B6C3F1 mouse and Fischer 344/N rat by DNA transfection techniques. A marked difference in the presence of activated oncogenes in spontaneous rat tumors versus spontaneous mouse liver tumors was observed in this study. All rat tumors tested failed to yield activated oncogenes (0/29), whereas 30% (3/10) of mouse hepatocellular adenomas and 77% (10/13) of hepatocellular carcinomas scored positive by DNA transfection. These transforming genes were identified as an activated Ha-*ras* gene in all the adenoma transfectants and in 8 of the 10 carcinoma transfectants. The two remaining hepatocellular carcinomas contained transforming genes that appear not to be members of the known *ras* gene family. The B6C3F1 mouse liver system might provide a very sensitive assay not only for assessing the potential of a chemical to activate a cellular proto-oncogene, but also for detecting various classes of proto-oncogenes that are susceptible to mutational activation.**

Increasing evidence has shown that a small set of cellular genes appear to be frequent targets for genetic alterations that lead cells along the pathway toward malignancy. Such genes, termed proto-oncogenes, were initially discovered as the transduced oncogenes of acute transforming retroviruses. Subsequent studies have established that proto-oncogenes can also be activated as oncogenes in naturally occurring tumor cells by mechanisms completely independent of retroviral involvement. These genetic alterations range from point mutations to gross DNA rearrangements such as translocation and gene amplification (1, 2).

The most frequently detected oncogenes to date are three highly conserved members of the *ras* gene family, Ha-, Ki-, and N-*ras* (3–12). *ras* oncogenes have been detected in 15–20% of human malignancies and at an even higher frequency in chemically induced tumors in animal systems (6–11). Normal cellular *ras* genes can acquire transforming activity by a single point mutation in their coding sequence (13–22). In primary rodent and human tumors, point mutations have been observed at codons 12 and 61, and these codons, for unknown reasons, appear to be "hot spots" for activation of the *ras* genes (6, 8, 10). These recent investigations on oncogenes have provided experimental support for the hypothesis that genetic alterations, either spontaneously occurring or secondary to interaction with environmental mutagens/carcinogens, are involved in cellular conversion to the neoplastic state.

A number of approaches have been utilized to identify environmental agents posing significant carcinogenic hazards to man. These include *in vitro* assays for mutagenesis and/or transformation and *in vivo* tumorigenicity assays. A commonly used animal model involves bioassay in rodents over the lifespan of the animal (23, 24). One extensively utilized mouse strain, the B6C3F1 hybrid, exhibits a high incidence of spontaneous hepatocellular adenoma (10%) and carcinoma (21%) in males and a lower but significant incidence of adenoma (4%) and carcinoma (4%) in females (23). In this study, we have analyzed spontaneously occurring tumors as well as normal tissues from this mouse strain for the presence of oncogenes detectable by transfection analysis. A variety of spontaneous tumors in the Fischer 344/N rat were also examined for oncogene activation to compare with mouse liver tumors. Our results provide insights into the molecular mechanisms involved in hepatic tumor formation in this mouse strain as well as a potentially important model system for detection of new cellular oncogenes.

MATERIALS AND METHODS

Source of Spontaneous Rodent Tumors. Spontaneous tumors were obtained from untreated B6C3F1 mice and Fischer 344/N rats at terminal sacrifice of the National Toxicology Program 2-year carcinogenesis tests. A representative portion of each tumor sample was fixed in 10% neutral buffered formalin for histopathologic examination. The remainder of each tumor specimen was quick frozen in liquid nitrogen within 10 min of euthanasia. Frozen tissues were stored at −70°C until processed for transfection assays. When available, representative portions of adjacent nontumor tissue were similarly frozen for transfection studies. Fixed tissues were embedded in paraffin and sectioned for histopathologic examination for conventional hematoxylin and eosin stained sections. Established criteria for diagnosis of tumors were followed (25, 26).

