Detection of a Cellular Oncogene in Spontaneous Liver Tumors of B6C3F1 Mice

Abstract. *An active cellular oncogene was demonstrated in hepatocellular neoplasms arising spontaneously in 24-month-old B6C3F1 mice. DNA isolated from the tumorous tissue and transfected into NIH 3T3 cells showed an 82 percent (9 of 11 animals) frequency of foci induction. In contrast, DNA isolated from the surrounding nontumorous hepatic tissue from the same animals and DNA from other 24-month-old B6C3F1 mice without tumors did not cause transformation in the NIH 3T3 cell assay. This strain of mouse is used extensively in carcinogen bioassays, and the observed high frequency of transformation (82 percent, compared to 10 to 20 percent in humans) supports the concept that the B6C3F1 mouse is hypersusceptible to liver tumor development. It also emphasizes the need to further understand the mechanisms of oncogene activation in animals used for long-term studies of toxicity and oncogenicity before evaluating potential human risk.*

While considerable effort has been expended in evaluating the role of oncogenes in human cancer, there are surprisingly few reports characterizing the presence or absence of cellular oncogenes in animals used for long-term studies of carcinogenicity. Yet most of the data on which decisions are made to protect the public from potential carcinogens are generated from animal bioassays. Understanding the potential role that cellular oncogenes play in tumor development in animals used for long-term studies is important for proper interpretation of the data and subsequent assessment of potential human risk.

The B6C3F1 mouse is used extensively in long-term bioassays to evaluate carcinogenicity. A recent compilation of these studies conducted under the direction of the National Toxicology Program reported an average incidence of 25 percent for spontaneous hepatocellular carcinomas in male B6C3F1 mice (*1*). The difficulty of interpreting bioassay data on animals that show a high spontaneous tumor incidence and the relevance of an increased incidence associated with chemical treatment to human risk assessment are areas of particular controversy (*2–4*). Resolution of this controversy necessitates a more detailed understanding of the cellular mechanisms of organ tumorigenesis.

The detection of cellular oncogenes in a variety of presumably spontaneous human tumors suggests that cellular oncogenes might also be associated with the high incidence of spontaneous liver tumors in the male B6C3F1 mouse. To test this hypothesis, we isolated high molecular weight DNA from spontaneous liver tumor tissue from untreated 24-month-old male B6C3F1 mice. The DNA was used to transfect NIH 3T3 cells (*5*). In initial experiments the DNA isolated from 6 of 11 animals with spontaneous liver tumors induced foci in NIH 3T3 cell cultures (Table 1). The specific transforming frequencies of the tumor cell DNA ranged from 0.005 to 0.02 focus-forming units per microgram of DNA. These values are in close agreement with the frequencies reported for DNA from human hematopoietic tumors (*6*). No foci were observed when NIH 3T3 cells were transfected with calf thymus DNA or DNA isolated from liver tissue from a 3-month-old male B6C3F1 mouse free of tumors.

To ensure reproducibility of these results, we conducted a second experiment using DNA isolated from the same 11 tumor-bearing animals. The number of control cultures was expanded to include liver DNA isolated from two 3- to 5-month-old and ten 24-month-old male B6C3F1 mice without tumors. The cultures were scored 2 to 3 weeks after transfection. Again, foci were detected in the cultures transfected with DNA derived from the tumor tissue. In this experiment 7 of the 11 tumor-bearing animals were positive for the ability to induce transformed foci. No foci were observed in the 43 cultures transfected with DNA isolated from young mice (3 to 5 months) without tumors (Table 1).

The development of spontaneous liver tumors in the B6C3F1 mouse is apparently an age-associated phenomenon. However, the lack of transforming activity of DNA derived from normal 24-month-old mice indicates that the transforming potential of the tumor DNA was not a function of age (Table 1). Rather, these experiments suggest that an active cellular oncogene is present in the liver tumor DNA of the B6C3F1 mouse.

These results are significant because, to our knowledge, no transforming activity has previously been detected with DNA isolated from human, mouse, or rat liver tumor tissue. In addition, 9 of 11 tumor-bearing animals demonstrated transforming activity in at least one of the two transformation assays; this represents an 82 percent frequency of transforming activity, which is appreciably higher than the 10 to 20 percent previously reported for DNA isolated from tumors in various human tissues (colon, lung, pancreas, and hematopoietic) (*6*, *7*). The high frequency of transformation

