Molecular Characterization and Biological Functions of Cytochrome P450s in CYP4A and CYP4B Subfamilies

2001

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PREFACE

Cytochrome P450 comprises a large superfamily of proteins that are of central importance in the metabolism of foreign compounds such as therapeutic drugs and environmental pollutants and in the metabolism of endogenous compounds such as fatty acids and steroids. Humans and rodents have CYP1 - CYP51 families of cytochrome P450. Cytochrome P450s in CYP1 - CYP3 families largely metabolize therapeutic drugs and activate chemical carcinogens. Cytochrome P450s in CYP5 - CYP51 families mainly metabolize endogenous compounds including steroids, vitamins, and prostaglandins. Cytochrome P450s in the CYP4 family can metabolize both fatty acids and chemical carcinogens. The members of the CYP4 family are widely distributed throughout mammalian and other species including insects. In mammals, cytochrome P450 content is the highest in the liver. However, cytochrome P450s in the CYP4 family are largely extrahepatic-type forms and are present in the lung and the kidney. In this paper, the cytochrome P450s in the CYP4A and CYP4B subfamilies are described.

The CYP4A2 was purified from rat renal microsomes and metabolized lauric acid and arachidonic acid at a ω-position, but not efficiently metabolized drugs such as aminopyrine and 7-ethoxycoumarin which are often used as substrates for hepatic cytochrome P450. CYP4A2 was a male-dominant form in the rat kidney, and expression of CYP4A2 was regulated by androgen as well as by the pituitary growth hormone which regulates expression of sex-specific forms of cytochrome P450 in rat liver. CYP4A2 was induced by diabetes, but increased levels of CYP4A2 were recovered by treatment with thyroid hormone which appeared to decrease in diabetes cases. CYP4A11 cDNA was first cloned from the human kidney library and is a homologue of rat CYP4A2. Human CYP4A11 was expressed in human renal microsomes. Recombinant CYP4A11 was expressed in Sf9 insect cells with baculovirus system. CYP4A11 catalyzed lauric acid and arachidonic acid hydroxylation in the manner of rat CYP4A2. Native human CYP4A11 was first purified from human renal microsomes. The N-terminal amino acid sequence of purified CYP4A11 was identical with the cDNA-deduced amino acid sequence of CYP4A11 cDNA cloned from the kidney library. CYP4A11 was a major cytochrome P450 in the human kidney.

The Cyp4b1 was purified from mouse renal microsomes. Rat CYP4B1 was also purified from rat pulmonary microsomes. Mouse Cyp4b1 cDNA was cloned from the mouse kidney library. Rat and mouse CYP4B1 activated carcinogenic amines such as naphthylamine and benzidine derivatives which cause bladder cancer in experimental animals and humans. Immunochemical study indicated the presence of CYP4B1 in rat bladder mucosa. Expression of CYP4B1 mRNA in the bladders of male rats was higher than that in female rats, and androgen regulated its expression. This finding

explains why male rats have a higher incidence of bladder cancer induced by carcinogenic amines than do female rats. In humans, males also show a higher incidence of bladder cancer than do females. CYP4B1 was detected in the human bladder by means of Cyp4b1 antibody, and it appeared that bladder-tumor patients showed a higher expression of CYP4B1 than did non-bladder tumor patients. As Western blotting requires large tissues, a sensitive assay of the expression of CYP4B1 with reverse transcriptase-polymerase chain reaction (RT-PCR) was developed. Using this method, the expression of CYP4B1 in biopsy samples from bladders was investigated. The results indicated that bladder-tumor patients had higher expression of CYP4B1 than did non-bladder tumor patients. Human CYP4B1 was very unstable and the heterologous expression of the form failed. A transgenic mouse with human CYP4B1 was then developed in order to study the function of human CYP4B1. Human CYP4B1 was able to efficiently activate 2-aminofluorene, a potent carcinogen. As well, human bladder activated 2-aminofluorene. These findings suggested that CYP4B1 contributes to bladder carcinogenesis.

Recently, ω-hydroxyarachidonic acid was reported to be a potent vasoconstrictor. In the present study, CYP4A2 and CYP4A11 appeared to produce ω-hydroxyarachidonic acid. These findings suggest that CYP4A2 and CYP4A11 contribute to the regulation of blood pressure. The findings described in this paper indicate that cytochrome P450s in CYP4A and 4B subfamilies play important roles in carcinogenesis and regulation of renal function.

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PART I
THE CYP4A SUBFAMILY

CHAPTER 1
CHARACTERIZATION OF THREE CYTOCHROME P450S PURIFIED FROM RAT KIDNEY AND COMPARISON OF THOSE WITH HUMAN RENAL CYTOCHROME P450

1.1 INTRODUCTION

Cytochrome P450 and its associated monooxygenase activities are predominantly found in the liver, but are also present in many other tissues, although usually at lower levels (1). There are species, sex, age, and tissue differences in cytochrome P450s (2-4). The balance of matabolic activation and detoxification of drugs and other chemicals by individual forms of cytochrome P450 in different tissues or species is an important factor in explaining organ- or species-specific toxicity. Cytochrome P450 also has organ-specific roles involving, for example, the synthesis of steroids by adrenal cytochrome P450 (5) and the synthesis of thromboxane by platelet cytochrome P450 (6). Most studies of cytochrome P450 have focused on hepatic forms in rats (7-13) and humans (14-20). Much less is known about of renal cytochrome P450s in rats and humans.

Renal cytochrome P450 efficiently analyzes the hydroxylation of prostaglandins and fatty acids such as lauric acid but metabolizes testosterone and drugs such as aminopyrine and 7-ethoxycoumarin little if at all (21-24). The substrate specificity of renal cytochrome P450 is different from that of hepatic cytochrome P450s. Arachidonic acid can be metabolized by renal cytochrome P450, being converted to ω- or (ω-1)-hydroxyeicosatetraenoic acids and epoxyeicosatrienoic acids (25, 26). We have investigated arachidonic acid metabolism by a cytochrome P450 purified from the rat renal microsomes, finding that purified renal cytochrome P450 produces epoxyeicosatrienoic acid and ω- and (ω-1)-hydroxyeicosatetraenoic acid (27). However, the contribution to renal function of arachidonic acid or prostaglandin metabolites arising from cytochrome P450-dependent monooxygenase is unknown. Further characterization of renal cytochrome P450 and a study of its regulation are needed.

The characterization of cytochrome P450 in humans has been limited by the difficulty in obtaining organs in good condition. Hepatic cytochrome P450 in humans has been purified and studied in detail (14-20). It has been compared with rat hepatic cytochrome P450, which has also been studied extensively. The study of extrahepatic cytochrome P450 in humans is only in microsomes, because the specific content of

cytochrome P450 in extrahepatic organs of humans is low (28-30) and the purification of cytochrome P450 is difficult. Because of the species differences in cytochrome P450, renal cytochrome P450 in humans must be purified for characterization.

In this study, we characterized P450 K-2 (CYP2C23), P450 K-4 (CYP4A8), and P450 K-5 (CYP4A2) purified to homogeneity from rat kidney by their NH_2-terminal sequence, spectral properties, and catalytic activity. Furthermore, we partially purified renal cytochrome P450 of humans and compared it with rat renal cytochrome P450s.