Transfection Assays. DNA transfection analysis was carried out by the calcium phosphate coprecipitation method (27) as described (3, 28). NIH 3T3 cells, seeded 24 hr earlier at 2×10^5 cells per 10-cm dish in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, were exposed for 22 hr to the DNA precipitate (20 μg per dish). Cultures were maintained with twice weekly changes of

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

†To whom reprint requests should be addressed.

DMEM containing 5% calf serum. Focus formation was scored at 14–21 days.

DNA Blotting Analysis. DNA samples were digested with appropriate restriction endonucleases, electrophoresed in horizontal agarose gels, and transferred to nitrocellulose as described by Southern (29). Filters were hybridized under stringent conditions (50% formamide/0.75 M NaCl/0.075 M Na citrate, 42°C) with nick-translated ^{32}P-labeled DNA probes for 24 hr. Blots were washed twice for 5 min at room temperature in 2× SSC/0.1% $NaDodSO_4$, then washed at a final stringency of 0.1× SSC/0.1% $NaDodSO_4$ at 50°C for at least 1 hr (1× SSC = 0.15 M NaCl/0.015 M Na citrate). Blots were air-dried and exposed to x-ray film at −70°C.

Metabolic Labeling and Immunoprecipitation Analysis. First cycle NIH 3T3 transformants were plated at 1–2 × 10^6 cells per 10-cm dish 24 hr before labeling. For [^{35}S]methionine labeling, the growth medium was removed and the cells were incubated with methionine-free DMEM (Met$^-$ DMEM) for 30 min. After removing Met$^-$ DMEM, cells were incubated for 4 hr with Met$^-$ DMEM (2.5 ml per dish) containing [^{35}S]methionine (New England Nuclear; >1100 Ci/mmol; 1 Ci = 37 GBq) at a concentration of 400 μCi/ml.

Cells were extracted with 1% Triton X-100/0.1% $NaDodSO_4$/0.5% sodium deoxycholate/0.1 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM phenylmethanesulfonyl fluoride/aprotinin (100 kallikrein inactivator units per ml). Extracts were cleared at 4°C by centrifugation at 30,000 rpm for 30 min in a type 50 Ti Beckman rotor and used immediately for immunoprecipitation analysis. Each immunoprecipitation sample contained 4 × 10^7 trichloroacetic acid-precipitable cpm. Cell lysates were incubated for 1 hr at 4°C with anti-Harvey-MSV-p21 monoclonal antibody Y13-259. This antibody is specific for p21 protein encoded by the three members of the *ras* gene family (30). The Y13-259 hybridoma line was provided by E. M. Scolnick (Merck Sharp & Dohme Research Laboratories). Protein A-Sepharose was swollen and washed as described (31) and coated with goat anti-rat IgG by the method of Furth *et al.* (32); 200 μl of a 1:20 (wt/vol) suspension of the coated protein A-Sepharose was added to each sample and the samples were shaken in an Eppendorf shaker at 4°C for 30 min. The immunocomplexes were washed and the final pellets were dissolved at 95°C for 3–5 min in electrophoresis sample buffer.

$NaDodSO_4$/PAGE of immunoprecipitated samples was done in 12.5% polyacrylamide gels with a Tris glycine buffer system (31). For fluorography, gels were treated with EN^{3}HANCE (New England Nuclear) before drying. Dried gels were exposed to x-ray film at −70°C.

RESULTS

Spontaneous Liver Tumors of the B6C3F1 Mouse Strain Contain Cellular Oncogenes Detected by DNA Transfection. In an effort to investigate the basis for the genetic predisposition of B6C3F1 mice to develop spontaneous hepatocellular tumors, we investigated such tumors for the presence of oncogenes detectable by DNA transfection of NIH 3T3 cells.

