Jpn. J. Cancer Res. 85, 510–514, May 1994

Absence of p53 Mutations in Rat Colon Tumors Induced by 2-Amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole, 2-Amino-3-methylimidazo[4,5-*f*]quinoline, or 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine

Hiroshi Makino,[1] Toshikazu Ushijima,[1] Hideki Kakiuchi,[1] Masahiko Onda,[2] Nobuyuki Ito,[3] Takashi Sugimura[1] and Minako Nagao[1]

[1]*Carcinogenesis Division, National Cancer Center Research Institute, 1-1 Tsukiji 5-chome, Chuo-ku, Tokyo 104,* [2]*First Department of Surgery, Nippon Medical School, 1-5 Sendagi 1-chome, Bunkyo-ku, Tokyo 116 and* [3]*First Department of Pathology, Nagoya City University Medical School, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467*

Colon tumors were induced in F344 rats by three heterocyclic amines (HCAs), 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) or 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), and examined for p53 mutations. Seven carcinomas induced by Glu-P-1, and nine carcinomas and two adenomas induced by IQ were examined by cDNA-polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis from codon 103 to 391 of p53, which encompasses the conserved regions II to IV. Nine carcinomas induced by PhIP were examined by genomic PCR-SSCP analysis of exons 5 to 7 (from codon 124 to 304), which encompasses the 3′ half of the conserved region II and all the conserved regions III–V. No band shifts were found in any of these tumors under at least two conditions of SSCP analysis. Our previous study had shown a Ki-*ras* mutation in only one Glu-P-1-induced adenocarcinoma among the same 27 colon tumors, and no other mutation of *ras* family genes had been found. HCA-induced rat colon tumors appear to represent a group of human colon tumors in which neither Ki-*ras* nor p53 is involved.

Key words: Heterocyclic amine — Genetic alteration — Chemically induced tumor — Colorectal neoplasm

2-Amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) are mutagenic heterocyclic amines (HCAs), which are present in normal human foods.[1] Glu-P-1 was first isolated from a glutamic acid pyrolysate,[2] and IQ was first isolated from broiled fish.[3] IQ has also been detected in cooked meat, fish and cigarette smoke.[4] Glu-P-1 and IQ induced colon tumors in F344 rats as well as liver and Zymbal gland tumors.[5,6] PhIP was first isolated from fried ground beef[7] and later found in substantial amounts in various cooked meats and fish.[1] It mainly induces colon tumors in male F344 rats and mammary carcinomas in female F344 rats.[8] The colon carcinogenesis induced by these HCAs in experimental animal is expected to be useful systems since the genetic alterations in these tumors are caused by carcinogens to which humans are exposed and since carcinogens tend to leave their mutational fingerprints in the genes they have affected.[9]

Human colon carcinogenesis has been shown to consist of multiple steps, and alterations of the APC, Ki-*ras*, p53 and DCC genes are supposed to take place sequentially along with the progression of carcinogenic stages in most human colon cancers.[10,11] Among the alterations, Ki-*ras* mutation is considered to take place in early adenomas and p53 mutation in later stages.[11,12]

We previously examined mutations in *ras* family genes of colon tumors induced by the three HCAs, Glu-P-1, IQ and PhIP.[13] Only one of seven Glu-P-1-induced colon adenocarcinomas had a G-to-T transversion at the second position of Ki-*ras*, while 11 IQ-induced tumors (two adenomas and nine adenocarcinomas) and nine PhIP-induced adenocarcinomas had no mutation in any *ras* family gene. Involvement of p53 mutation, which is observed in approximately 70% of human colon cancers,[11] in these *ras*-negative tumors is of interest.

In this study, we examined these HCA-induced colon tumors for p53 mutations by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis.

MATERIALS AND METHODS

Tumors and cell lines Details of tumor induction were described elsewhere. Seven adenocarcinomas induced by 0.05% Glu-P-1 in the diet,[13] 11 tumors (two adenomas and nine adenocarcinomas) induced by 0.03% IQ in the

diet[13] and nine adenocarcinomas induced by 0.04% PhIP in the diet[8] were used. The liver of an untreated F344 male rat was used as a negative control.

