Jpn. J. Cancer Res. **89**, 1028–1032, October 1998

Expression of Cytochrome P450 3A4 in Foveolar Epithelium with Intestinal Metaplasia of the Human Stomach

Tomoyuki Yokose,[1,5] Mikio Doy,[2] Motoharu Kakiki,[3] Tohru Horie,[3] Yasushi Matsuzaki[4] and Kiyoshi Mukai[1]

[1]Pathology Division, National Cancer Center Research Institute East, 5-1 Kashiwanoha 6-chome, Kashiwa, Chiba 277-8577, [2]Pathology Division, Tsukuba Medical Center, 3-1 Amakubo 1-chome, Tsukuba, Ibaraki 305-8588, [3]Strategic Drug Metabolism Research Unit, Eisai Co., Ltd., 1-3 Tokodai 5-chome, Tsukuba, Ibaraki 300-2635 and [4]Department of Internal Medicine, Tsukuba University, 1-1 Tennodai 1-chome, Tsukuba, Ibaraki 305-8575

The expression of cytochrome P450 3A4 (CYP3A4) in the foveolar epithelium of the human stomach with intestinal metaplasia was studied using immunohistochemistry, western blotting and reverse transcription polymerase chain reaction (RT-PCR). CYP3A4 was immunohistochemically detected in the foveolar epithelium with intestinal metaplasia, but was not detected in foveolar epithelium without intestinal metaplasia, in the pyloric gland or in the fundic gland of the stomach. Western blotting and RT-PCR demonstrated that CYP3A4 protein and mRNA were expressed in the liver and pyloric gland mucosa with intestinal metaplasia, but not in the fundic gland mucosa without intestinal metaplasia. Possible roles of CYP expression in the gastric mucosa with intestinal metaplasia in human stomach carcinogenesis are briefly discussed.

Key words: Human — Stomach — Cytochrome P450 3A4 — Intestinal metaplasia — Reverse transcription polymerase chain reaction

CYPs are important in the oxidative, peroxidative and reductive metabolism of endogenous and exogenous materials. They metabolize a wide range of foreign chemicals, including drugs, environmental pollutants, natural plant products and alcohols. CYP functions not only to detoxify internal biological substances and xenobiotics, but also to metabolically activate carcinogens; thus CYP may be responsible for tumor initiation, promotion and progression.[1)] Degawa *et al.*[2)] reported that DNA adducts detected in human larynx are largely generated via metabolic activation by CYP2C, 3A4, and/or 1A1 of polycyclic aromatic hydrocarbons in cigarette smoke. Moreover, specific CYPs have recently been shown to be involved in the metabolism of several essential anticancer agents.[3)]

CYP is most abundant in the liver. CYP3A is the major component of human liver microsomes.[4)] The CYP3A subfamily accounts for about 25% of the total CYP present in the liver microsomes.[5)] It is well-known that CYP3A4 not only metabolizes testosterone and cortisol, but also actively metabolizes polycyclic aromatic hydrocarbons and AFB1, a well-known environmental carcinogen.[6)] Contamination of human food by AFB1 is associated with high incidence of hepatocellular carcinoma in parts of Africa and Asia.[7, 8)]

Intestinal metaplasia of the stomach is now thought to be a precancerous lesion.[9)] Foveolar epithelium with intestinal metaplasia of the stomach resembles small intestinal mucosa in morphology and in the character of the mucus.[10)] CYP3A4 is abundant in the small intestine, where it has been shown to play an important role in intestinal drug metabolism.[11)] On the other hand, CYP has also been shown to play an important role in chemical carcinogenesis and cancer therapy.[12)] Therefore, there is a great deal of interest in the presence and role of human CYP3A in foveolar epithelium with intestinal metaplasia of the stomach.

The aim of the present study was to investigate the expression of CYP3A4 in foveolar epithelium of the stomach with intestinal metaplasia using immunohistochemistry, western blotting and RT-PCR methods. Based on the results, the possible carcinogenic role of CYP in gastric mucosa with intestinal metaplasia will be discussed.