As shown in Table 1, DNA from 10 of 13 spontaneous mouse hepatocellular carcinomas were found to be capable of inducing foci formation in the NIH 3T3 transfection assay. The efficiencies of transformation by individual tumor cell DNAs varied from 0.012 to 0.037 focus-forming units per microgram of DNA (Table 1). Six of eight carcinomas from male mice and four of five carcinomas from female mice scored positive by DNA transfection. Based on this limited sampling, there were no obvious sex differences with respect to the presence of activated oncogenes in spontaneous hepatocellular carcinomas. No foci were observed when NIH 3T3 cells were transfected with NIH 3T3 DNA or with DNA isolated from the liver tissues of non-tumor-bearing B6C3F1 mice (Table 1), indicating that oncogene activation in the liver tumors was most likely the result of a somatic alteration rather than genetic transmission of activated oncogenes in this strain.

Table 1. Transforming genes in spontaneous hepatocellular tumors of the B6C3F1 mouse

DNA source	Frequency*	Transformation efficiency, foci per μg of DNA: Tumor DNA	Transformation efficiency, foci per μg of DNA: Transfectant DNA, first cycle
Normal liver	0/4	0.00	—
Hepatocellular carcinoma	10/13	0.012–0.037	0.25–0.79
Hepatocellular adenoma	3/10	0.012–0.025	0.25–0.79

*(Number positive)/(number tested).

Transfection analysis of a similar series of spontaneous adenomas also revealed the presence of transforming genes in some of these benign tumors. The frequency of transfection positive adenomas, 3 of 10 analyzed, was significantly lower than was observed with the malignant carcinomas analyzed. The possibility that the tumors diagnosed as adenomas may contain focal areas of malignantly transformed cells in histologically unexamined areas cannot be excluded. However, the transforming efficiencies of the transfection-positive adenoma DNAs were comparable to the transfection-positive carcinoma DNAs (Table 1). This indicates that the transfection results for the positive adenomas cannot be attributed to the presence of a small minority of carcinoma cells within these adenomas. Since nine of the adenomas were from male mice, no sex comparisons could be made as to the presence of oncogenes in the adenomas.

Primary transfectants were grown up to mass culture and their high molecular weight DNAs were analyzed in the NIH 3T3 transfection assay. In each case, the transforming gene(s) was found to be stable upon serial transfer. The efficiencies of transformation in the second cycle of transfection ranged from 0.25 to 0.79 focus-forming units per microgram of DNA (Table 1), an approximate 20-fold increase in transfection efficiency over that of tumor DNA. These results implied amplification of the transforming gene in the first cycle transfectants, a phenomenon previously observed to occur during the transfection process (7, 9, 33).

Spontaneous Tumors of the Fischer 344/N Rat Do Not Contain Cellular Oncogenes Detectable by DNA Transfection. In striking contrast to our results with spontaneous mouse liver tumors, oncogenes detectable by transfection analysis were absent in 9 malignant and in 20 benign spontaneous tumors from Fischer 344 rats. These tumors included five testicular interstitial cell adenomas, four subcutaneous fibromas or fibroadenomas, two fibrosarcomas, eight mammary adenomas or fibroadenomas, three mononuclear cell leukemias, and a single adrenal pheochromocytoma, mammary adenocarcinoma, pancreatic acinar adenoma, pancreatic islet cell adenoma, pituitary adenoma, splenic hemangiosarcoma, and prostatic adenocarcinoma.

Identification of Oncogenes in Spontaneous B6C3F1 Mouse Liver Tumors. Genes introduced into cells of the same species by DNA transfection can often be detected by Southern blot analysis because of the presence of additional restriction fragments containing the exogenous gene resulting from a loss of restriction sites upon integration during the transfection process. Moreover, an increase in the hybridization intensity of the added gene, in the absence of any detectable DNA rearrangement, can often be observed because of its amplification during the transfection process. In

