Jpn. J. Cancer Res. 81, 129–134, February 1990

Effects of a Choline-deficient Diet and a Hypolipidemic Agent on Single Glutathione *S*-Transferase Placental Form-positive Hepatocytes in Rat Liver

Kinichi Yokota,[1] Ushasi Singh and Hisashi Shinozuka[2]

Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261

Using the placental form of glutathione *S*-transferase (GST-P) as a marker of carcinogen-initiated hepatocytes, we investigated how a choline-deficient (CD) diet and BR931, a carcinogenic hypolipidemic agent, modify populations of single GST-P-positive hepatocytes. The liver of male Fischer rats (6–7 weeks old) fed a CS or basal diet contained mostly single or double GST-P-positive hepatocytes. Feeding a CD diet for 2–4 weeks led to increases in the number of aggregates of two and three GST-P-positive hepatocytes. By 8–12 weeks, there was an emergence of discrete foci of GST-P-positive hepatocytes consisting of more than 20 hepatocytes. Feeding a BR931 diet for 4–8 weeks resulted in no significant change in the number of single GST-P-positive hepatocytes in the liver as compared to feeding a basal diet. It is suggested that single GST-P-positive hepatocytes in the liver of relatively young rats maintained on a commercial diet may represent endogenously initiated cells. A CD diet promotes endogenously initiated cells to form larger aggregates or foci of GST-P-positive cells.

Key words: Choline deficiency — Hypolipidemic agent — Liver carcinogenesis — GST-P-positive cells

A choline-deficient (CD) diet is a powerful promoting regimen of the emergence of preneoplastic foci and the development of hepatomas in experimental liver carcinogenesis.[1,2] It is uncertain whether the diet acts as a complete carcinogen. Several investigators reported that chronic feeding of a CD diet without administration of known chemical carcinogens led to the development of hepatocellular carcinomas.[3–5] It is now well documented that many strains of rats develop spontaneous preneoplastic lesions as well as hepatomas during their life spans.[6–12] Tumor promoters may enhance the development of hepatocellular carcinomas by promoting these spontaneous preneoplastic lesions. Thus, a stringent distinction between the carcinogenic and promotional effects of many types of tumor promoters is often difficult.[13]

Among many histological markers used to identify precursor lesions of hepatocellular carcinomas in rats, the placental form of glutathione *S*-transferase (GST-P) has been shown to be extremely sensitive,[14–16] and it has been used to identify even single putative initiated hepatocytes shortly after a single dose of various carcinogens.[17,18] From these studies, it became evident that a small number of single or a few clusters of GST-P-positive hepatocytes are present in the liver of rats maintained on a commercial diet. It is not known whether these GST-P-positive cells represent spontaneously (endogenously) initiated cells which will evolve into spontaneous liver tumors in aged rats. In the present study, we investigated how a CD diet and a selected hypolipidemic agent might modify the progression of single GST-P-positive hepatocytes to minifoci of a few cells to larger discrete foci. Although many hypolipidemic agents are known to induce hepatocellular carcinomas in rats and mice, their mechanisms of action are currently unknown.[19,20]

MATERIALS AND METHODS

Animals and diets Male Fischer rats (Harlan Sprague Dawley Inc., Indianapolis, IN) weighing 120–30 g (6–7 weeks old) at the beginning of experiments were used. They were housed individually in metal wire cages in a room under a 12 h light-dark cycle and maintained at a constant temperature (20±2°C) and humidity (50±10%). Semisynthetic, semipurified CD or CS diets were prepared according to the basal B diet of Young *et al.*[21] and their composition has been previously described.[22] BR931 (LPB Instituto Farmaceutico S.P.A., Milan, Italy) was incorporated into a basal diet (Dyets Inc., Bethlehem, PA) at the concentration of 0.16%. Diets and water were given *ad libitum*.

In the first experiment a total of 40 rats were divided into two groups. One group received a CS diet and the

[1] Present address: 3rd Department of Internal Medicine, Asahikawa Medical College, Asahikawa, Japan.

[2] To whom correspondence and requests for reprints should be sent.

[3] The abbreviations used are: CD, choline deficient; CS, choline supplemented; BR931, 4-chloro-6-(2,3-xylidino)pyrimidinylthio-(N-hydroxyethyl)acetamide; GGT, γ-glutamyltranspeptidase; GST-P, glutathione *S*-transferase, placental form.