MATERIALS AND METHODS

Gastric tissue Fresh 1×1 cm tissue samples of the mucosa propria of the stomach were obtained from gastrectomy specimens less than 5 min old taken from patients with gastric cancer. As positive controls, fresh liver specimens were also prepared from the surgically resected materials obtained from patients with metastatic colon cancer. Informed consent was obtained from each

[5] To whom requests for reprints should be addressed.

The abbreviations used are: CYP, cytochrome P450; RT-PCR, reverse transcription polymerase chain reaction; PBS, phosphate-buffered saline; AFB1, aflatoxin B1.

patient. Portions of each tissue sample were frozen in liquid nitrogen and used for western blotting and RT-PCR. The remaining tissues were fixed in 10% neutral-buffered formalin for 24 h, then embedded in paraffin and used for hematoxylin-eosin staining and immunohistochemistry.

Immunohistochemistry Sections from each paraffin block, 4 μm thick on 3-aminopropyltriethoxysilane-coated slides (Muto, Tokyo), were dewaxed by passage through xylene and hydrated in graded ethanols. The deparaffinized sections were immersed in methyl alcohol containing 3% H_2O_2 for 30 min to minimize endogenous peroxidase activity. Then the sections were incubated in normal swine serum for 30 min at room temperature in a humidified chamber. The sections were immunostained with primary antibody to CYP3A (NF-2, gift from Dr. T. Taniguchi, Eisai Co.) diluted at 1:2000, using the avidin biotin-peroxydase complex method (ABC kit, Dakopatts, Glostrup, Copenhagen, Denmark).[13] The color reaction was developed with 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Dojindo, Kumamoto) and 0.005% hydrogen peroxide in 0.05 *M* Tris solution (pH 7.6). Finally, the sections were counterstained with hematoxylin. By substituting normal rabbit serum or PBS for antibody, serial sections were stained as negative controls.

Western blotting of CYP3A4 Frozen liver tissue specimens were rinsed in ice-cold 0.9% NaCl solution. The tissues were minced with scissors and homogenized in 10 m*M* Tris-HCl (pH 7.5). Microsomes were prepared from the homogenates by differential centrifugation.[14] Microsomal pellets were suspended in phosphate buffer 100 m*M*, pH 7.4 containing 20% glycerol and 10 m*M* $MgCl_2$. Microsomal proteins (50 μg) were separated by electrophoresis on sodium dodecyl sulfate polyacrylamide gels as described by Laemmli.[15] Resolved proteins were electrotransferred to nitrocellulose membranes, which were probed with the antibody NF-2 and stained as described previously.[16] We used 50 μl of microsomal protein solution containing human CYP3A4 as a positive control (GENTEST, Woburn, MA).

Amplification of CYP3A4 mRNA using RT-PCR Total RNA was isolated from 100–200 mg of homogenized stomach tissue using the Trizol Total RNA Extraction Agent (Gibco BRL Life Technologies, Eggenstein, Germany). Each tissue sample was cut into smaller pieces and kept frozen until it was homogenized. The RNA was quantified with the GeneQuant photometer (Pharmacia, Freiburg, Germany) and its integrity was checked by agarose gel electrophoresis in 3-[N-morpholino]-2-hydroxypropanesulfonic acid buffer. The purity of the RNA preparations was high, as demonstrated by the 260 nm/280 nm ratio (range, 1.8–2.0). cDNA (RT-product) was synthesized from 5 μg of the total RNA using random hexadeoxynucleotide[17] to prime 2 units of M-MLV reverse transcriptase (Amersham, Buckinghamshire, UK) in 10 μl of reaction mixture (final volume). Control reactions were identical except for the omission of the reverse transcriptase in one set of reaction mixtures (to control for the amplification of genomic DNA) and the omission of RNA in another set (to control for DNA contamination of reagents).