L6TG, a rat myeloblast cell line, and SCC131, a rat Zymbal gland tumor cell line, were obtained from the Japanese Cancer Research Resources Bank, and maintained as recommended. L6TG was previously shown to have no mutations in p53, and SCC131 to have a G-to-A transition at the first position of the p53 codon 171.[14]

Preparation of cDNA and genomic DNA Total cellular RNAs from Glu-P-1- and IQ-induced tumors and the L6TG cell line were prepared by the acid guanidinium thiocyanate-phenol-chloroform extraction method.[15] Trace amounts of contaminating DNA in 50 μg samples of RNA were eliminated by digestion at 37°C for 30 min with 2 units of RNase-free DNase (RQ1, Promega) in the presence of ribonuclease inhibitor (RNasin, Toyobo). After phenol/chloroform extraction, RNA was concentrated by ethanol precipitation.[14] cDNA was synthesized from 1 μg of total cellular RNA with a random hexamer and 200 units of Moloney murine leukemia virus RNaseH$^-$ reverse transcriptase (Superscript, Gibco-BRL) in a mixture of 20 μl.

DNA of PhIP-induced tumors was extracted from paraffin-embedded samples.[16] The region of the tumor in 5 μm thick sections was trimmed on a slide glass, stripped off the glass and deparaffinized in xylene. DNA was then extracted with PCR buffer with the non-ionic detergents Nonidet P-40 (Sigma) and Tween 20 (Bio-Rad). The DNA in each section was prepared in a volume of 90 μl.

PCR-SSCP analysis PCR-SSCP analysis was performed by a reported method.[17] For cDNA-PCR, primers were designed to cover codons 103 to 391, which encompassed the conserved regions II to V (Y, Z and W; Fig. 1, bottom; Table I). For genomic DNA-PCR, primers were designed to cover exons 5 through 7, which covers the 3′ half of the conserved region II and all the conserved regions III to V (E5, E6-1, E6-2 and E7; Fig. 1, top; Table I). Exon 6 of rat p53 is known to correspond to exons 6 and 7 of human p53.[18] At least one of a pair of primers was based on the intron sequence. Primers were synthesized by the phosphoroamidite method in a 381A DNA synthesizer (Applied Biosystems).

Samples of 1 μl of the cDNA reaction mixture or 2 μl of the DNA mixture were amplified by PCR with 15 pmol of primers end-labeled with [γ-^{32}P]ATP (ICN Radiochemicals) and 0.25 unit of *Taq* polymerase. Thirty cycles of the reaction at 94°C, 55°C and 72°C for 0.5, 0.5, and 1 min, respectively, were run in a thermal cycler (Perkin-Elmer Cetus). The total PCR reaction mixture (5 μl) was mixed with 45 μl of 0.1% SDS and 10

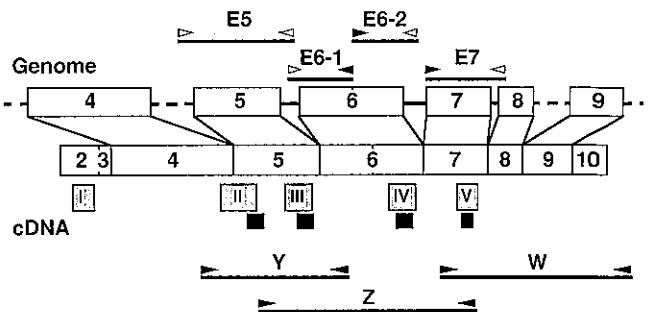

Fig. 1. Design of primers for genomic and cDNA-PCR-SSCP analyses. Rat exon 6 of p53 corresponds to human exons 6 and 7. In genomic PCR-SSCP analysis, exons 5, 6 and 7 were analyzed. At least one of each pair of PCR primers was located within an intron. In cDNA-PCR-SSCP analysis, exons 5 through 10 were analyzed. Closed boxes, mutational hot spots in humans; shaded boxes I–V, conserved regions among species; closed arrowheads, primers based on exon sequences; open arrowheads, primers based on intron sequences.