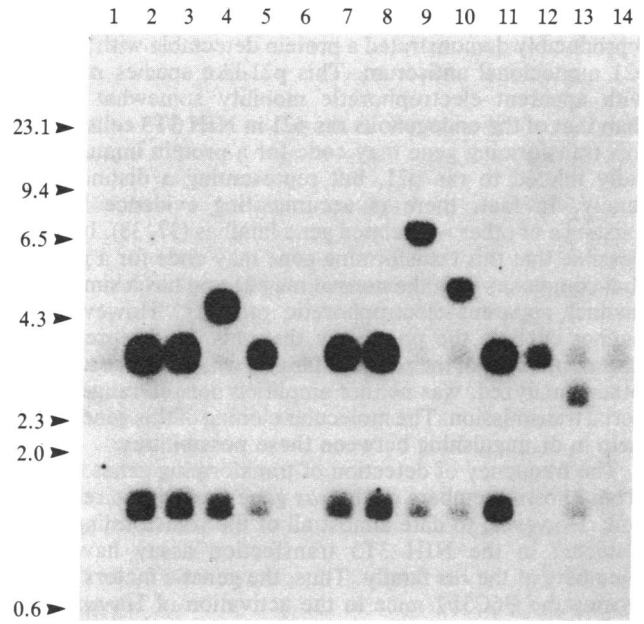

FIG. 1. Southern blot analysis of sequences related to the Ha-*ras* gene (V-bas) of BALB murine sarcoma virus in NIH 3T3 transformants induced by DNA of individual mouse spontaneous liver tumors. Normal and transfectant DNAs (20 μg of each) were digested with restriction endonuclease *Bam*HI, fractionated by electrophoresis through a 0.8% agarose gel, and transferred to nitrocellulose paper. Filters were incubated with ^{32}P-labeled probe (5×10^6 cpm per ml of hybridization buffer; specific activity, $>10^8$ cpm/μg). The probe was a 0.6-kilobase-pair *Hin*dIII/*Bam*HI fragment of the mouse viral Ha-*ras* gene designated as pHB1 (34). Lanes: 1, NIH 3T3 DNA; 2–4, NIH 3T3 transformants derived from transfection with liver adenoma DNAs; 5–14, transformants derived from transfection with liver carcinoma DNAs. Numbers on the left are kilobases.

view of the high frequency at which *ras* oncogenes are detected by transfection, we initially analyzed primary transfectant DNAs by Southern blotting and hybridization with probes specific for Ha-, Ki-, and N-*ras*.

Hybridization of the transfectant DNAs with Ki-*ras*- or N-*ras*-specific probes gave identical intensities and patterns of hybridization relative to normal NIH 3T3 DNA (data not shown). However, using Ha-*ras* as a probe, DNAs from representative foci induced by each of the adenomas (Fig. 1, lanes 2–4) as well as 8 of the 10 transfectants induced by individual carcinomas (lanes 5, 7–13) exhibited a striking increase in hybridization intensity as compared to normal NIH 3T3 DNA (lane 1). The endogenous NIH 3T3 Ha-*ras* gene was observed as 3.8- and 1.5-kilobase fragments after *Bam*HI digestion. Several transfectants (lanes 4, 9, 10, and 12) also demonstrated additional Ha-*ras* amplified DNA fragments of varying size. From these findings, we conclude that Ha-*ras* oncogenes were activated at high frequency in both spontaneous hepatocellular adenomas and carcinomas of this mouse strain.

Since DNA transfectants of two of the carcinomas (lanes 6 and 14) lacked evidence of amplified or rearranged *ras* genes, we sought further evidence that these tumors contained oncogenes. Repeated analysis demonstrated that these tumor DNAs possessed transforming activity with stable transmission of the transformed phenotype through at least two subsequent cycles of DNA transfection. Amplified or rearranged DNA fragments of any of the three known *ras* genes were not detected in the second or third cycle transfectants derived from these foci. Our findings implied that these two oncogenes were unrelated to the known members of the *ras*-gene family or represented *ras* oncogenes that were not amplified or rearranged during the transfection process.

Heterogeneity in Electrophoretic Mobilities of p21 ras Proteins Encoded by Ha-*ras* Oncogenes of Spontaneous B6C3F1 Mouse Liver Tumors. Recent reports have shown that *ras* genes that have undergone mutational activation frequently produce p21 proteins with altered electrophoretic mobilities. Characteristic alterations of either slower or faster mobility appear to accompany many of the activating lesions at positions 12 and 61, respectively (30). Therefore, first cycle DNA transfectants were analyzed for the presence of ras proteins with altered electrophoretic mobility.