1.2 MATERIALS AND METHODS

1.2.1 Chemicals

Testosterone, sodium laurate, and ω-hydroxylauric acid were obtained from Sigma Chemical Co. (St. Louis, MO). (ω-1)-Hydroxylauric acid was the kind gift of Dr. Y. Imai of Osaka University. Dilauroylphosphatidylcholine was obtained from Sigma. NADPH was obtained from Oriental Yeast Co. (Tokyo, Japan). Emulgen 911 was a gift of the Kao Corp. (Tokyo, Japan). 9-Anthryldiozomethane was obtained from Funakoshi Chemical Industry (Tokyo, Japan). Coomassie brilliant blue R-250 was obtained from Bio-Rad Laboratories (Richmond, CA). A Vectastain ABC kit containing the reagent consisting of avidin and biotinylated horseradish peroxidase was obtained from Vector Laboratories (Burlingame, CA). Other reagents and organic solvents were obtained from Wako Pure Chemical Industries (Tokyo, Japan).

1.2.2 Purification of renal cytochrome P450

Male Sprague-Dawley rats weighing 200-250 g were obtained from Japan Clea (Tokyo, Japan). The microsomes from the whole kidneys of untreated male rats were prepared as described elsewhere (31). Purification of renal cytochrome P450s was done by the method described before (27, 32, 33). Renal microsomes containing 7 g of protein (specific content, 0.12 nmol of P450/mg of protein) were solubilized on ice with 10% sodium cholate to give 10 mg of protein/ml in 0.1 M potassium phosphate buffer, pH 7.4, containing 30% glycerol, 1 mM EDTA, and 1 mM dithiothreitol (buffer A) and 5 to 15% polyethylene glycol fractionation was done. The fraction, which contained 3.2 g of protein, was homogenized in 0.1 M potassium phosphate buffer, pH 7.2, containing 1 mM EDTA, 0.5 mM dithiothreitol, 20% glycerol, and 0.7% sodium cholate (buffer B) in the final concentration of 7 mg of protein/ml and put on a column of octylamino-Sepharose 4B (5 x 15 cm) equilibrated with 0.1 M potassium phosphate buffer, pH 7.2, containing 1 mM EDTA, 0.5 mM dithiothreitol, 20% glycerol, and 0.5% sodium cholate (buffer C). After the column was washed with 3 to 4 column volume of buffer C, cytochrome P450 was eluted with 0.1 M potassium phosphate buffer, pH 7.2, containing 1 mM EDTA, 0.5 mM dithiothreitol, 20% glycerol, 0.4% sodium cholate, and 0.2% Emulgen 911 (buffer D). The specific content of cytochrome P450 in the fraction eluted was 0.74 nmol of cytochrome P450/mg of protein. The yield of cytochrome P450

from microsomes was 36%. Then eluate containing 430 mg of protein (500 ml) was concentrated to 20 ml with an ultrafiltration membrane (UK-50, 10 cm in diameter, Toyo Roshi, Tokyo). The dark red, syrupy solution was diluted five times with 0.02 M Tris-acetate buffer, pH 7.5, containing 20% glycerol (buffer E) and put on a preparative anion-exchange column (DEAE-5PW, 2.15 x 15 cm, Tosoh Corp., Tokyo). The chromatography was done at a flow rate of 2 ml/min with a linear gradient of sodium acetate from 0 to 0.2 M over 180 min in buffer E containing Emulgen 911. In this step, renal cytochrome P450 was resolved into three peaks. The first peak was the pass-through fraction containing 18 nmol of cytochrome P450. The cytochrome P450s in the second and the third peaks were designated P450 K-4 and K-5, respectively (27, 32). P450 K-4 and K-5 fractions contained 24 nmol and 197 nmol of cytochrome P450, respectively. The specific contents of cytochrome P450 in P450 K-4 and K-5 fractions were 2.3 and 2.9 nmol of cytochrome P450/mg of protein, respectively. P450 K-5 was the major cytochrome P450 in rat renal microsomes and was further purified to homogeneity by rechromatography with DEAE-5PW and hydroxylapatite HPLC with KB-column (0.6 x 10 cm, Koken, Tokyo, Japan) as described previously (32). The P450 K-4 fraction (40 ml) was concentrated to 5 ml with the UK-50 membrane, diluted five times with 0.02 M sodium phosphate buffer, pH 7.5, containing 20% glycerol (buffer F), and put on a preparative cation-exchange column (Asahipak, ES-502CP, 2.15 x 10 cm, Asahi Chemical Industry Co., Tokyo, Japan). The chromatography was done at a flow rate of 2 ml/min with a linear gradient of sodium acetate from 0 to 0.5 M over 180 min in buffer F containing 0.4% Emulgen 911. The eluate was further purified by hydroxylapatite HPLC with KB-column. The chromatography was done at a flow rate of 0.5 ml/min with a linear gradient of sodium phosphate buffer, pH 7.4, from 0.01 to 0.35 M over 70 min. This phosphate buffer contained 0.4% Emulgen 911, 0.2% sodium cholate, and 20% glycerol. In this step, P450 K-4 was homogeneous by SDS-polyacrylamide gel electrophoresis.

The pass-through fraction (the first peak) from a preparative DEAE-5PW column (the specific content of cytochrome P450 was 0.62 nmol/mg, 100 ml) was concentrated to 10 ml with the UK-50 membrane, diluted five times with buffer F, and put on an ES-502CP column. The chromatography was done under the same conditions as in the purification of P450 K-4. In this step, cytochrome P450 was resolved into three peaks, designated P450 K-1, K-2, and K-3. The P450 K-2 fraction was further purified by hydroxylapatite with KB-column. The chromatography was done under the same conditions as in the purification of P450 K-4 and K-5. In this step, P450 K-2 was homogeneous by SDS-polyacrylamide gel electrophoresis. The concentration of cytochrome P450 in the K-1 and K-3 fractions was low and further purification was not done. Purified cytochrome P450 fractions eluted from KB-column (2-3 ml) were diluted five times with 0.01 M sodium phosphate buffer, pH 7.4, containing sodium cholate and

20% glycerol (buffer G) and put on a hydroxylapatite open column (1.0 x 2.0 cm, Bio-Gel HT, Bio-Rad Laboratories) to remove the Emulgen. After the column was washed with buffer G until absorption by Emulgen at 280 nm disappeared, the cytochrome P450 was eluted with 0.35 M potassium phosphate buffer, pH 7.4, containing 20% glycerol and 0.05% sodium cholate.

Human kidney microsomes were prepared from samples obtained at the surgery from two patients with tumors. The renal tissues apart from the tumors were quickly frozen in liquid nitrogen and were kept at −70 °C until use. The two specimens were combined and used in this study. Frozen kidney (200 g) was cut into small pieces and renal microsomes were prepared by the method as described for the preparation of rat renal microsomes (31). Human renal microsomes containing 2200 mg of protein (specific content, 9.1 pmol/mg) were used for purification. The human cytochrome P450 was partially purified by the same method as in the purification of the rat renal cytochrome P450, P450 K-5 (32). The human renal microsomes were diluted to 15 mg protein/ml with buffer A, solubilized with sodium cholate at the final concentration of 2%, and 5 to 15% polyethylene glycol fractionation was done. The fraction, which contained 619 mg of protein, was solubilized with buffer B in the final concentration of 5 mg of protein/ml and put on a column of octylamino-Sepharose 4B (2.5 x 15 cm). Cytochrome P450 was eluted with buffer D. The eluate (100 ml) was concentrated to 10 ml with an ultrafiltration membrane and diluted five times with buffer E. The diluted fraction was divided into three portions and each was put on an analytical DEAE-5PW column (7.5 x 75 mm, Tosoh). The chromatography was done at a flow rate of 1.0 ml/min with a linear gradient of sodium acetate from 0 to 0.2 M over 30 min in buffer E containing 0.4% Emulgen 911. The cytochrome P450 eluted was further purified with hydroxylapatite HPLC with KB-column under the same conditions as in the purification of P450 K-4.