Table 1. Detection of transforming genes in spontaneous hepatic tumors from 24-month-old male B6C3F1 mice. Excised tissues were immediately frozen on dry ice. Tissues were homogenized with a Dounce homogenizer in 10 ml of 50 m*M* tris, 50 m*M* NaCl, and 5 m*M* EDTA (*p*H 8.0). Cells were lysed by addition of sodium dodecyl sulfate to 0.5 percent and the lysate was treated overnight with proteinase K (0.2 mg/ml). Proteins and lipids were removed by extracting once with phenol, phenol-chloroform-isoamyl alcohol (25:24:1), and chloroform-isoamyl alcohol (24:1). DNA was precipitated with ethanol, dried, and resuspended in 10 m*M* tris and 1 m*M* EDTA (*p*H 7.2) and incubated for 1 hour with α-amylase (400 μg/ml) and ribonuclease (100 μg/ml). The DNA was again extracted as described above and precipitated with ethanol, dried, and resuspended in 10 m*M* tris and 1 m*M* EDTA (*p*H 7.2). The DNA transfection assay was performed (*5*). Approximately 20 hours before transfection 1.5×10^5 NIH 3T3 cells were seeded in 10-cm culture dishes (Corning). DNA (50 μg) was suspended in 250 m*M* $CaCl_2$ and the DNA-Ca-PO_4 precipitate was formed by slow addition with gentle agitation to an equal volume of 50 m*M* Hepes, 280 m*M* NaCl, and 1.5 m*M* Na_2PO_4. After 1 hour the precipitate was resuspended and applied to the NIH 3T3 monolayer. After 20 hours of incubation at 37°C the precipitate was removed. The medium (Dulbecco's modified Eagle medium with 5 percent calf serum and 1 μ*M* dexamethasone) was changed three times weekly. Foci were counted 14 to 21 days after transfection by individuals having no knowledge of the DNA sources represented. Transfection-positive animals gave 0.25 to 1.00 focus-forming units per plate, with four plates assayed per animal.

DNA source	Age (months)	Number of cultures	Total number of foci observed	Number of animals whose DNA induced transformation
		Experiment 1		
Mice with tumors	24	44	10	6 of 11
Mouse without tumors	3	4	0	0 of 1
Calf thymus		4	0	
		Experiment 2		
Mice with tumors	24	44	14	7 of 11
Mice without tumors	3 to 5	43	0	0 of 2
Mice without tumors	24	40	0	0 of 10

in the B6C3F1 mouse liver suggests that a common mechanism of oncogene activation is occurring in tumor tissue. Such a common mechanism is predictable because of the high degree of genetic similarity characteristic of inbred strains. Notably, the C3H mouse (the paternal genetic contributor in the B6C3F1 hybrid cross) has an even higher incidence of spontaneous hepatocellular tumors than does the B6C3F1 mouse (*4*).

One possible mechanism accounting for both the high spontaneous tumor incidence in the B6C3F1 mouse and the observed high transforming activity could be germ line transmission of the oncogene, resulting in a genetic predisposition to neoplasia. To evaluate this possibility, we isolated DNA from nontumorous liver tissue from eight animals whose tumor DNA induced foci in the NIH 3T3 assay. This DNA was transfected into NIH 3T3 cells as before. An equal number of control cultures were treated with calf thymus DNA. After 3 weeks no foci were observed in any of the cultures.

Thus the cellular oncogene detected in the spontaneously occurring hepatocellular carcinomas is not attributable to germ line transmission. Similar results have been reported for human patients having familial syndromes associated with a greatly increased cancer risk (*8*). This suggests that the active cellular oncogene found in the spontaneous liver tumors arose from alterations in individual somatic cells. In addition, the observation that DNA from 9 of 11 tumor-bearing animals produced transforming activity implies that some factor predisposing to malignancy is genetically transmitted in this strain of mouse. These results indicate that the B6C3F1 mouse is dissimilar to the genetically diverse human population in its ability to activate, with a very high frequency, a specific tumor-associated oncogene.

The detection of a cellular oncogene in the B6C3F1 mouse liver provides the opportunity to address questions of significant toxicological interest. For example, several studies have shown an increase in the incidence of liver tumors in the B6C3F1 mouse after prolonged exposure to chemical agents. Many of these agents have demonstrated genotoxic activity (defined as a direct chemical interaction with DNA), and it is generally concluded that this enhancement occurs through a somatic mutational mechanism. However, increased incidences of hepatic tumors in the B6C3F1 mouse have also been observed after treatment with agents that appear to be nongenotoxic in nature but that increase cellular proliferation (*9–13*). Such conflicting results create a considerable dilemma in interpreting bioassay results.

It is attractive to hypothesize that substances that increase tumor development by apparently increasing cellular proliferation do so by altering the quantitative or temporal expression of cellular oncogenes. Recent data from several laboratories have shown a quantitative increase in oncogene expression in response to proliferative stimuli (*14–16*). In addition, there is evidence associating quantitative changes in oncogene expression with tumor development (*17–19*). Whether similar changes are important in the increased induction of liver tumors in the B6C3F1 mouse after chemical exposure remains to be determined.