Cells were labeled with [^{35}S]methionine and analyzed for p21 expression by immunoprecipitation with monoclonal antibody Y13-259 (32). Altered p21 proteins could be classified as slow- or fast-moving species (Fig. 2) when compared with mouse endogenous p21 bands (Fig. 2, lane 1). NIH 3T3 cells transformed with the 12th codon activated c-Ha-*ras* T24 oncogene (14) produced a p21 that migrated as a slow-moving species (lane 2). In contrast, NIH 3T3 cells transformed with the 61st codon activated c-Ha-*ras* Hs242 gene (17) produced a p21 that migrated as a fast-moving species (lane 3). As shown in Fig. 2, each liver tumor DNA transfectant containing an activated c-Ha-*ras* gene produced a p21 that migrated as a fast-moving species (lanes 4–7, 9–15). These results implied that these activated c-Ha-*ras* genes possessed similar structural lesions, most likely mutations of their 61st codon.

Transfectants containing the other two transforming genes were also analyzed for altered p21 electrophoretic mobility. One transfectant yielded only p21 proteins with the expected normal electrophoretic mobility (Fig. 2, lane 8). However, the other transformant contained a p21 that migrated as a slow-moving species (lane 16). Although this slow-moving p21 species was present in lower amounts than the endogenous NIH 3T3 p21 bands as detected by immunoprecip-

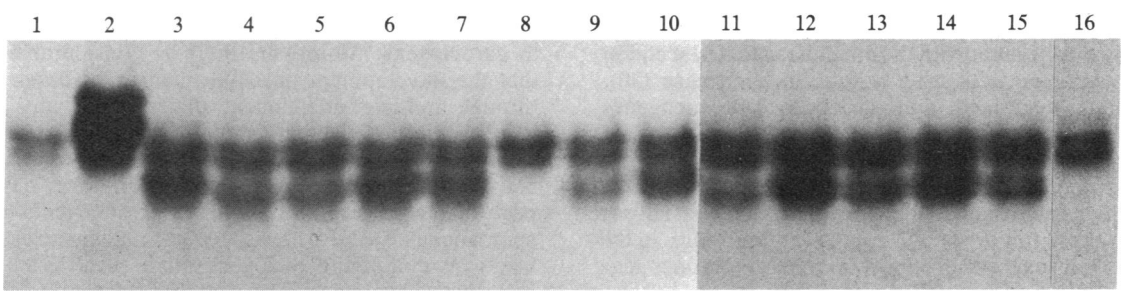

FIG. 2. Heterogeneity in the electrophoretic mobilities of p21 ras proteins from NIH 3T3 transformants induced by DNAs of spontaneous mouse liver tumors. [^{35}S]Methionine-labeled cell extracts from NIH 3T3 cells and different NIH 3T3 transformants induced by spontaneous mouse liver tumor DNAs were immunoprecipitated with p21 monoclonal antibody Y13-259 and analyzed by NaDodSO$_4$/12.5% PAGE and fluorography. Lanes: 1, NIH 3T3 cells; 2, NIH 3T3 cells transformed by T24 oncogene; 3, NIH 3T3 cells transformed by Hs242 oncogene; 4–6, NIH 3T3 transformants of liver adenoma DNAs; 7–16, NIH 3T3 transformants of liver carcinoma DNAs.

itation, this band persisted in each second and third cycle transfectant analyzed.

DISCUSSION

In this report, we examined spontaneous hepatocellular adenomas and carcinomas in the B6C3F1 mouse, as well as a variety of spontaneous tumors in the Fischer 344/N rat, for the presence of oncogenes detectable by DNA transfection. The frequency of oncogenes in spontaneous hepatocellular carcinomas of the B6C3F1 mice was 77%, the highest incidence so far observed in any spontaneous tumor of animal or human origin. This is even more striking when contrasted with the absence of transforming genes in other liver tumors so far reported (35) and with spontaneous rat malignancies analyzed under identical conditions in our present studies. All of these findings imply that spontaneous hepatocellular carcinomas of the B6C3F1 mouse involved a different mechanism than is responsible for the majority of naturally occurring liver tumors.