1.2.3 Immunochemical methods

Antibody against purified cytochrome P450 was raised as described previously (34) in female Japanese white rabbits obtained from Biotech (Saga, Japan). The rabbits were immunized with 100 μg of purified P450 K-5 in Freund's complete adjuvant (Calbiochem, San Diego, CA). The rabbits were given one booster at 2 weeks or three boosters at 2, 4, and 6 weeks with 50 μg of P450 K-5 in Freund's incomplete adjuvant (Difco Laboratories, Detroit, MI) after immunization. Antiserum was collected at 2 weeks after the last booster and immunoglobulin G was prepared described previously (34). The specificity of antibodies was tested by Western blotting (34). The specificity of antibody obtained from the rabbit given one booster was higher than that of antibody obtained from the rabbit that was given three boosters.

1.2.4 Assay of metabolic activity

Aminopyrine N-demethylation, 7-ethoxycoumarin O-dealkylation, testosterone

hydroxylation, lauric acid hydroxylation, arachidonic acid hydroxylation and prostaglandin A_1 hydroxylation activities were measured as described previously (33, 35, 36). Purification of NADPH-cytochrome P450 reductase and cytochrome b_5 was reported elsewhere (37).

1.2.5 Other methods

Sequencing of the NH_2-terminal amino acid of purified cytochrome P450 was done by a method reported before (34). As partially purified human renal cytochrome P450 revealed two bands on SDS-polyacrylamide gel electrophoresis, cytochrome P450 was blotted to PVDF membrane electrically. The corresponding band was analyzed by a protein sequencer (377A, Applied Biosystems, Foster City, CA). The amount of cytochrome P450 was estimated by the method of Omura and Sato (38). The protein concentration was measured by the method of Lowry *et al.* (39).

1.3 RESULTS

1.3.1 Purification of rat renal cytochrome P450s

Rat renal microsomes were solubilized, fractionated with polyethylene glycol, and separated by octylamino-Sepharose. The eluted fraction was applied onto DEAE-5PW anion-exchange column. In this step, cytochrome P450s were separated to three peaks, PT-fraction, P450 K-4, and K-5 fractions (Fig.1). The PT-fraction was applied to

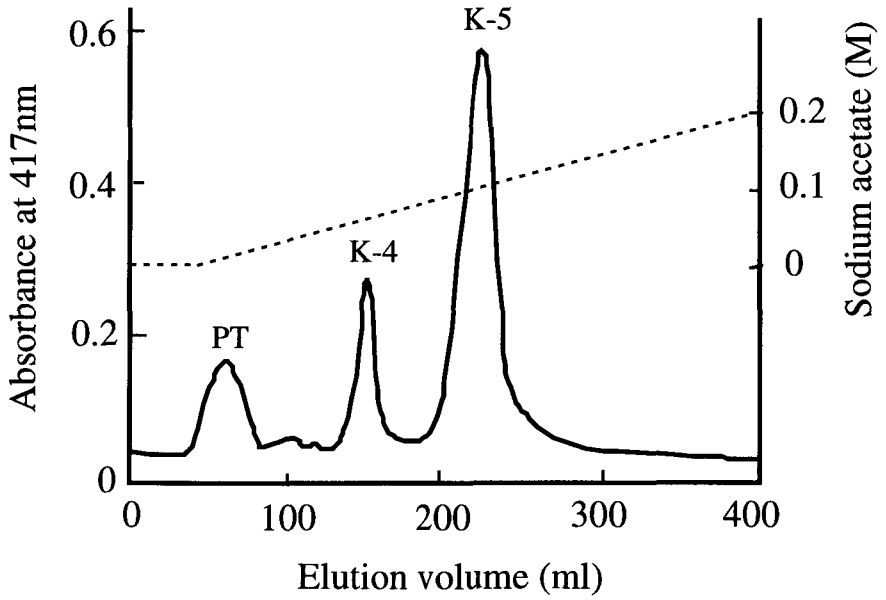

Fig 1 Chromatographic profile of rat renal cytochrome P450 by HPLC with a DEAE-5PW anion-exchange column. The fraction eluted from octylamino-Sepharose 4B was put on a DEAE-5PW column (2.15 x 15 cm). Chromatography was done at the flow rate of 2.0 ml/min with a linear gradient of sodium acetate from 0 to 0.2 M over 180 min in 0.02 M Tris-acetate buffer, pH 7.5, containing 20% glycerol and 0.4% Emulgen 911 at room temparature. PT, pass-through fraction.

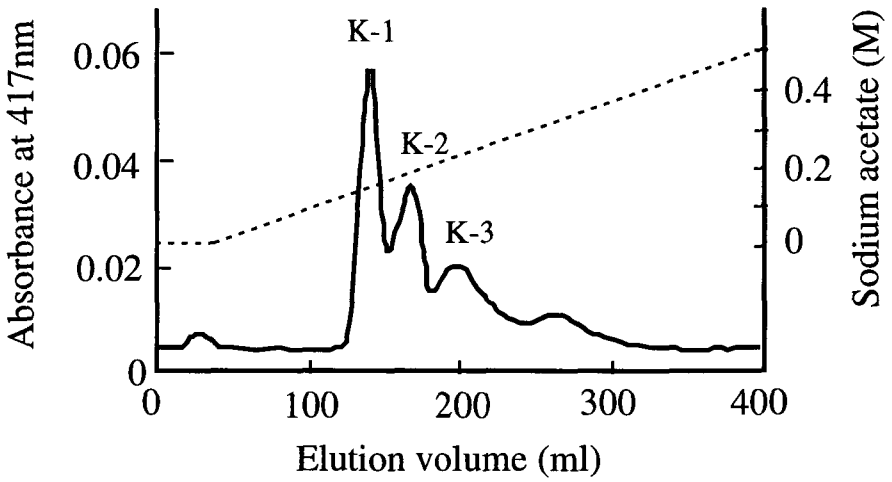

Fig 2 Chromatographic profile of rat renal cytochrome P450 by HPLC with a cation-exchange column. (ES-502 CP). The pass-through fraction eluted from DEAE-5PW was put on a ES-502CP column (2.15 x 10 cm). Chromatography was done at the flow rate of 2.0 ml/min with a linear gradient of sodium acetate from 0 to 0.5 M over 180 min in 0.02 M sodium phosphate buffer, pH 6.5, containing 20% glycerol and 0.4% Emulgen 911 at room temparature.