Table I. Oligonucleotide Primers

	Region	Name	Sequence
cDNA	Y	P 15	5′-GGCAACTATGGCTTCCACCT-3′
		P 7	5′-AGCATACGGATTTCCTTCCA-3′
	Z	P 16	5′-CCTCCACCTGGTACCCGTGT-3′
		P 11	5′-CAATGCTCTTCTTTTTTGCG-3′
	W	P R11	5′-GGACAGAGGAAGAAAATTTC-3′
		P FP-2	5′-GTGATGGGGACAGGATGCAG-3′
Genome	Exon 5	P I4-2	5′-GATTCTTTCTCCTCTCCTAC-3′
		P I5-1	5′-ACAGGCAGTGCCAGTGCTCA-3′
	Exon 6-1	P I5-3	5′-CCCGGCCTCTGACTTATTCT-3′
		P 19	5′-CTCAGGTGGCTCATACGGTA-3′
	Exon 6-2	P Z	5′-TATACCACTATCCACTACAAG-3′
		P I7-4	5′-CCCAACCTGGCACACAGCTT-3′
	Exon 7	P 18	5′-GGGAATCTTCTGGGACGGGA-3′
		P I8-2	5′-CTTCTTTGTCCTGCCTGCTC-3′

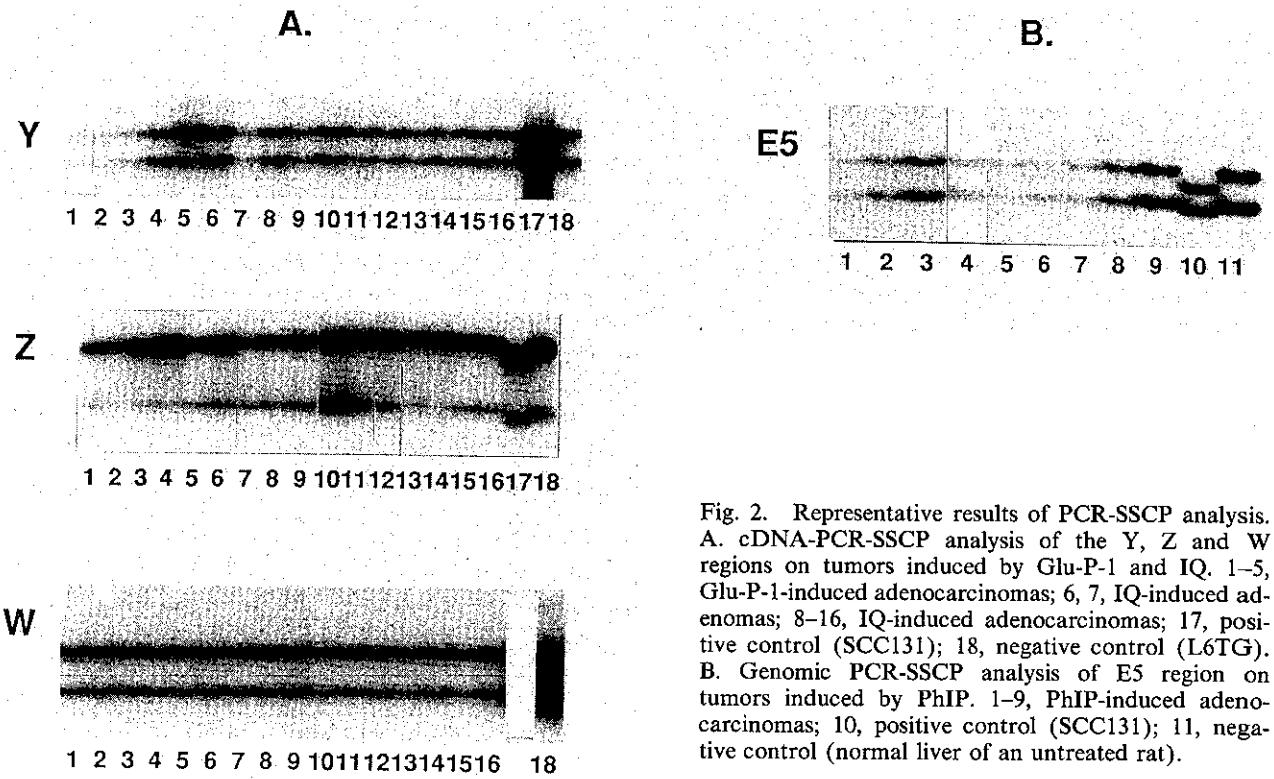

Fig. 2. Representative results of PCR-SSCP analysis. A. cDNA-PCR-SSCP analysis of the Y, Z and W regions on tumors induced by Glu-P-1 and IQ. 1–5, Glu-P-1-induced adenocarcinomas; 6, 7, IQ-induced adenomas; 8–16, IQ-induced adenocarcinomas; 17, positive control (SCC131); 18, negative control (L6TG). B. Genomic PCR-SSCP analysis of E5 region on tumors induced by PhIP. 1–9, PhIP-induced adenocarcinomas; 10, positive control (SCC131); 11, negative control (normal liver of an untreated rat).