Adenomas, which occur at high frequency in this strain, also contained oncogenes that could be detected by the DNA transfection assay. Although the incidence of transfection-positive adenomas (30%) was lower than that observed with carcinomas, the comparably higher transforming efficiencies of the transfection-positive adenoma DNAs suggest that the oncogenes must be present in the vast majority of cells in these benign tumors.

Mouse hepatocellular adenomas and carcinomas represent different stages in the process of neoplastic transformation. Although it is not known if all carcinomas arise within adenomas, it is believed that hepatocellular adenomas have the potential to progress to carcinomas because morphologic features of malignancy may be found in as many as 40% of mouse hepatocellular adenomas (36). The presence of activated Ha-*ras* genes in benign liver tumors indicates that *ras* gene activation can occur at a nonmalignant stage of liver tumor development. Similar findings have been reported by Balmain *et al.* (33) who have demonstrated the presence of an activated Ha-*ras* gene in mouse skin papillomas. If the liver adenoma were the direct precursor of the liver carcinoma, our studies imply that those adenomas possessing activated *ras* oncogenes have a much higher probability of neoplastic progression.

In those adenomas and carcinomas possessing oncogenes, most were identified as activated alleles of Ha-*ras*, one of the highly conserved members of the *ras* gene family. Ha-*ras* oncogenes have been previously detected in several chemically induced rodent tumors and in spontaneous human tumors (9, 14, 17, 21, 33). *ras* oncogenes capable of registering in the NIH 3T3 transfection assay are most commonly activated by point mutation in either the 12th or 61st codon (6, 8, 10). In the present studies, p21 molecules encoded by each of the Ha-*ras* oncogenes of the spontaneous liver tumors demonstrated faster electrophoretic mobilities than normal p21 molecules, consistent with the conclusion that 61st codon alterations were the activating lesions in each case (30). These findings, combined with the absence of oncogenes detectable by transfection of normal liver DNA of B6C3F1 mice, lead us to conclude that the Ha-*ras* oncogenes were activated by somatic events within the liver of this mouse strain. The high frequency of detection of an activated Ha-*ras* oncogene in these tumors and the apparent uniformity in the activating lesion suggests an important role for this oncogene in mouse liver tumorigenesis.

Transforming genes that did not appear to be members of the known *ras* gene family were detected in two spontaneous liver carcinomas. This evidence was based on the absence of either rearrangements or amplification of any of the three known *ras* genes in serial transfectants induced by these oncogenes. Transfectants induced by one of the genes reproducibly demonstrated a protein detectable with anti-ras p21 monoclonal antiserum. This p21-like species migrated with apparent electrophoretic mobility somewhat slower than that of the endogenous ras p21 in NIH 3T3 cells. Thus, this transforming gene may code for a protein immunologically related to ras p21, but representing a distinct gene family. In fact, there is accumulating evidence for the existence of other *ras*-related gene families (37, 38). It is also possible that this transforming gene may code for a product that complexes with the normal ras p21 and has a similar but distinct apparent electrophoretic mobility. However, we cannot exclude the possibility that this gene represents a known member of the *ras* gene family, which, unlike all of the others analyzed, was neither amplified nor rearranged upon serial transmission. The molecular cloning of this gene should help in distinguishing between these possibilities.

The frequency of detection of transforming genes distinct from known members of the *ras* gene family was relatively low. However, to date almost all of the transforming genes detected in the NIH 3T3 transfection assay have been members of the *ras* family. Thus, the genetic factors predisposing the B6C3F1 mice to the activation of Ha-*ras* oncogenes in spontaneous liver adenomas and carcinomas may be associated with a higher incidence of activation of other genes capable of scoring in this assay as well. In support of this possibility, we have observed a high incidence of apparent non-*ras* transforming genes in chemically induced hepatocellular carcinomas of the B6C3F1 mouse (unpublished observations). If so, this mouse model may be very useful in the detection of the various classes of proto-oncogenes that are susceptible to mutational activation.