Table I Specific content, recovery, absorption maxima, and molecular weight of purified cytochrome P450

Cytochrome P450 (CYP)	Specific content (nmol/mg)	Recovery (%)	Absorption maxima (nm)			Molecular weight
			Oxidized	Reduced	CO-reduced	
CYP2C23 (K-2)	11.0	0.6	418	418	449	52,000
CYP4A8 (K-4)	10.0	1.1	418	sh	451	52,000
CYP4A2 (K-5)	12.6	12.7	419	421	452	52,000

The absorption spectra were measured in 0.1 M sodium phosphate buffer, pH 7.4, containing 20%glycerol. Monomeric molecular weights of cytochrome P450 were measured by SDS-polyacrylamide gel electrophoresis.

a cation-exchange column (ES-502CP) and separated to three peaks, P450 K-1, K-2, and K-3 fractions (Fig.2). Only P450 K-2 fraction included significant amount of cytochrome P450. Table I summarizes the specific content, the recovery, spectral properties, and molecular weight of purified rat renal cytochrome P450s. Overall yields of P450 K-2 and K-4 from the renal microsomes of untreated male rats were 0.6 and 1.1% respectively. Their yields were low compared to that of P450 K-5 (12.7%); these forms were minor. P450 K-2, K-4, and K-5 gave single bands on SDS-polyacrylamide gel electrophoresis and had very similar mobility (Fig. 3). These forms had the same molecular weight, 52000, but different CO-reduced absorption maxima, at 449 nm for

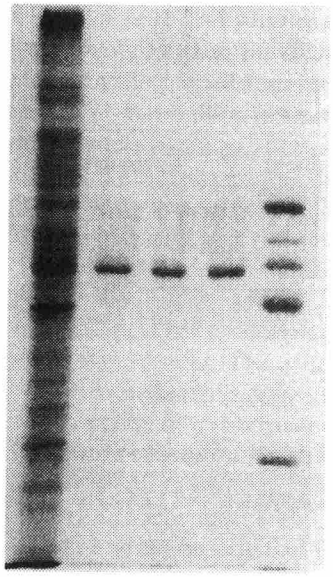

Fig. 3 SDS-polyacrylamide gel electrophoresis of purified cytochrome P450 K-2, K-4, and K-5. The electrophoresis was done on 10% acrylamide gel. Lane 1 contains rat renal microsomes (10 μg). Lanes 2, 3, and 4 contain P450 K-2 (0.5 μg), P450 K-4 (0.5 μg), and P450 K-5 (0.5 μg), respectively. Lane 5 contains standard proteins including bovine serum albumin (MW, 68,000), catalase (57,000), glutamate dehydrogenase (53,000), ovalbumin (45,000), and α-chymotrypsinogen (25,000). Proteins were stained with Coomassie brilliant blue.

P450 K-2, 451 nm for P450 K-4, and 452 nm for P450 K-5 (Table I). Also, P450 K-2, K-4, and K-5 contained the low-spin state of heme, as suggested by the Soret peak at 418-419 nm for the oxidized forms.

1.3.2 Partial purification of cytochrome P450 from human renal microsomes

The fraction of human cytochrome P450 eluted from octylamino-Sepharose 4B was applied onto a DEAE-5PW column and resolved into three peaks. Figure 4 shows the chromatographic profile of cytochrome P450 by DEAE-5PW. Peak 2 was mostly cytochrome P450, which was designated P450 HK. Peaks 1 and 3 contained very little

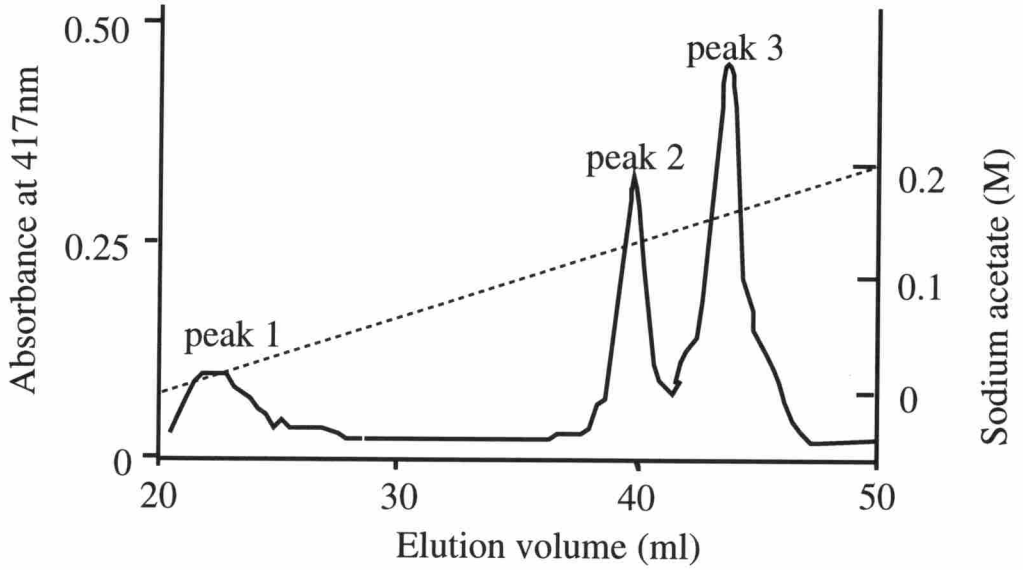

Fig. 4 Chromatographic profile of human renal cytochrome P450 by HPLC with a DEAE-5PW anion-exchange column. The fraction eluted from octylamino-Sepharose 4B was put on a DEAE-5PW column (7.5 x 75 mm). Chromatography was done at the flow rate of 1.0 ml/min with a linear gradient of sodium acetate from 0 to 0.2 M over 30 min in 0.02 M Tris-acetate buffer, pH 7.5, containing 20% glycerol and 0.4% Emulgen 911 at room temparature.

cytochrome P450. Therefore, peak 2 was further purified by hydroxylapatite HPLC. In rat kidney, renal microsomes contain one major cytochrome P450 (P450 K-5) and other minor forms. As in human kidney, only one major cytochrome P450 was detected in renal microsomes. The recovery and specific content of cytochrome P450 in each step are listed in Table II. The concentration of cytochrome P450 in the human renal

Table **II** Purification of P450 HK from human renal microsomes

Purification steps	Protein (mg)	P450 (nmol)	Specific content (nmol/mg)	Yield (%)
Microsomes	2200	20.2	0.0091	100
PEG	619	12.0	0.019	59.4
OA-Sepharose	22	4.9	0.22	24.3
HPLC				
DEAE-5PW	–	1.2	–	5.9
KB-column	0.49	1.0	2.0	5.0

PEG, polyethylene glycol fractionation (5-15%).
OA, octylamino.

microsomes was very low (0.0091 nmol/mg of protein), so 5-15% fractionation of polyethylene glycol was used instead of the 7-15% fractionation used in the purification of rat hepatic cytochrome P450 (33), and the rechromatography with DEAE-5PW was omitted to increase recovery. Polyethylene glycol fractionation was not effective in increasing the specific content but was useful in removing phospholipids, reducing the

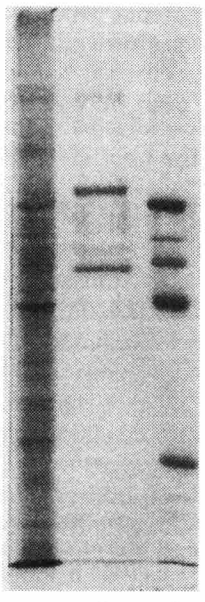

Fig. 5 SDS-polyacrylamide gel electrophoresis of partially purified P450 HK. The electrophoresis was done under the same conditions as those described in the legend to Fig. 4. Lanes 1, 2, and 3 contain human renal microsomes (10 μg), P450 HK (1 μg), and standard proteins, respectively.

damage caused to the HPLC column. Cytochrome P450 was purified 24-fold from microsomes in the octylamino-Sepharose chromatography. Renal cytochrome P450 was further purified by HPLC with DEAE-5PW anion-exchange column and KB-column. The overall yield of cytochrome P450 from microsomes was 5.0% and cytochrome P450 was purified 220-fold from microsomes. However, its preparation had more than one band on SDS-polyacrylamide gel electrophoresis (Fig. 5) and its specific content was 2.0 nmol of P450/mg of protein. The Soret maxima of P450 HK were at 418 nm for the oxidized form, 416 nm for the reduced form, and 450 nm for the CO-reduced form.