m*M* EDTA. Then 2 μl of this mixture was mixed with 2 μl of formamide dye (95% formamide, 20 m*M* EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol blue). After heat denaturation at 80°C, 1 μl of sample was applied to 6% polyacrylamide gel with or without 5% glycerol, and run at 20°C. The gel was dried on filter paper and exposed to X-ray film (Kodak) at −80°C for 3–12 h with an intensifying screen (Dupont).

RESULTS

Regions Y, Z and W of the cDNA of seven Glu-P-1-induced colon carcinomas and 11 IQ-induced tumors (nine carcinomas and two adenomas) were amplified by PCR. In SSCP analysis of the Y, Z and W region, all the samples showed the same mobility as that of a negative control, L6TG, under two conditions (with or without 5% glycerol). On the other hand, a positive control, SCC131, showed a mobility shift in the Y and Z region under both conditions (Fig. 2A).

Regions E5, E6-1, E6-2 and E7 of genomic DNAs of PhIP-induced colon carcinomas were amplified. None of the nine samples showed a mobility shift of any fragment relative to that of the negative control (the liver of an untreated F344 rat) under two conditions of SSCP analysis, as illustrated in Fig. 2B, for E5.

DISCUSSION

No band shift was detected in the regions examined by SSCP analysis under at least two conditions. When a fragment analyzed by SSCP is less than 300 base pairs in length, the possibility of a false negative result is quite low under two conditions.[19,20] In our experiments, all the fragments analyzed were shorter than 300 base pairs, and the probability of a false negative result in SSCP analysis was very low. In fact, in our experimental systems, both genomic and cDNA-PCR-SSCP analyses were effective for detecting p53 mutations in rat Zymbal gland tumors induced by IQ.[14]

IQ, Glu-P-1 and PhIP are known of form DNA adducts with guanine bases, and mutations induced by these compounds are considered to take place mostly at guanine bases.[21] Since guanine bases are distributed evenly throughout the p53 gene and the conserved regions of p53 are functionally important,[22] the probability that mutations had clustered in the regions which were not examined in this study should be very low.

Although the rat colon carcinomas induced by the three HCAs clearly show marked atypia and invasive features in microscopic analysis,[8] most of them are macroscopically of polypoid type and resemble human adenomatous colon polyps of small size, which are often

histologically benign.[23] Since p53 mutation is rarely found in human colon adenomas and is considered to take place in a late step of human colon carcinogenesis,[11, 12, 24] we still cannot rule out the possibility of p53 mutations in HCA-induced colon tumors if the tumors were allowed to grow further.

p53 mutation was found to be involved in rat carcinogenesis of the Zymbal gland, liver and nasal cavity.[14, 21, 25, 26] As regards colon carcinogenesis, this is the first report of analysis of p53 mutations. Although no conclusion about the involvement of p53 mutations can be drawn at this stage, the absence of p53 mutations in the HCA-induced rat colon tumors does not seem simply due to species differences, since involvements of Ki-*ras* and APC even in rodent colon carcinogenesis have been demonstrated.[27–31]

The finding of genes involved in HCA-induced rat colon carcinogenesis should lead to identification of molecular mechanisms of human colon carcinogenesis not involving Ki-*ras* or p53.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid from the Ministry of Health and Welfare for the Comprehensive 10-Year Strategy for Cancer Control, Japan and a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture. H. Makino and H. Kakiuchi are recipients of Research Resident Fellowships from the Foundation of Cancer Research. The authors thank M. Ochiai and H. Kushida for expert help in animal experiments.