The potential human health hazards from chemicals in the environment are often assessed on the basis of epidemiologic evidence from exposed human populations with supportive evidence being derived from short-term tests that correlate with carcinogenicity (39). However, most chemicals are considered potential human carcinogens on the basis of long-term bioassays in rodent models. One model, the hybrid B6C3F1 mouse, has been used in National Toxicology Program/National Cancer Institute (NTP/NCI) bioassay studies since 1971. The liver of this strain not only has a high spontaneous incidence of hepatocellular tumors but also is the most frequent tumor target tissue endpoint in NTP/NCI carcinogenesis studies (24). Of 278 compounds tested and evaluated through June 1984, 141 (51%) were found to be carcinogenic. Of these, 50% (70/141) involved mouse liver tumors. At present, any relationship between spontaneous tumors and the high frequency of chemically induced tumors in the liver of the B6C3F1 mouse is unclear.

Our present findings suggest two possible explanations for the increased incidence of hepatocellular tumors in response to putative carcinogens. Whatever genetic predisposing factors lead to spontaneous activation of oncogenes may make the liver cell more susceptible to genetic lesions in response to carcinogens. Moreover, there is considerable evidence that the development of a fully malignant tumor proceeds through multiple independent stages. Thus, any agent that induced additional somatic mutational events may accelerate the malignant process of liver cells that already contain activated oncogenes such as Ha-*ras*. In either case, knowledge of the pattern and identities of oncogenes present in spontaneous B6C3F1 hepatocellular malignancies should be useful in comparing oncogenes that result from chemical exposure. The detection of new or different oncogenes after chemical treatment could provide further confirmation of the validity of this bioassay system.

While this paper was in preparation, Fox and Watanabe (40) reported that DNA from B6C3F1 mouse liver tumors induced foci in NIH 3T3 cells. This report did not attempt to

identify the nature of the oncogenes or to distinguish between adenomas and carcinomas.