1.3.3 NH_2-terminal amino acid sequences of purified renal cytochrome P450s

Table III shows the results of the analysis of the NH_2-terminal amino acid sequences of P450 K-2, K-4, K-5, and HK. As the P450 HK did not show a single band on SDS-polyacrylamide gel electrophoresis, it was blotted to PVDF membrane electrophoretically and the corresponding band was analyzed. Usually, the NH_2-terminal sequences of constitutive hepatic cytochrome 450s begin with methionine and hepatic cytochrome P450s have hydrophobic NH_2-teminal sequence that contains a cluster of leucines or valines (9, 12, 33-35, 40). However, the first residues of these cytochrome P450s were not methionine and did not have a cluster of hydrophobic amino acids. Similarity was slight between the NH_2-terminal amino acid sequences of P450 K-2, K-4, and K-5. Their NH_2-terminal amino acid sequences were different from the corresponding sequences reported for rat liver cytochrome P450 (9, 12, 33-35, 40). These are novel cytochrome P450s. Purification of human renal cytochrome P450 was first reported in this study. The NH_2-terminal amino acid sequence of P450 HK had similarity with rat P450 K-5; five amino acid residues identified were identical between these cytochrome P450s. Human renal P450 HK was a homologue of rat renal P450 K-5. Later, cDNAs of these cytochrome P450s were isolated by other groups (41, 42) and our group (43) and P450 K-2, K-4, K-5, and HK designated CYP2C23, 4A8, 4A2, and 4A11 by the nomenclature of cytochrome P450 (44).

Table III NH_2-terminal sequences of purified renal cytochrome P450

Residue	1	5	10	15
CYP2C23 (K-2)	Leu-Arg-Thr- x -Gly- x -Leu-Pro-Pro-Gly-Pro-Thr-Pro-Leu-Pro			
CYP4A8 (K-4)	Phe-Thr-Ile-Phe-Pro- x -Ser-Ile-Leu-Gly-Phe-Leu-Gln-Ile-Ala			
CYP4A2 (K-5)	Val-Phe- x -Pro-Thr- x -Ser-Leu-Asp-Gly-Val-Ser-Gly-Phe-Phe			
CYP4A11 (HK)	Val-Leu- x -Pro- x -Arg-Leu-Leu-Gly-Asp-Val- x -Gly-Ile-Leu			

In cycles indicated by an x, the residue was not identified.

1.3.4 Catalytic activity of rat and human renal cytochrome P450s

The substrate specificities of purified CYP2C23 (K-2), CYP4A8 (K-4), CYP4A2 (K-5) and partially purified CYP4A11 (HK) in a reconstituted system were compared (Table IV). CYP2C23 and 4A8 were minor forms in rat kidney and were efficient catalysts in the N-demethylation of aminopyrine which is a typical substrate for hepatic cytochrome P450s (37). On the other hand, CYP4A2, the major form in rat kidney, and CYP4A11, the human renal cytochrome P450, did not have significant aminopyrine N-demethylation activity. All of these forms of cytochrome P450 could catalyze the O-dealkylation of 7-ethoxycoumarin. CYP4A11 was the most efficient for 7-ethoxycoumarin. CYP4A2 was the poorest catalyst for 7-ethoxycoumarin. Testosterone was poorly metabolized by rat and human renal cytochrome P450s. Usually, renal microsomes efficiently catalyze the hydroxylation of fatty acids compared to drugs (21-24). Likewise, all cytochrome P450s purified in this study were efficient catalysts for lauric and arachidonic acids. However, the regiospecificity of these forms

Table IV Catalytic activities of purified renal cytochrome P450

Cytochrome P450	CYP2C23 (K-2)	CYP4A8 (K-4)	CYP4A2 (K-5)	CYP4A11 (HK)
Aminopyrine N-demethylation	7.1	6.9	–	–
7-Ethoxycoumarin O-dealkylation	1.1	1.3	0.2	1.7
Testosterone hydroxylation	–	–	–	–
Lauric acid				
ω-hydroxylation	–	26.0	7.5	25.1
(ω-1)-hydroxylation	1.8	10.2	1.0	1.0
Arachidonic acid				
ω-hydroxylation	–	5.7	1.6	3.0
(ω-1)-hydroxylation	–	0.9	0.5	–
Prostaglandin A_1				
ω-hydroxylation	–	2.2	–	–
(ω-1)hydroxylation	–	0.1	–	–

The catalytic activities toward aminopyrine(2 mM), 7-ethoxycoumarin (1 mM), testosterone (1 mM), lauric acid (0.4 mM), arachidonic acid (0.025 mM), and prostaglandin A_1 (0.2 mM) were assayed in a reconstituted system containing purified cytochrome P450 (30 pmol), NADPH-cytochrome P450 reductase (0.3 unit), cytochrome b_5 (30 pmol), and dilauroylphosphatidylcholine (5 μg). The values are expressed as nmol of product/min/nmol of P450. – , not detected (less than 0.05 nmol/min/nmol).

to lauric and arachidonic acids were different. CYP2C23 hydroxylated lauric acid only at the (ω-1)-position and produced epoxyeicosatrienoic acid (EET) from arachidonic acid; the reaction is epoxydation but not hydroxylation (data not shown). Other rat renal cytochrome P450s and the human renal cytochrome P450 metabolized lauric and arachidonic acids predominantly at the ω-position. The regiospecificity of ω-hydroxylation was the highest for CYP4A11. Prostaglandin A_1 was metabolized at ω-position only by CYP4A8.