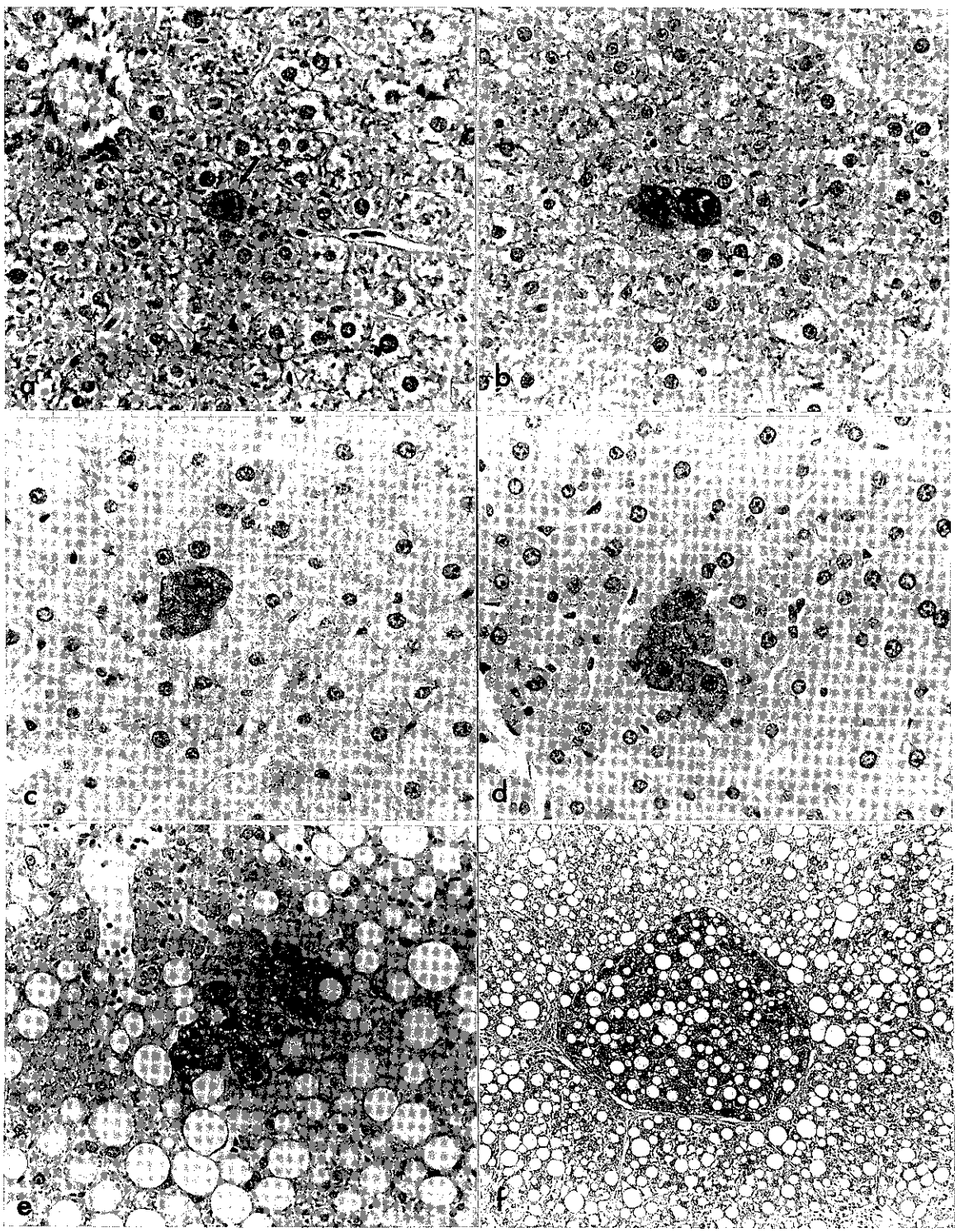

Fig. 1. a) A single GST-P-positive hepatocyte (arrow) in the liver of a rat fed a CS diet for 2 weeks. ×500. b) Doublet of GST-P-positive hepatocytes in the liver of a rat fed a CS diet for 2 weeks. ×500. c) Doublet of GST-P-positive hepatocytes in the liver of a rat fed a basal diet for 4 weeks. ×500. d) An aggregate of 4–5 GST-P-positive hepatocytes in the liver of a rat fed a CS diet for 2 weeks. ×500. e) An aggregate of GST-P-positive hepatocytes in the liver of a rat fed a CD diet for 2 weeks. ×500. f) A discrete focus of GST-P-positive hepatocytes in the liver of a rat fed a CD diet for 8 weeks. ×130.