1. Land, H., Parada, L. & Weinberg, R. A. (1983) *Science* **222,** 771–778.
2. Bishop, M. J. (1983) *Annu. Rev. Biochem.* **52,** 301–354.
3. Pulciani, S., Santos, E., Lauver, A. V., Long, L. K., Robbins, K. C. & Barbacid, M. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 2845–2849.
4. Eva, A., Tronick, S. R., Gol, R. A., Pierce, J. H. & Aaronson, S. A. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 4926–4930.
5. Parada, L. F. & Weinberg, R. A. (1983) *Mol. Cell. Biol.* **3,** 2298–2301.
6. Santos, E., Martin-Zanca, D., Reddy, E. P., Pierotti, M. A., Porta, D. E. & Barbacid, M. (1984) *Science* **223,** 661–664.
7. Eva, A. & Aaronson, S. A. (1983) *Science* **220,** 955–956.
8. Sukumar, S., Notario, V., Martin-Zanca, D. & Barbacid, M. (1983) *Nature (London)* **306,** 658–661.
9. Balmain, A. & Pragnell, I. (1983) *Nature (London)* **303,** 72–74.
10. Guerrero, I., Villasante, A., Corces, V. & Pellicer, A. (1984) *Science* **225,** 1159–1162.
11. Guerrero, I., Calzada, P., Mayer, A. & Pellicer, A. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 202–205.
12. Schechter, A. L., Stern, D. F., Vaidyanathan, L., Decker, S. J., Drebin, J. A., Greene, M. I. & Weinberg, R. A. (1984) *Nature (London)* **312,** 513–516.
13. Tabin, C. J., Bradley, S. M., Bargmann, C. I., Weinberg, R. A., Papageorge, A. G., Scolnick, E. M., Dhar, R., Lowy, D. R. & Chang, E. H. (1982) *Nature (London)* **300,** 143–149.
14. Reddy, E. P., Reynolds, R. K., Santos, E. & Barbacid, M. (1982) *Nature (London)* **300,** 149–152.
15. Taparowsky, E., Suard, Y., Fasano, O., Shimizu, K., Goldfarb, M. & Wigler, M. (1982) *Nature (London)* **300,** 762–765.
16. Capon, D. J., Chen, E. Y., Levinson, A. D., Seeburg, P. H. & Goeddel, D. V. (1983) *Nature (London)* **302,** 33–37.
17. Yuasa, Y., Srivastava, S. K., Dunn, C. Y., Rhim, J. S., Reddy, E. P. & Aaronson, S. A. (1983) *Nature (London)* **303,** 775–779.
18. Shimizu, K., Birnbaum, D., Ruley, M. A., Fasano, O., Suard, Y., Edlund, L., Taparowsky, E., Goldfarb, M. & Wigler, M. (1983) *Nature (London)* **304,** 497–500.
19. Capon, D. J., Seeburg, P. H., McGrath, J. P., Hayflick, J. S., Edman, U., Levinson, A. D. & Goeddel, D. V. (1983) *Nature (London)* **304,** 507–513.
20. Yuasa, Y., Gol, R. A., Chang, A., Chiu, I.-M., Reddy, E. P., Tronick, S. R. & Aaronson, S. A. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 3670–3674.
21. Kraus, M., Yuasa, Y. & Aaronson, S. A. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 5384–5388.
22. Nakano, H., Yamamoto, F., Neville, C., Evans, D., Mizumo, T. & Perucho, M. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 71–75.
23. Haseman, J. K., Huff, J. & Boorman, G. A. (1984) *Toxicol. Pathol.* **12,** 126–135.
24. Maronpot, R. R. & Boorman, G. A. (1982) *Toxicol. Pathol.* **10,** 71–80.
25. Turusov, V. S., ed. (1976) *Pathology of Tumours in Laboratory Animals*, Tumours of the Rat (International Agency for Research on Cancer, Lyon, France), Vol. 1.
26. Turusov, V. S., ed. (1979) *Pathology of Tumours in Laboratory Animals*, Tumours of the Mouse (International Agency for Research on Cancer, Lyon, France), Vol. 2.
27. Graham, F. L. & van der Eb, A. J. (1973) *Virology* **52,** 456–467.
28. Wigler, M., Silverstein, S., Lee, L. S., Pellicer, A., Cheng, Y. & Axel, R. (1977) *Cell* **11,** 223–232.
29. Southern, E. M. (1975) *J. Mol. Biol.* **98,** 503–517.
30. Srivastava, S. K., Yuasa, Y., Reynolds, S. H. & Aaronson, S. A. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 38–42.
31. Barbacid, M., Lauver, A. V. & Devare, S. G. (1980) *J. Virol.* **33,** 196–207.
32. Furth, M. E., Davis, L. J., Fleurdelys, B. & Scolnick, E. M. (1982) *J. Virol.* **43,** 294–304.
33. Balmain, A., Ramsden, M., Bowden, G. T. & Smith, J. (1984) *Nature (London)* **307,** 658–660.
34. Anderson, P. R., Tronick, S. R. & Aaronson, S. A. (1981) *J. Virol.* **40,** 431–439.
35. Farber, E. (1984) *Cancer Res.* **44,** 5463–5474.
36. Ward, J. N. (1984) in *Mouse Liver Neoplasia: Current Perspectives*, ed. Popp, J. A. (Hemisphere, New York), pp. 1–26.
37. Padua, R. A., Barrass, N. & Currie, G. A. (1984) *Nature (London)* **311,** 671–673.
38. Madaule, P. & Axel, R. (1985) *Cell* **41,** 31–40.
39. Interagency Regulatory Liaison Group (1979) *J. Natl. Cancer Inst.* **63,** 241–268.
40. Fox, T. R. & Watanabe, P. G. (1985) *Science* **228,** 596–597.