The primers were designed for specific amplification of CYP3A4.[18–20] Oligonucleotide primers were used to amplify 323 and 424 base pair fragments of the rat CYP3A4 from the RT products. The sequences of the CYP3A4 primers were 5′-CCAAGCTATGCTCTTCACCG-3′ (sense) and 5′-TCAGGCTGGACTTACGGTGC-3′ (anti-sense). CYP3A4 cDNA were amplified for a total of 35 cycles in subsequent assays.

The PCR amplification was performed under the following reaction conditions: 30 μl of buffer (10 m*M* Tris-HCl, 2.5 m*M* $MgCl_2$, 50 m*M* KCl, 0.001% gelatin) containing 0.2 m*M* each of dATP, dCTP, dGTP and dTTP, 45 p*M* of each PCR primer in water, 0.01% bovine serum albumin, 0.3 μl of [α-^{32}P]dCTP (Amersham), 11.25 U of *Taq* DNA polymerase (Promega, Madison, WI), and 10 μl of RT sample were added to 500-μl microcentrifuge tubes. Each mixture was brought to a final volume of 50 μl with distilled water, overlaid with 30 μl of mineral oil, and amplified in a thermal cycler (Perkin-Elmer/Cetus, Norwalk, CT). The amplification of cDNA products involved denaturation at 94°C for 1 min, and extension at 72°C for 1.5 min. An aliquot (20 μl) of each PCR reaction mixture was subjected to electrophoresis at 100 V on a 2% agarose gel in 1× TAE buffer. Amplification of the appropriate segment was confirmed by visualizing a band of appropriate length under UV light.

RESULTS

Immunohistochemistry Cytoplasm of the gastric foveolar epithelium with intestinal metaplasia showed a positive immunoreaction for CYP3A (Fig. 1A). Some of the glands with intestinal metaplasia showed the immunoreaction only in the superficial part, while others showed it along almost the whole length. Foveolar epithelium without intestinal metaplasia, and fundic and pyloric gland epithelium showed no immunoreactivity to the antibody (Fig. 1, A and B).

Western blotting of CYP3A4 Microsomal proteins from both the pyloric gland mucosa with intestinal metaplasia and the liver reacted immunochemically to the antibody NF-2, but those from fundic gland mucosa without intestinal metaplasia did not. Each of the visible bands was located on the membrane at the 60 kD position, identical to the position of control CYP3A4 from human microsomes containing CYP3A4 (Fig. 2).

RT-PCR CYP3A4 mRNA was detected in the pyloric gland mucosa with intestinal metaplasia and the liver tis-