other a CD diet. Five rats from each group were killed at 2, 4, 8 and 12 weeks after the beginning of the diet feeding. In the second experiment, 18 rats were divided into two groups. One group received a basal diet and the other a BR931 diet. Five rats from each group were killed after 4 weeks and 4 rats from each group were killed after 8 weeks.

GST-P immunohistochemistry Anti rat GST-P antibody was obtained from Bio Prep (Dublin, Ireland), and the ABC method was used to stain GST-P-positive hepatocytes.[23] Affinity-purified biotin-labeled goat anti-rabbit immunoglobulin IgG and avidin-biotin-peroxidase complex (Vectastain ABC kit) was obtained from Vector Laboratories Inc. (Burlingame, CA). Two tissue blocks from each lobe of the liver were fixed in Bouin's solution, embedded in paraffin and sectioned at 5–6 μm thickness. Deparaffinized sections were treated sequentially with normal goat serum, anti GST-P, biotin-labeled goat anti-rabbit IgG and avidin biotin peroxidase (ABC). The site of peroxidase binding was detected by the diaminobenzidine method of Graham and Karnovsky,[24] and the sections were counterstained with hemotoxylin. As a negative control, pre-immune rabbit serum was used instead of anti GST-P. GST-P-positive hepatocytes were quantitated on two slides from each rat covering 3.5–5.3 cm^2 of the tissue sections and classified as single (S), double (D) and triple (T) GST-P-positive hepatocytes, lesions consisting of 4 to 19 GST-P-positive hepatocytes (Q) and foci consisting of more than 20 GST-P positive hepatocytes (F). Data were expressed as number per 5 cm^2 of the sections and were analyzed statistically by the use of Student's t test.

RESULTS

The livers of rats fed a CS or basal diet contained mostly single or double GST-P-positive hepatocytes (Fig. 1 a, b) and aggregates of more than 3 GST-P-positive hepatocytes were extremely rare (Tables I and II, Fig. 1 c, d). Within the time frame of the present experiments (up to 12 weeks), there was no indication that the number of single GST-P-positive hepatocytes increases with the age of rats. Feeding a CD diet for 2 to 4 weeks led to increases in the number of two or three cell aggregates of GST-P-positive hepatocytes. A few aggregates consisting of 4 to 19 cells were observed (Fig. 1 e). By 8 weeks, there was an emergence of discrete foci of GST-P-positive hepatocytes consisting of more than 20 hepatocytes (Fig. 1 f). The number of single to triplet GST-P-positive hepatocytes remained relatively constant in the liver of rats fed a CD diet. Feeding a BR931 diet for 4–8 weeks resulted in no significant change in the

Table II. GST-P-positive Hepatocytes after BR931

Diet	Duration (weeks)	Subpopulations[a]			
		S	D	T	Q
Basal	4	2.0±0.7[b]	1.0±0.4	0	0
BR931	4	3.8±0.6	1.4±0.8	0	0
Basal	8	4.1±0.6	0.6±0.6	1.2±0.6	0
BR931	8	5.3±0.9	1.1±0.6	1.2±0.6	0

a, b) See footnotes *a* and *b* of Table I.

Table I. GST-P-positive Hepatocytes after CD or CS Diet

Diet	Duration (weeks)	Subpopulations[a]				
		S	D	T	Q	F
CS	2	7.1±2.4[b]	1.1±0.7	0	0.8±0.8	0
CD	2	11.9±2.9	6.4±1.2[c]	3.6±0.8[c]	3.9±1.3	0
CS	4	24.7±5.7	2.4±1.1	0	0	0
CD	4	20.5±2.6	8.7±3.2	5.1±0.9[c]	7.3±0.4[c]	0
CS	8	8.8±2.5	1.7±0.8	0.6±0.4	0.4±0.4	0
CD	8	16.3±4.4	5.9±2.0	2.9±0.9[d]	11.5±2.7[c]	15.6±3.6[c]
CS	12	9.3±1.6	0.8±0.5	0	0	0
CD	12	9.0±2.2	6.4±1.7[c]	1.7±1.2	12.7±2.6[c]	12.7±1.2[c]

a) S, single; D, double; T, triple; Q, 4 to 19 cells; F, foci consisting of more than 20 cells.
b) Each value represents the mean number per 5 cm^2 section±SEM of 5 rats.
c) $P<0.01$ against CS.
d) $P<0.05$ against CS.

number of single GST-P-positive hepatocytes as compared to feeding a basal diet (Table II). Only occasional doublets or triplets were observed and no discrete foci were found.