Detection of a cellular oncogene in the B6C3F1 liver might provide insight into the unusually high frequency of spontaneous tumors in this strain of mouse. An understanding of this process may have important implications for the interpretation of animal bioassay data and may provide for more informed estimates of risk from chemical exposure in humans.

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References and Notes

1. K. C. Chu, C. Cueto, J. Ward, *J. Toxicol. Environ. Health* **8**, 251 (1981).
2. Interdisciplinary Panel on Carcinogenicity, *Science* **225**, 682 (1984).
3. Task Force of Past Presidents of the Society of Toxicology, *Fundam. Appl. Toxicol.* **2**, 101 (1982).
4. International Expert Advisory Committee to the Nutrition Foundation, *The Relevance of Mouse Liver Hepatoma to Human Carcinogenic Risk* (Nutrition Foundation, Inc., Washington, D.C., 1983).
5. S. Pulciani *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 2845 (1982).
6. A. Eva, S. R. Tronick, R. A. Gol, J. H. Pierce, S. A. Aaronson, *ibid.* **80**, 4926 (1983).
7. S. Pulciani *et al.*, *Nature (London)* **300**, 539 (1982).
8. S. W. Needleman, Y. Yuasa, S. Srivastava, S. A. Aaronson, *Science* **222**, 173 (1983).
9. F. F. Becker, *Cancer Res.* **42**, 3918 (1982).
10. National Cancer Institute, *DHEW Publ.* 77-813 (1977).
11. National Cancer Institute, *Carcinogenesis Bioassay of Chloroform* (TID PB264018/AS, National Technical Information Service, Springfield, Va., 1976).
12. R. H. Reitz, T. R. Fox, J. F. Quast, *Environ. Health Perspect.* **46**, 163 (1982).
13. A. M. Schumann, J. F. Quast, P. G. Watanabe, *Toxicol. Appl. Pharmacol.* **55**, 207 (1980).
14. M. Goyette, C. J. Petropoulos, P. R. Shank, N. Fausto, *Science* **219**, 510 (1983).
15. K. Kelly, B. H. Cochran, C. D. Stiles, P. Leder, *Cell* **35**, 603 (1983).
16. J. Campisi, H. E. Gray, A. B. Pardee, M. Dean, G. E. Sonenshein, *ibid.* **36**, 241 (1984).
17. M. Schwab, K. Alitalo, H. E. Varmus, J. M. Bishop, D. George, *Nature (London)* **303**, 497 (1983).
18. K. Alitalo, M. Schwab, C. C. Lin, H. E. Varmus, J. M. Bishop, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1707 (1983).
19. D. Kozbor and C. M. Croce, *Cancer Res.* **44**, 438 (1984).
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Brain Dopamine and Serotonin Receptor Sites Revealed by Digital Subtraction Autoradiography

Abstract. *Autoradiography combined with image analysis permitted quantitative visualization of dopamine (D_2) and serotonin (S_2) binding sites in rat brain. Forebrain sections were incubated with tritiated spiroperidol alone or with tritiated spiroperidol plus unlabeled compounds that saturated the D_2 or S_2 sites. By subtracting the digitized image of an autoradiograph derived from the latter sections from that of the former, the D_2 or S_2 sites were specifically revealed. The resulting quantitative images demonstrate the differing anatomical distributions of these sites. The D_2 site is largely restricted to the striatal complex (caudate-putamen, nucleus accumbens septi, and olfactory tubercle), whereas the S_2 site is enriched in layer 5 of motor cortex, the perirhinal and cingulate cortices, and the claustrum.*

The action of dopamine in the forebrain of humans and other mammals has been linked to such diverse functions as sensorimotor coordination, affect, and cognition (*1*). Because dopamine seems to modulate sensorimotor functions and cognitive processes through its interaction with the D_2 class of dopamine receptors (*2*), anatomically distinct populations of D_2 receptors are likely to subserve these specific dopamine-dependent behaviors. When brain sections are incubated with radioligands that bind with high affinity to the D_2 receptor, the resulting autoradiographs can reveal the distribution of these sites in the forebrain with high resolution (*3, 4*). In this report we describe the use of computer-based imaging techniques to analyze [^{3}H]spiroperidol autoradiographs and provide what appears to be the first quantitative visualization of D_2 and serotonin S_2 sites in the mammalian forebrain.

Tritiated spiroperidol labels both D_2 and S_2 sites with nanomolar affinities in brain homogenates or thin sections (*3–6*). The [^{3}H]spiroperidol binding to these two sites is distinguished by using unla-