(Received October 2, 1993/Accepted January 26, 1994)

REFERENCES

1) Wakabayashi, K., Nagao, M., Esumi, H. and Sugimura, T. Food-derived mutagens and carcinogens. *Cancer Res.*, **52**, 2092s–2098s (1992).
2) Yamamoto, T., Tsuji, K., Kosuge, T., Okamoto, T., Shudo, K., Takeda, K., Iitaka, Y., Yamaguchi, K., Seino, Y., Yahagi, T., Nagao, M. and Sugimura, T. Isolation and structure determination of mutagenic substances in L-glutaminic acid pyrolysate. *Proc. Jpn. Acad.*, **54**, 248–250 (1978).
3) Kasai, H., Yamaizumi, Z., Wakabayashi, K., Nagao, M., Sugimura, T., Yokoyama, S., Miyazawa, T., Springarn, N. E., Weisburger, J. H. and Nishimura, S. Potent novel mutagen produced by broiling fish under normal conditions. *Proc. Jpn. Acad.*, **56B**, 278–283 (1980).
4) Yamashita, M., Wakabayashi, K., Nagao, M., Sato, S., Yamaizumi, Z., Takahashi, M., Kinae, N., Tomita, I. and Sugimura, T. Detection of 2-amino-3-methylimidazo-[4,5-*f*]quinoline in cigarette smoke condensate. *Jpn. J. Cancer Res.*, **77**, 419–422 (1986).
5) Takayama, S., Masuda, M., Mogami, M., Ohgaki, H., Sato, S. and Sugimura, T. Induction of cancers in the intestine, liver and various other organs of rats by feeding mutagens from glutamic acid pyrolysate. *Gann*, **75**, 207–213 (1984).
6) Takayama, S., Nakatsuru, Y., Masuda, M., Ohgaki, H., Sato, S. and Sugimura, T. Demonstration of carcinogenicity in F344 rats of 2-amino-3-methylimidazo[4,5-*f*]-quinoline from broiled sardine, fried beef and beef extract. *Gann*, **75**, 467–470 (1984).
7) Felton, J. S., Knize, M. G., Shen, N. H., Lewis, P. R., Andresen, B. D., Happe, J. and Hatch, F. T. The isolation and identification of a new mutagen from fried ground beef: 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). *Carcinogenesis*, **7**, 1081–1086 (1986).
8) Ito, N., Hasegawa, R., Sano, M., Tamano, S., Esumi, H., Takayama, S. and Sugimura, T. A new colon and mammary carcinogen in cooked food, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). *Carcinogenesis*, **12**, 1503–1506 (1991).
9) Vogelstein, B. and Kinzler, K. W. Carcinogens leave fingerprints. *Nature*, **355**, 209–210 (1992).
10) Vogelstein, B., Fearon, E. R., Hamilton, S. R., Kern, S. E., Preisinger, A. C., Leppert, M., Nakamura, Y., White, R., Smits, A. M. M. and Bos, J. L. Genetic alterations during colorectal-tumor development. *N. Engl. J. Med.*, **319**, 525–532 (1988).
11) Fearon, E. R. and Vogelstein, B. A genetic model for colorectal tumorigenesis. *Cell*, **61**, 759–767 (1990).
12) Ichii, S., Takeda, S., Horii, A., Nakatsuru, S., Miyoshi, Y., Emi, M., Fujiwara, Y., Koyama, K., Furuyama, J., Utsunomiya, J. and Nakamura, Y. Detailed analysis of genetic alterations in colorectal tumors from patients with and without familial adenomatous polyposis (FAP). *Oncogene*, **8**, 2399–2405 (1993).
13) Kakiuchi, H., Ushijima, T., Makino, H., Ishizaka, Y., Hasegawa, R., Ito, N., Sugimura, T. and Nagao, M. Rare frequency of activation of Ki-*ras* in rat colon carcinomas induced by heterocyclic amines, suggesting alternative mechanisms of human colon carcinogenesis. *Mol. Carcinog.*, **8**, 44–48 (1993).
14) Makino, H., Ishizaka, Y., Tsujimoto, A., Nakamura, T., Onda, M., Sugimura, T. and Nagao, M. Rat p53 gene mutations in primary Zymbal gland tumors induced by 2-amino-3-methylimidazo[4,5-*f*]quinoline, a food mutagen. *Proc. Natl. Acad. Sci. USA*, **89**, 4850–4854 (1992).
15) Chomczynski, P. and Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, **162**, 156–159 (1987).
16) Impraim, C. C., Saiki, R. K., Erlich, H. A. and Teplitz,