DISCUSSION

The results of the present experiments demonstrate that there are substantial numbers of single GST-P-positive hepatocytes in the liver of relatively young rats maintained on commercial diets. The significance of these single cells with altered phenotype is not known. Rat GST-P has been shown to be elevated in putative neoplastic hepatocellular foci, nodules and hepatomas and used to identify histogenetic stages in the development of hepatomas.[14, 16, 17] Based on the observation that administration of a hepatocarcinogen to rats gave rise to dose-dependent increases in the number of single GST-P-positive hepatocytes, Moore *et al.*[18] postulated that single GST-P-positive hepatocytes may represent putative initiated hepatocytes which progress to hepatomas, even though some single GST-P-positive hepatocytes and small aggregates of GST-P-positive cells were observed in the liver of control animals in their experiments. It is, thus, attractive to suggest that single hepatocytes expressing the placental form of GST in the liver of rats without exposure to known chemical carcinogens represent the earliest precursor cells (endogenously initiated cells) of spontaneously developing hepatomas. On the basis of data obtained with less sensitive markers for preneoplastic lesions and hepatomas, such as γ-glutamyltranspeptidase (γ-GGT), it was concluded that altered hepatocytes suggestive of spontaneously initiated cells appear only in aged rats.[8, 12]

It is evident from the present study that feeding a CD diet to young rats resulted in a striking shift in the populations of GST-P-positive hepatocytes. While single GST-P-positive cells represent the majority of positive cells in the liver of control groups, two to four weeks of a CD diet led to significant increases in aggregate forms of two or three GST-P-positive hepatocytes (Table I). Aggregates of more than four GST-P-positive cells were also present but no discrete foci, consisting of twenty or more cells, were observed. Discrete foci of GST-P-positive hepatocytes began to appear after 8 weeks of the dietary feeding. It is not clear whether these small aggregates of GST-P-positive hepatocytes in the liver of rats fed a CD diet are the result of amplification of single GST-P-positive cells present in control animals or represent *de novo* initiation of hepatocytes by a CD diet. Ghoshal *et al.*[25] concluded that possible initiation of hepatocytes by a diet low in methionine and choline became apparent only after 10–11 weeks of feeding the diet. Lombardi[1] also noted that 6 months' feeding of a CD diet is needed to observe the emergence of enzyme-altered foci in the liver as determined by γ-GGT staining. The presence of single GST-P-positive hepatocytes in the liver of young rats without carcinogen exposure and the progressive increases in small aggregates of GST-P-positive cells shortly after the start of CD diet feeding suggest a possible selective effect of the diet on preexisting GST-P-positive hepatocytes. The concept that tumor promoters may act on endogenously initiated cells to develop hepatocellular carcinomas has been introduced by several investigators.[1, 13] It would be worth examining how other types of liver tumor promoters affect single GST-P-positive cells present in the liver of rats not exposed to known chemical carcinogens.

In contrast to the effect of the CD diet, feeding of BR931, a carcinogenic hypolipidemic agent, for four to eight weeks resulted in no significant alteration in the distribution of GST-P-positive hepatocytes (Table II). This finding is consistent with the earlier observations by several investigators that carcinogenic hypolipidemic agents suppressed the induction of enzyme-altered putative preneoplastic foci in carcinogen-initiated rat liver.[26–29] Although the hepatocarcinogenicity of hypolipidemic agents has been well established,[19, 20] the mechanisms of tumor induction remain unclear. Unlike hepatomas and their precursor lesions induced by classical hepatocarcinogens, those induced by hypolipidemic agents do not express γ-GGT or GST-P.[30, 31] Thus, the cellular lineage for the development of hepatocellular carcinomas induced by hypolipidemic agents may differ from that of the carcinomas induced by classical carcinogens. Based on the present study, it is not likely that hypolipidemic agents induce liver tumors by promoting endogenously initiated cells. The concept of single GST-P-positive hepatocytes as putative "initiated" cells may be useful in further mechanistic studies of liver carcinogenesis.