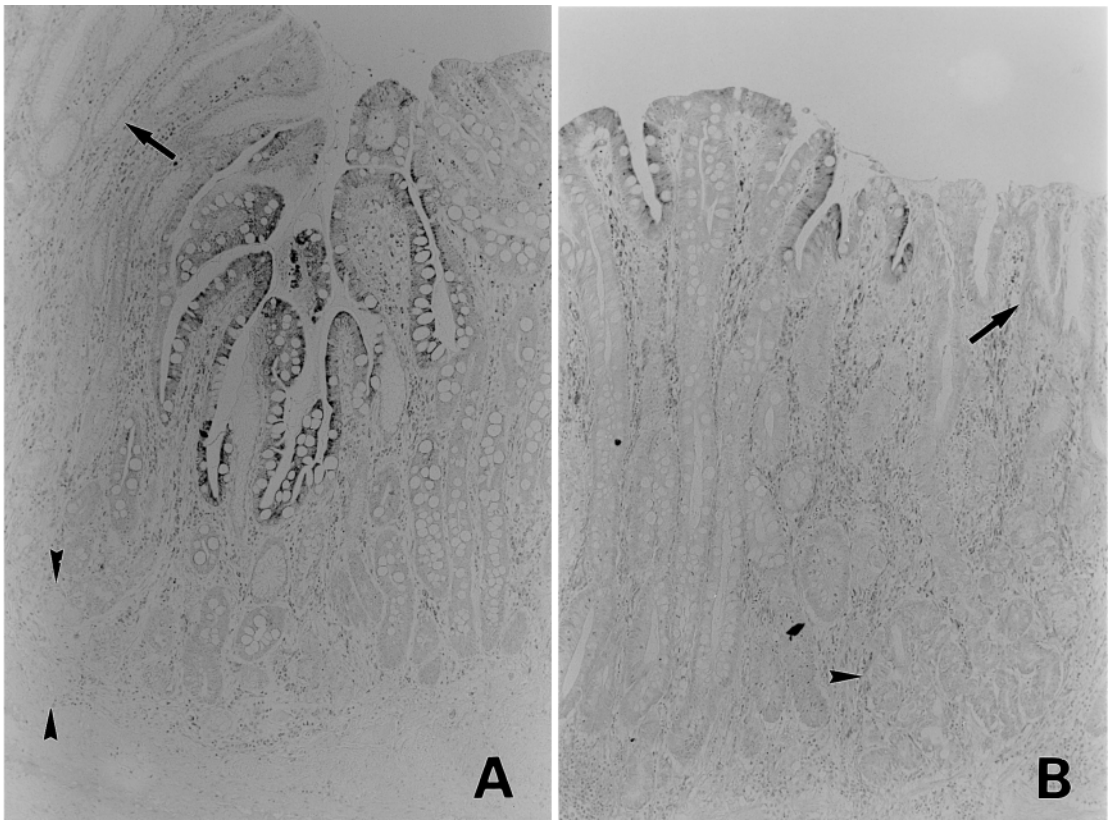

Fig. 1. Immunohistochemistry of CYP3A4 in the stomach. Cytoplasm of the foveolar epithelium with intestinal metaplasia of the stomach showed a positive immunoreaction to CYP3A (A, B). Foveolar epithelium without intestinal metaplasia (A, B, arrow) and pyloric gland epithelium (A, arrow head) showed no reaction to the antibody. Fundic gland epithelium (B, arrow head) also showed no immunoreactivity to the antibody.

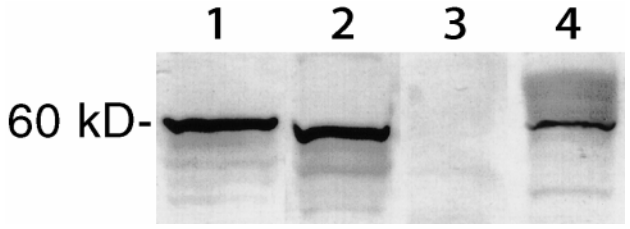

Fig. 2. Western blotting of CYP3A4 in the liver and stomach. Proteins from the microsomes of the liver (lane 2) and pyloric gland mucosa with intestinal metaplasia (lane 4) immunochemically reacted with the antibody NF-2, but those of the fundic gland mucosa of the stomach without intestinal metaplasia (lane 3) did not. Each of the visible bands was located on the membrane at a position of 60 kD, identical with that of control CYP3A4 from human microsomes (lane 1).

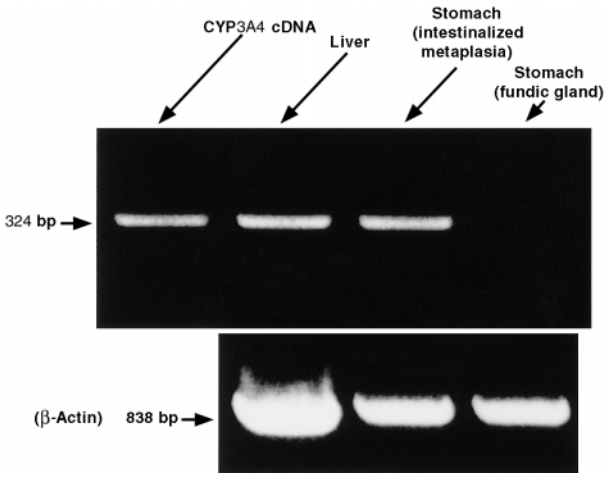

Fig. 3. Detection of CYP3A4 in the liver and stomach by RT-PCR. The control CYP3A4 cDNA was successfully amplified and its band was located at the position of 324 bp (lane 1). The same bands were detected in the liver tissue (lane 2) and pyloric gland mucosa with intestinal metaplasia (lane 3), but not in the fundic gland mucosa without intestinal metaplasia (lane 4).

sue, but not in the fundic gland mucosa without intestinal metaplasia (Fig. 3). The control CYP3A4 cDNA was successfully amplified, confirming the correct functioning of the PCR assay.