1.3.5 Comparison of rat and human renal cytochrome P450s

The regiospecificity of CYP4A11 toward ω-hydroxylation was most similar to that to CYP4A2, and both were major renal cytochrome P450s in humans and rats, respectively. So, the immunochemical relatedness of CYP4A11 and CYP4A2 was investigated with anti-CYP4A2 (P450 K-5) antibody (Fig.6). We prepared anti-CYP4A2 antibody in rabbits and obtained antibodies that had different specificities. We used antibody of high or low specificity. The highly specific antibody reacted with CYP4A2 only. The immunoblot of the rat renal microsomes stained with highly specific antibody gave a single band and this antibody did not react with any proteins in human renal microsomes (Fig. 6). The antibody of low specificity reacted with CYP4A8 and CYP4A11 as well as CYP4A2. CYP4A8, 4A2, and 4A11 have similar structures. This antibody reacted with proteins in both rat and human renal microsomes and gave a single staining band. Partially purified CYP4A11 also gave a single staining band with this antibody and molecular weight was calculated to 52,000. The content of CYP4A2 in rat renal microsomes used in this study were calculated to 104 pmol/mg protein by

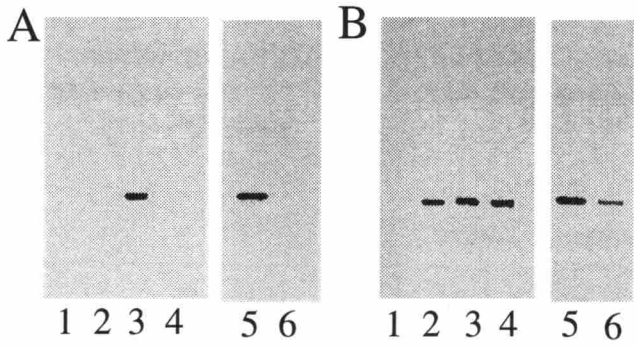

Fig. 6 Immunostained Western blots of purified cytochrome P450. Proteins were transferred from the acryl amide gel to nitrocellulose sheets that were reacted with highly specific anti-CYP4A2 (P450 K-5) antibody (A) and anti-CYP4A2 antibody of low specificity (B). Lanes 1, 2, 3, and 4 contain 0.5 pmol of P450 K-2 (CYP2C23), K-4 (CYP4A8), K-5 (CYP4A2), and P450 HK (CYP4A11), respectively. Lanes 5 and 6 contain rat renal microsomes (5 μg) and human renal microsomes (20 μg), respectively.

densitometory of the nitrocellulose membranes stained with anti-CYP4A2 antibody of high specificity. Known amounts of purified CYP4A2 were used on each nitrocellulose membrane to construct a standard curve. The content of CYP4A11 was 3.1 pmol/mg in the human renal microsomes used in this study, according to results of quantitative immunoblotting with anti-CYP4A2 antibody of low specificity and partially purified CYP4A11 for the standard.

1.4 DISCUSSION

Many forms of cytochrome P450 have been purified from hepatic microsomes and characterized, but few have been purified from renal microsomes; they include pig P450 (24), rabbit P448 (45), P450ka, and P450kb (46), and rat P450k-1 and P450 k-2 (47). Here, we purified three different forms of cytochrome P450, CYP2C23, 4A8, and 4A2, from renal microsomes of untreated male rats and partially purified human renal cytochrome P450, CYP4A11. To the best of our knowledge, human renal cytochrome P450 has not been isolated before. The purified renal cytochrome P450s, pig P450, rabbit P450ka, rat P450k-1, and k-2, are all efficient in the hydroxylation of fatty acids or prostaglandins (14, 45-47). Our cytochrome P450s, CYP2C23, 4A8, 4A2, and 4A11, were efficient catalysts of the hydroxylation of lauric acid and oxidation of arachidonic acid. However, these cytochrome P450s have catalytic properties. Only CYP2C23 catalyzed epoxydation of arachidonic acid. Only CYP4A8 catalyzed prostaglandin A1 hydroxylation. Our CYP4A8 and 4A2 may correspond to the P450k-1 and k-2 of Yoshimoto *et al.* (47), respectively, judging from chromatographic behavior, spectral properties, and catalytic activities. However, the regiospecificity of CYP4A8 and CYP4A2 toward lauric acid was different from that of P450k-1 and k-2. Rabbit P450ka and pig P450 are forms similar to rat P450k-1(our CYP4A8), based on their catalytic activity; these forms can metabolize both fatty acids and prostaglandins (24, 46, 47). Also, these forms had a similar ratio of lauric acid ω- and (ω-1)-hydroxylation activity [ω/(ω–1) = 1 to 3] to each other. On the other hand, P450k-2 (our CYP4A2) acted only on fatty acid (47), and the ratio of ω/(ω–1) lauric acid hydroxylation was larger than that of pig P450 or CYP4A8. Judging from the regiospecificity toward lauric acid (the ratio of ω/(ω–1) was high), human CYP4A11 may be a form similar to rat CYP4A2. Also, our CYP2C23 was very different from CYP4A8 and 4A2. CYP2C23 was efficient in (ω-1)-hydroxylation of lauric acid and epoxydation of arachidonic acid. Rat hepatic cytochrome P450s have such catalytic properties (48). Especially, cytochrome P450s placed in CYP2 family such as CYP2B1 and 2C11 have high activity. CYP2C23 is renal cytochrome P450 but its properties resemble those of hepatic cytochrome P450. Of hepatic cytochrome P450s, CYP4A1 and 4F1 catalyze arachidonic and lauric acids ω-hydroxylation and CYP4F1 also hydroxylates prostaglandin A_1 (48). These forms resemble renal CYP4A8 and 4A2.

Renal cytochrome P450 is known to have strong hydroxylation activities toward prostaglandins, saturated fatty acids such as lauric acid, and polyunsaturated fatty acids such as arachidonic acid, but its biological role has not been clearly understood. Recently, renal cytochrome P450 has been found to produce several regioisomeric epoxyeicosatrienoic acids as well as ω- and (ω-1)-hydroxyarachidonic acids (25, 26). Important for the evaluation of the possible role of cytochrome P450 in renal physiology is the recent finding that epoxyeicosatrienoic acids are endogenous constituents of rabbit kidney and human urine (49, 50). We have shown that CYP2C23 produces epoxyeicosatrienoic acids and that CYP4A8 and 4A2 produce ω- and (ω-1)-hydroxyarachidonic acids from arachidonic acid in a reconstituted system. Also, cytochrome P450 seems to be involved in the generation of two oxygenated arachidonate metabolites in incubates containing a preparation of cells isolated from the thick ascending limb of Henle's loop with arachidonic acid added. One of the products inhibits Na^+-K^+-ATPase, and the other causes relaxation of isolated blood vessels (51). Usually, ω- and (ω-1)-hydroxylation of prostaglandins and leukotrienes are thought of an inactivation pathway of these autacoids (52-55). Here, we found that renal cytochrome P450 was quite different from hepatic cytochrome P450. The renal cytochrome P450 may have functions different from those of hepatic cytochrome P450.

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CHAPTER 2
HORMONAL REGULATION OF RAT RENAL CYTOCHROME P450S BY ANDROGEN AND THE PITUITARY

2.1 INTRODUCTION

Sex-related differences of a number of cytochrome P450s in the hepatic microsomes of rats have been studied in detail. The male-specific form, CYP2C11, and the female-specific form, CYP2C12, are regulated by sex steroids and pituitary growth hormone, the secritory pattern of which is sex-associated in rats (1, 2). A pulsatile pattern of growth hormone secretion is required for expression of CYP2C11 and a continuous pattern of growth hormone secretion is required for expression of CYP2C12 (2). Other cytochrome P450s in rat liver are also affected by growth hormone (3-5). The regulation of the expression of hepatic cytochrome P450 has been much studied but little is known about extrahepatic tissues, including the kidney, which in rats has the next highest amount of cytochrome P450 after the liver. The characteristics of rat renal cytochrome P450s are different from those of rat hepatic cytochrome P450s (6). For example, fatty acids are better substrates for rat renal cytochrome P450s than drugs such as aminopyrine and 7-ethoxycoumarin, which are often used for the characterization of hepatic cytochrome P450, and the induction of cytochrome P450 by phenobarbital is low in rat kidneys (7). Therefore, the regulation of renal cytochrome P450 is probably different from that of hepatic cytochrome P450.