ACKNOWLEDGMENTS

The work was supported by grants from the National Institutes of Health (CA 26556 and CA 40062). We thank Ms. Christine M. Tappe for technical assistance and Ms. Christel Lollo for help with the preparation of the manuscript.

(Received November 1, 1989/Accepted December 19, 1989)

REFERENCES

1) Lombardi, B. The choline-devoid diet model of hepatocarcinogenesis in the rat. *In* "Chemical Carcinogenesis," ed. F. Feo, P. Pani, A. Columbano and R. Garcia, pp. 563–581 (1988). Plenum Press, New York.
2) Shinozuka, H., Katyal, S. L. and Perera, M. I. R. Choline deficiency and chemical carcinogenesis. *In* "Essential Nutrients in Carcinogenesis," ed. L. A. Poirier, P. M. Newberne, and M. W. Pariza, pp. 253–267 (1986). Plenum Press, New York.
3) Ghoshal, A. K. and Farber, E. Induction of liver cancer by a dietary deficiency of choline and methionine without added carcinogens. *Carcinogenesis,* **5,** 1367–1370 (1984).
4) Mikol, Y. G., Hoover, K. L., Creasia, D. and Poirier, L. A. Hepatocarcinogenesis in rats fed methyl-deficient amino acid-defined diets. *Carcinogenesis,* **4,** 1619–1629 (1983).
5) Yokoyama, S., Sells, M. A., Reddy, T. V. and Lombardi, B. Hepatocarcinogenic and promoting action of a choline-devoid diet in the rat. *Cancer Res.,* **45,** 2834–2842 (1985).
6) Goodman, D. G., Ward, J. M. and Squire, R. A. Neoplastic and nonneoplastic lesions in aging Osborne Mendel rats. *Toxicol. Appl. Pharmacol.,* **55,** 433–447 (1980).
7) Goodman, D. G., Ward, J. M., Squire, R. A., Chu, K. C. and Linhart, M. S. Neoplastic and nonneoplastic lesions in aging F344 rats. *Toxicol. Appl. Pharmacol.,* **48,** 237–248 (1979).
8) Ogawa, K., Onoe, T. and Takeuchi, M. Spontaneous occurrence of γ-glutamyl transpeptidase positive hepatocytic foci in 105 week old Wistar and 72 week old Fischer 344 male rats. *J. Natl. Cancer Inst.,* **67,** 407–412 (1981).
9) Pollard, M. and Luchert, P. H. Spontaneous liver tumors in aged germ free Wistar rats. *Lab. Anim. Sci.,* **29,** 74–77 (1979).
10) Popp, J. A., Scartichini, B. H. and Garvey, L. K. Quantitative evaluation of hepatic foci of cellular alteration occurring spontaneously in Fischer 344 rats. *Fundam. Appl. Toxicol.,* **5,** 314–319 (1985).
11) Ward, J. M. Morphology of foci of altered hepatocytes and naturally-occurring hepatocellular tumors in F344 rats. *Virchows Arch. A.,* **390,** 339–345 (1981).
12) Ward, J. M. Increased susceptibility of livers of aged F344INCr rats to the effects of phenobarbital on the incidence, morphology and histochemistry of hepatocellular foci and neoplasms. *J. Natl. Cancer Inst.,* **71,** 815–823 (1983).
13) Schulte-Hermann, R., Timmermann-Trosiener, I. and Schuppler, J. Promotion of spontaneous preneoplastic cells in rat liver as a possible explanation of tumor production by nonmutagenic compounds. *Cancer Res.,* **43,** 839–844 (1983).
14) Sato, K., Kitahara, A., Satoh, K., Ishikawa, T., Tatematsu, M. and Ito, N. The placental form of glutathione *S*-transferase as a new marker protein for preneoplasia in rat chemical hepatocarcinogenesis. *Gann,* **75,** 199–202 (1984).
15) Satoh, K., Kitahara, A., Soma, Y., Inaba, Y., Hatayama, I., and Sato, K. Purification, induction and distribution of placental glutathione transferase: a new marker enzyme for preneoplastic cells in the rat chemical hepatocarcinogenesis. *Proc. Natl. Acad. Sci. USA,* **82,** 3964–3968 (1985).
16) Tatematsu, M., Mera, Y., Ito, N., Satoh, K., and Sato, K. Relative merits of immunohistochemical demonstrations of placental, A, B and C forms of glutathione *S*-transferase and histochemical demonstration of γ-glutamyltransferase as markers of altered foci during liver carcinogenesis in rats. *Carcinogenesis,* **6,** 1621–1626 (1985).
17) Cameron, R. G. Comparison of GST-P versus GGT as markers of hepatocellular lineage during analyses of initiation of carcinogenesis. *Cancer Invest.,* **6,** 725–734 (1988).
18) Moore, M. A., Nakagawa, K., Satoh, K., Ishikawa, T. and Sato, K. Single GST-P positive liver cells — putative initiated hepatocytes. *Carcinogenesis,* **8,** 483–486 (1987).
19) Reddy, J. K., Azarnoff, D. L. and Hignite, C. E. Hypolipidaemic hepatic peroxisome proliferators form a novel class of chemical carcinogens. *Nature,* **283,** 397–398 (1980).
20) Reddy, J. K. and Lalwani, N. D. Carcinogenesis by hepatic peroxisome proliferators: evaluation of the risk of hypolipidemic drugs and industrial plasticizers. *Crit. Rev. Toxicol.,* **12,** 1–58 (1983).
21) Young, R. J., Lucas, C. C., Patterson, I. M. and Best, C. H. Lipotropic dose response studies in rats: comparison of choline, betaine and methionine. *Can. J. Biochem. Physiol.,* **34,** 713–726 (1956).
22) Perera, M. I. R., Demetris, A. J., Katyal, S. K. and Shinozuka, H. Lipid peroxidation of liver microsome membranes induced by choline deficient diet and its relationship to the diet-induced promotion of the induction of γ-glutamyl transpeptidase-positive foci. *Cancer Res.,* **45,** 2533–2538 (1985).
23) Hsu, S. M., Raine, L. and Fanger, H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques. *J. Histochem. Cytochem.,* **29,** 577–580 (1981).
24) Graham, R. C., Jr. and Karnovsky, M. J. The early stage of absorption of injected horseradish peroxidase in the proximal convoluted tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.,* **14,** 291–302 (1966).
25) Ghoshal, A. K., Rushmore, T. H. and Farber, E. Initiation of carcinogenesis by a dietary deficiency of choline in the absence of added carcinogens. *Cancer Lett.,* **36,** 289–296 (1987).
26) De Angelo, A. B. and Garret, C. T. Inhibition of development of preneoplastic lesions in the livers of rats fed a weekly carcinogenic environmental contaminant. *Cancer Lett.,* **20,** 199–205 (1983).
27) Popp, J. A., Garvey, L. K., Hamm, T. E., Jr. and Swenberg, J. A. Lack of hepatic promotional activity by