DISCUSSION

There are few reports about CYP3A expression in the stomach. Murray *et al*. found that CYP3A was immunohistochemically absent in the stomach.[21] The present study did not demonstrate CYP3A expression in the parietal cells or CYP3A4 mRNA in the fundic gland of the stomach. To our knowledge, there is no previous report of CYP3A4 expression in intestinal metaplasia of the stomach. Using immunohistochemistry, western blotting and RT-PCR, we have demonstrated here that CYP3A4 was exclusively expressed in the foveolar epithelium with intestinal metaplasia of the stomach, but not in the non-metaplastic gastric mucosa. Kolars *et al*. reported that mRNAs of CYP3A4 and 3A5 were amplified by RT-PCR in the antrum and body.[22] The results of the present study suggest that the tissues which Kolars *et al*. used in their study might have included intestinal metaplasia.

CYP3A4 is thought to participate in the metabolic activation of carcinogens such as AFB1. AFB1 has been consistently associated with high incidences of hepatocellular carcinoma in studies from Kenya, Swaziland, Mozambique and Asian countries.[23] The molecular mechanism of the carcinogenic effect of AFB1 is thought to involve the structural modification of the host DNA by covalent adduct formation and consequent alteration of the functional properties of the host DNA.[23]

Intestinal metaplasia of the stomach is defined as a replacement of antral or fundic gastric mucosa by glands composed of epithelium resembling the small intestine.[24] The enzymatic activity of the metaplastic glands is essentially the same as that of the small intestinal epithelium.[10] The prevalence of intestinal metaplasia in the tissue surrounding gastric carcinoma is reported to be 87.3%, while the association of non-neoplastic gastritis with carcinoma is only 12.7%.[9] Ochiai *et al*. also reported that 4 out of 10 non-neoplastic intestinal metaplasia associated with chronic atrophic gastritis showed p53 alteration, as detected by PCR-single strand conformation polymorphism.[25] On this basis, intestinal metaplasia is regarded as one of the precursors of gastric cancer. However, the reason for the frequent genetic alterations in the metaplastic gastric mucosa has not yet been elucidated. From the results of the current study, it seems reasonable to hypothesize that CYP3A4 induced in the metaplastic gastric mucosa activates carcinogens prior to metabolic activation in the liver and causes genetic alterations of the intestinalized mucosa.

Small-intestinal CYP has been postulated to be the principal agent of initial biotransformation of ingested xenobiotics.[26] Among several CYPs expressed in the small intestine, CYP3A is the predominant component, as it is in the liver. Within the small intestine, the duodenum shows the highest expression of CYP3A by northern blot analysis.[27] The immunohistochemical study by Murray *et al*.[21] demonstrated a CYP3A staining pattern in the columnar epithelium of the small intestine characterized by cytoplasmic localization and a gradient from positive villi to negative crypts. The explanation commonly given for why small intestinal cancer rarely occurs is that xenobiotics pass more quickly through the small intestine than through the stomach, and that there is a more rapid turnover of exfoliating villous epithelium than of the metaplastic foveolar epithelium of the stomach.[28] Under these conditions, even if DNA adducts are produced, they would be rapidly removed from the small intestine.

In conclusion, genotypic and phenotypic expression of CYP3A was demonstrated in the foveolar epithelium with intestinal metaplasia of the stomach. This finding suggests to us that prehepatic activation of carcinogens by CYP3A could lead to the transformation of intestinalized mucosa to cancer in the stomach, although the direct relationship between gastric carcinogenesis and CYP3A4 in the metaplastic gastric mucosa remains to be proven.