R. L. Analysis of DNA extracted from formalin-fixed, paraffin-embedded tissues by enzymatic amplification and hybridization with sequence-specific oligonucleotides. *Biochem. Biophys. Res. Commun.*, **142,** 710–716 (1987).
17) Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K. and Sekiya, T. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proc. Natl. Acad. Sci. USA,* **86,** 7652–7656 (1989).
18) Hulla, J. E. and Schneider, R. P. Structure of the rat p53 tumor suppressor gene. *Nucleic Acids Res.*, **21,** 713–717 (1993).
19) Hayashi, K. PCR-SSCP: a simple and sensitive method for detection of mutations in the genomic DNA. *PCR Methods Appl.*, **1,** 34–38 (1991).
20) Hayashi, K. and Yandell, D. W. How sensitive is PCR-SSCP? *Hum. Mutat.*, **2,** 338–346 (1993).
21) Ushijima, T., Makino, H., Kakiuchi, H., Inoue, R., Tsujimoto, A., Sugimura, T. and Nagao, M. Genetic alterations in HCA-induced tumors. *Proc. 23rd Int. Symp. Princess Takamatsu Cancer Res. Fund,* (1993), in press.
22) Caron de Fromental, C. and Soussi, T. TP53 tumor suppressor gene: a model for investigating human mutagenesis. *Genes Chromosomes Cancer,* **4,** 1–15 (1992).
23) Muto, T., Bussey, H. J. R. and Morson, B. C. The evolution of cancer of the colon and rectum. *Cancer,* **36,** 2251–2270 (1975).
24) Baker, S. J., Preisinger, A. C., Jessup, J. M., Paraskeva, C., Markowitz, S., Willson, J. K., Hamilton, S. and Vogelstein, B. p53 gene mutations occur in combination with 17p allelic deletions as late events in colorectal tumorigenesis. *Cancer Res.*, **50,** 7717–7722 (1990).
25) Smith, M. L., Yeleswarapu, L., Scalamogna, P., Locker, J. and Lombardi, B. p53 mutations in hepatocellular carcinomas induced by a choline-devoid diet in male Fischer 344 rats. *Carcinogenesis,* **14,** 503–510 (1993).
26) Recio, L., Sisk, S., Pluta, L., Bermudez, E., Gross, E. A., Chen, Z., Morgan, K. and Walker, C. *p53* mutations in formaldehyde-induced nasal squamous cell carcinomas in rats. *Cancer Res.*, **52,** 6113–6116 (1992).
27) Jacoby, R. F., Llor, X., Teng, B. B., Davidson, N. O. and Brasitus, T. A. Mutations in the K-*ras* oncogene induced by 1,2-dimethylhydrazine in preneoplastic and neoplastic rat colonic mucosa. *J. Clin. Invest.*, **87,** 624–630 (1991).
28) Jacoby, R. F., Alexander, R. J., Raicht, R. F. and Brasitus, T. K-*ras* oncogene mutations in rat colon tumors induced by *N*-methyl-*N*-nitrosourea. *Carcinogenesis,* **13,** 45–49 (1992).
29) Llor, X., Jacoby, R. F., Teng, B. B., Davidson, N. O., Sitrin, M. D. and Brasitus, T. A. K-*ras* mutations in 1,2-dimethylhydrazine-induced colonic tumors: effects of supplemental dietary calcium and vitamin D deficiency. *Cancer Res.*, **51,** 4305–4309 (1991).
30) Stopera, S. A., Murphy, L. C. and Bird, R. P. Evidence for a *ras* gene mutation in azoxymethane-induced colonic aberrant crypts in Sprague-Dawley rats: earliest recognizable precursor lesions of experimental colon cancer. *Carcinogenesis,* **13,** 2081–2085 (1992).
31) Su, L.-K., Kinzler, K., Vogelstein, B., Preisinger, A. C., Moser, A. R., Luongo, C., Gould, K. A. and Dove, W. F. Multiple intestinal neoplasia caused by a mutation in the murine homologue of the APC gene. *Science,* **256,** 668–670 (1992).