In mouse kidneys, sex differences in monooxygenase dependent on cytochrome P450 have been found (8-13). Microsomal catalytic activity toward some chemicals and carcinogens such as lauric acid and N,N-diethylnitrosoamine is higher in male mice than in female mice, and is affected by androgen (8-13). Henderson *et al.* (10, 13) reported that there is a sex difference in proteins in mouse renal microsomes cross-reacted with antibodies against rat hepatic cytochrome P450, and that some of these proteins are induced by androgen. Squires and Negishi (11) found that the expression of cytochrome P450 purified from male mouse kidneys is androgen-dependent. Recently, Sundseth and Waxman (14) reported that levels of CYP4A2 mRNA in rat kidneys are not affected by growth hormone but are increased by testosterone.

We have purified three cytochrome P450s from rat renal microsomes, CYP2C23 (P450 K-2), 4A8 (P450 K-4), and 4A2 (P450 K-5) (6). CYP4A2 is a major cytochrome P450 in the renal microsomes of male rats. CYP4A2 is similar to a major renal cytochrome P450, CYP4A11 (P450HK), in humans (6). These forms have high catalytic activity toward lauric acid and arachidonic acid. Arachidonic acid is metabolized by cytochrome P450s to bioactive substances including ω-hydroxyeicosatetraenoic acid (20-HETE) and epoxyeicosatrienoic acids (EETs);

ω-hydroxyeicosatetraenoic acid is a potent vasoconstrictor and epoxyeicosatrienoic acids are an inhibitor of Na^+-K^+-ATPase (15-17). These findings suggest that renal cytochrome P450 may contribute to the regulation of renal function. Therefore, the regulation of cytochrome P450 in the kidney is of interest.

Here, we investigated the regulation of three forms of cytochrome P450, CYP2C23, 4A8, and 4A2 in rat kidney by androgen and pituitary growth hormone, which affect the expression of several forms of cytochrome P450 in rat liver.

2.2 MATERIALS AND METHODS

2.2.1 Treatment of animals

Sprague-Dawley rats were obtained from Japan Clea (Tokyo). Hypophysectomy was done at 7 weeks of age. The rats were left to recover for at least 1 week and were then given a subcutaneous injection (0.2 IU/100g body wt) twice a day or an osmotic infusion (0.01 IU/h) of human growth hormone (Kabi Vitrum, Stockholm, Sweden) for seven days as described previously (2). Castration and sham-operation were performed at 8 weeks of age. Testosterone propionate (10 mg/kg) in corn oil was given subcutaneously to castrated male rats, intact female rats, or hypophysectomized female rats every other day for 2 weeks after castration or sham operation (1,2) and the rats were killed at 10 weeks of age.

2.2.2 Purification of cytochrome P450s and preparation of antibodies.

CYP2C23, 4A8, and 4A2 were purified from renal microsomes of male rats as described previously (6). Antibodies against these cytochrome P450s were prepared with Japanese white rabbits (Biotech, Saga, Japan). Characterization of antibodies was described elsewhere (6, 18).

2.2.3 Immunochemical assay of cytochrome P450

SDS-polyacrylamide gel electrophoresis was done with 7.5% gels for analysis of microsomal cytochrome P450 (18, 19). Microsomal proteins (5-20 μg) were electrophoresed and electrophoretically transferred to a nitrocellulose membrane. The transferred cytochrome P450 was reacted with a specific antibody from a rabbit and stained with a Vectastain ABC kit with horseradish peroxidase (Vector Laboratory). Measurement was done by densitometry of the stained band (19). The microsomal levels of individual cytochrome P450 forms were estimated relative to purified cytochrome P450 as a standard. Statistical analysis was performed using Student's *t*-test.

2.2.4 Other methods

The protein concentration was assayed by the method of Lowry *et al.* (20). Cytochrome P450 was assayed by the method of Omura and Sato (21). Lauric acid hydroxylation activity was measured as follows (6). The reaction mixture (0.5 ml) containing renal microsomes (100 μg), 100 mM potassium phosphate (pH 7.2), sodium laurate (100

μM), and NADPH (200 μM) was incubated at 37 °C for 20 min. The reaction was stopped by the addition of 2 M HCl (10 μl). The hydroxylauric acid produced was extracted with ethyl acetate (2 ml). The organic layer was transferred to another test tube and distilled water (1 ml) was added to it for removing excess HCl. Again, organic layer was transferred to new test tube and the ethyl acetate was evaporated at 40 °C under reduced pressure. 9-Anthryldiazomethane (Funakoshi, Tokyo, Japan) dissolved in ethyl acetate (0.1 ml, 1 mg/ml) was added to the resulting residue. The reaction mixture was kept at 4 °C overnight. Then a 10-μl portion of solution was analyzed by HPLC with a reverse-phase column (ODS-120A, 4.6 x 250 mm, Tosoh, Tokyo, Japan). The column was developed with acetonitrile-H_2O (78:22) at 40 °C. The flow rate was 1.2 ml/min. ω- And (ω-1)-hydroxylauric acids were eluted separately at the retention time of 13-15 min. Then the column was washed for 5 min with 100% acetonitrile for elution of lauric acid ester. These processes were done automatically by a programmable HPLC system. Derivatives of ω- and (ω-1)-hydroxylauric acid were detected fluorometrically with the excitation wavelength of 365 nm and the emission wavelength of 412 nm. Measurement was done by comparison of the peak areas with those of authentic ω-hydroxylauric acid (Sigma Chemical Co., St. Louis, MO) and (ω-1)-hydroxylauric acid synthesized as described previously (18).

2.3 RESULTS

2.3.1 Effects of androgen and pituitary growth hormone on rat renal cytochrome P450s

The contribution of androgen and the pituitary growth hormone to the expression of the rat renal cytochrome P450s, CYP2C23, 4A8, and 4A2 was assessed by a series of castration or hypophysectomy and hormone replacement experiments (Table I). CYP4A2 is a major cytochrome P450 in male rat kidney. The level of CYP4A2 in male rat kidneys was five times that in female rats. CYP4A2 was a male-dominant form, and CYP2C23 and 4A8 were abundant in female rat kidneys. Castration of male rats resulted in a decrease in the renal CYP4A2 level, suggesting the involvement of androgen for expression of CYP4A2 in rat kidneys. The administration of testosterone to castrated rats increased the levels of CYP4A2 to twice the control level. Castration of male rats also decreased CYP4A8 and testosterone recovered its level of castrated male rats. On the other hand, castration did not change the level of CYP2C23, although it was female-dominant form. Castration plus testosterone treatment decreased the renal level of CYP2C23. Sex-specific cytochrome P450s in rat liver are indirectly regulated by androgen through changes in the secretory pattern of pituitary growth hormone. Therefore, we examined the possible role of pituitary growth hormone on the expression of CYP4A2, 4A8, and 2C23. Hypophysectomy of male rats decreased the level of CYP4A2 in renal microsomes. Administration of growth

Table I Effects of testosterone and growth hormone on renal cytochrome P450s in male rats