the peroxisomal proliferating hepatocarcinogen di(2-ethylhexyl)phthalate. *Carcinogenesis,* **6,** 141–144 (1985).
28) Shinozuka, H., Abanobi, S. E. and Lombardi, B. Modulation of tumor promotion in liver carcinogenesis. *Environ. Health Perspect.,* **50,** 163–168 (1983).
29) Stäubli, W., Bentley, P., Bieri, F., Trohlich, E. and Walchter, F. Inhibitory effect of nafenopin upon the development of diethylnitrosamine-induced altered foci within the rat liver. *Carcinogenesis,* **5,** 41–46 (1984).
30) Rao, M. S., Lalwani, N. D., Scarpelli, D. G. and Reddy, J. K. The absence of γ-glutamyltranspeptidase activity in putative preneoplastic lesions and in hepatocellular carcinomas induced in rats by the hypolipidemic peroxisome proliferator. Wy-14,643. *Carcinogenesis,* **3,** 1231–1233 (1982).
31) Rao, M. S., Tatematsu, M., Subbarao, V., Ito, N. and Reddy, J. K. Analysis of peroxisomal proliferator-induced preneoplastic and neoplastic lesions of rat liver for placental form of glutathione-*S*-transferase and γ-glutamyltranspeptidase. *Cancer Res.,* **46,** 5287–5290 (1986).