ACKNOWLEDGMENTS

The authors thank Drs. Hiroyasu Esumi and Tsutomu Ogura for their scientific advice, and Dr. T. Taniguchi for providing the primary antibody against CYP3A.

(Received June 1, 1998/Revised July 6, 1998/Accepted July 14, 1998)

REFERENCES

1) Nelson, D. R., Kamataki, T., Waxman, D. J., Guengerich, F. P., Estabrook, R. W., Feyereisen, R., Gonzalez, F. J., Coon, M. J., Gunsalus, I. C., Gotoh, O., Okuda, K. and Nebert, D. W. The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA Cell Biol.*, **12**, 1–51 (1993).
2) Degawa, M., Stern, S. J., Martin, M. V., Guengerich, F. P., Fu, P. P., Ilett, K. F., Kaderlik, R. K. and Kadlubar, F. F. Metabolic activation and carcinogen-DNA adduct detection in human larynx. *Cancer Res.*, **54**, 4915–4919 (1994).
3) Kivistoe, K. T., Fritz, P., Linder, A., Friedel, G., Beaune, P. and Kroemer, H. K. Immunohistochemical localization of cytochrome P450 3A in human pulmonary carcinomas

and normal bronchial tissue. *Histochemistry*, **103**, 25–29 (1995).
4) Murray, M. and Reidy, G. F. Selectivity in the inhibition of mammalian cytochrome P-450 by chemical agents. *Pharmacol. Rev.*, **42**, 85–101 (1990).
5) Watkins, P. B., Wrighton, S. A., Maurel, P., Schetz, E. G., Mendez-Picon, G., Parker, G. A. and Guzelian, P. S. Identification of an inducible form of cytochrome P-450 in human liver. *Proc. Natl. Acad. Sci. USA*, **82**, 6310–6314 (1985).
6) Miura, S. M., Mori, M., Takiguchi, M., Tatibana, M., Furuta, S., Miyazawa, S. and Hashimoto, T. Biosynthesis and intracellular transport of enzymes of peroxisomal beta-oxidation. *J. Biol. Chem.*, **259**, 6397–6402 (1984).
7) Peers, F. G. and Linsell, C. A. Dietary aflatoxins and liver cancer: population based study in Kenya. *Br. J. Cancer*, **27**, 473–481 (1973).
8) Shank, R. C., Bhamarapravati, N., Gordon, J. E. and Wogan, G. N. Dietary aflatoxins and human liver cancer (IV): incidence in Thailand. *Food Cosmet. Toxicol.*, **10**, 171–179 (1972).
9) Hirota, T., Okada, T., Itabashi, M., Yoshida, H., Matsukura, H., Kitaoka, H. and Hirayama, T. Significance of intestinal metaplasia as a precancerous condition of the stomach. *In* "Precursors of Gastric Cancer," ed. S. C. Ming, pp. 179–193 (1984). Praeger, New York.
10) Matsukura, N., Suzuki, K., Kawachi, T., Aoyagi, M., Sugimura, T., Kitaoka, H., Numajiri, H., Shirota, A., Itabashi, M. and Hirota, T. Distribution of marker enzymes and mucin in intestinal metaplasia in human stomachs and relation of complete and incomplete types of metaplasia to minute gastric carcinoma. *J. Natl. Cancer Inst.*, **65**, 231–240 (1980).
11) Thummel, K. E., O'Shea, D., Paine, M. F., Shen, D. D., Kunze, K. L., Perkins, J. D. and Wilkinson, G. R. Oral first-pass elimination of midazolam involves both gastrointestinal and hepatic CYP3A-mediated metabolism. *Clin. Pharmacol. Ther.*, **59**, 491–502 (1996).
12) Guengerich, F. P. Roles of cytochrome P-450 enzymes in chemical carcinogenesis and cancer chemotherapy. *Cancer Res.*, **48**, 2946–2954 (1988).
13) Hsu, S. M., Raine, L. and Fange, H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J. Histochem. Cytochem.*, **29**, 577–580 (1981).
14) Kremers, P., Beaune, P., Cresteil, T., De Graeve, J., Columelli, S., Leroux, J. P. and Gielen, J. E. Cytochrome P-450 monooxygenase activity in human and rat liver microsomes. *Eur. J. Biochem.*, **118**, 599–606 (1981).
15) Laemmli, U. K. Cleavage of structural properties during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685 (1970).
16) Beaune, P., Flinois, J. P., Kiffel, L., Kremers, P. and Leroux, J. P. Purification of a new cytochrome P 450 from human liver microsomes. *Biochim. Biophys. Acta*, **860**, 364–370 (1985).
17) Noonan, K. E. and Robinson, I. B. mRNA phenotyping by enzymatic amplification of randomly primed cDNA. *Nucleic Acids Res.*, **16**, 10366 (1988).
18) Gonzalez, F. J., Schmid, B. J., Umeno, M., McBride, O. W., Hardwick, J. P., Meyer, U. A., Gelboin, H. V. and Idle, J. R. Human p450PCN1: sequence, chromosomal localization, and direct evidence through cDNA expression that p450PCN1 is nifedipine oxidase. *DNA*, **7**, 79–86 (1988).
19) Komori, M., Nishio, K., Ohi, H., Kitada, M. and Katamiki, T. Molecular cloning and sequence analysis of cDNA containing the entire coding region for human fetal liver cytochrome p450. *J. Biochem.*, **105**, 161–163 (1989).
20) Molowa, D. T., Schuetz, E. G., Wrighton, S. A., Watkins, P. B., Kremers, P., Mendez-Picon, G., Parler, G. A. and Guzelian, P. S. Complete cDNA sequence of a cytochrome P-450 inducible by glucocorticoids in human liver. *Proc. Natl. Acad. Sci. USA*, **83**, 5311–5315 (1986).
21) Murray, G. I., Barnes, T. S., Sewell, H. F., Ewen, S. W. B., Melvin, W. T. and Burke, M. D. The immunohistochemical localization and distribution of cytochrome P-450 in normal human hepatic and extrahepatic tissues with monoclonal antibody to human cytochrome P-450. *Br. J. Clin. Pharmacol.*, **25**, 465–475 (1988).
22) Kolars, J. C., Lown, K. S., Schmiedlin-Ren, P., Ghosh, M., Fang, C., Wrighton, S. A., Merion, R. M. and Watkins, P. B. CYP3A gene expression in human gut epithelium. *Pharmacogenetics*, **4**, 247–259 (1994).
23) Lee, H. S., Sarosi, I. and Vyas, G. N. Aflatoxin B1 form-amidopyrimidine adducts in human hepatocarcinogenesis: a preliminary report. *Gastroenterology*, **97**, 1281–1287 (1989).
24) Stemmermann, G. N. Intestinal metaplasia of the stomach: a status report. *Cancer*, **74**, 556–564 (1994).
25) Ochiai, A. and Hirohashi, S. Genetic alterations in the precursors of gastric cancer. *In* "Molecular Pathology of Gastroenterological Cancer: Application to Clinical Practice," ed. E. Tahara, pp. 43–53 (1997). Springer-Verlag, Tokyo.
26) Kaminsky, L. S. and Fasco, M. J. Production and purification of antibodies to rat liver cytochrome P-450. *Crit. Rev. Toxicol.*, **21**, 407–422 (1992).
27) McKinnon, R. A. and McManus, M. E. Function and localization of cytochromes P450 involved in the metabolic activation of food-derived heterocyclic amines. *Proc. 23rd Int. Symp. Princess Takamatsu Cancer Res. Fund*, 145–153 (1995).
28) Kolars, J. C., Benedict, P., Schmiedlin-Ren, P. and Watkins, P. B. Aflatoxin B1-adduct formation in rat and human small bowel enterocytes. *Gastroenterology*, **106**, 433–439 (1994).