Treatment	n	CYP2C23	CYP4A2	CYP4A8
Controls, male	5	7.7 ± 2.2	74.5 ± 8.4	1.5 ± 0.27
Sham, male	5	6.6 ± 0.6	72.8 ± 1.6	1.3 ± 0.45
Castrated male	5	7.4 ± 1.4	32.8 ± 5.2**	0.5 ± 0.10 **
Cast. + T	6	4.0 ± 1.3**,†	131.7 ± 18.1 **, †	1.2 ± 0.28 †
Sham, male	5	5.7 ± 0.8	78.6 ± 12.8	1.4 ± 0.36
Hyp, male	3	10.1 ± 2.1**	41.4 ± 13.2 **	0.7 ± 0.26 *
Hyp + GH (inj)	3	5.6 ± 0.4 §	89.6 ± 8.6 §	0.9 ± 0.33
Hyp + GH (inf)	3	6.9 ± 1.5 §	47.8 ± 9.4 *	0.7 ± 0.16
Controls, female	4	10.8 ± 1.7**	14.4 ± 2.1 **	2.0 ± 0.27 *

Values (means ± SD) are expressed as pmol of P450/mg of microsomal protein. The levels of cytochrome P450 were assayed by immunoblotting. n, numbers of rats; sham, sham operated; Cast. + T, castrated male rats treated with testosterone; Hyp + GH (inj), hypophysectomized male rats treated with growth hormone by intermittent injection, male-type pattern; GH(inf), treated with growth hormone by infusion, female type pattern.
* Significantly different from sham, male, at $P < 0.05$.
** Significantly different from sham, male, at $P < 0.01$.
† Significantly different from castrated male, at $P < 0.01$.
§ Significantly different from Hyp, male, at $P < 0.01$.

hormone by intermittent injections, to mimic the male secretory pattern, reverse the decrease of CYP4A2; no reversal occurred with continuous infusion, which mimicked the female secretory pattern. Level of CYP4A8 also decreased by hypophysectomy but decreased CYP4A8 could not be recovered by the growth hormone administered by intermittent injections or continuous infusion. CYP2C23 in male rats was elevated to the female level upon hypophysectomy, and the elevation of CYP2C23 was reversed by growth hormone administered by intermittent injections or continuous infusion.

2.3.2 Effects of androgen and growth hormone on the activity of lauric acid hydroxylation of rat renal microsomes

The effects of testosterone and growth hormone on the lauric acid ω- and (ω-1)-hydroxylation of rat renal microsomes were investigated by castration and hypophysectomy (Table II). There was a sex difference in the activities of lauric acid ω- and (ω-1)-hydroxylation; both activities in the male rats were higher than those in the female rats. The ratio of ω/ω–1 in lauric acid hydroxylation of the male rats was larger than that of the female rats. Castration of male rats decreased the lauric acid

Table II Effects of testosterone and growth hormone on lauric acid hydroxylation activity of male rat kidneys

Treatment	n	ω-hydroxylation	(ω-1)-hydroxylation	ω/ω-1	Total P450
Controls, male	5	0.752 ± 0.176	0.222 ± 0.021	3.39	0.080 ± 0.018
Sham, male	5	0.708 ± 0.127	0.250 ± 0.037	2.83	0.089 ± 0.010
Castrated male	5	0.288 ± 0.028 **	0.173 ± 0.012**	1.66	0.079 ± 0.008
Cast. + T	6	1.027 ± 0.110 **, †	0.292 ± 0.039 †	3.52	0.095 ± 0.015
Sham, male	5	0.782 ± 0.133	0.290 ± 0.043	2.70	0.071 ± 0.008
Hyp, male	3	0.356 ± 0.054 **	0.200 ± 0.044 *	1.78	0.063 ± 0.011
Hyp + GH (inj)	3	0.633 ± 0.047 §	0.255 ± 0.027 §	2.48	0.078 ± 0.008
Hyp + GH (inf)	3	0.374 ± 0.070**	0.186 ± 0.054*	2.01	0.059 ± 0.014
Controls, female	4	0.339 ± 0.050**	0.199 ± 0.030**	1.70	0.074 ± 0.009

Catalytic activities are expressed as nmol of product/min/mg of microsomal protein. Total cytochrome P450 was measured photometrically and is expressed as nmol of P450/ mg of microsomal protein. All values are shown as means ± SD. Abbreviations are the same as in the footnote of Table I.

* Significantly different from sham, male, at $P < 0.05$.

** Significantly different from sham, male, at $P < 0.01$.

† Significantly different from castrated male, at $P < 0.01$.

§ Significantly different from Hyp, male, at $P < 0.01$.

hydroxylation activity and the ω/ω–1 ratio of the renal microsomes to the level of female rats. The administration of testosterone to castrated male rats reversed those changes. Hypophysectomy of male rats resulted in a feminization of the lauric acid hydroxylation activity. The decrease in lauric acid hydroxylation in renal microsomes was reversed by growth hormone administered by intermittent injections but not by continuous infusion.

2.3.3 Contribution of CYP4A2 to lauric acid ω- and (ω-1)-hydroxylation in rat renal microsomes

There were sex differences in the levels of CYP4A2 and the lauric acid hydroxylation activity of rat renal microsomes. So, anti-CYP4A2 antibody was added to the reaction mixture of renal microsomes to evaluate the contribution of this cytochrome P450 to renal lauric acid hydroxylation (Fig. 1). Both lauric acid ω- and (ω-1)-hydroxylation activities were completely inhibited by the addition of anti-CYP4A2 antibody (1mg IgG/mg of microsomal protein), indicating that CYP4A2 or the closely related form mainly contributes to lauric acid hydroxylation in rat kidney. Neither anti-CYP2C23 nor anti-CYP4A8 antibodies inhibited lauric acid hydroxylation of rat renal microsomes, even when 2 mg IgG/mg of microsomal protein was added (data not shown).

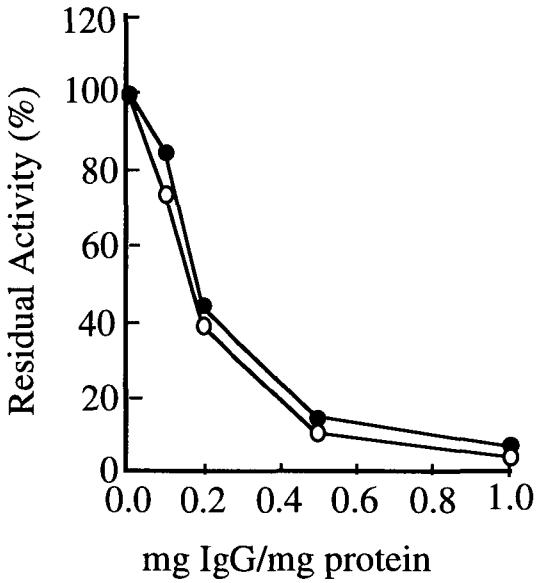

Fig. 1 Effects of anti-CYP4A2 antibody on the lauric acid ω- and (ω-1)-hydroxylation activities of renal microsomes of male rats. Open circles and closed circle indicate lauric acid ω- and (ω-1)-hydroxylation activities, respectively. Renal microsomes (100 μg) were reacted with lauric acid (0.2 mM) in the presence of anti-CYP4A2 antibody. The catalytic activity in the presence of control immunogloblin G (IgG) was set at 100%.

2.3.4 Effects of androgen on renal cytochrome P450 in female rats

To confirm whether the effects of androgen on the expression of CYP4A2 were direct or not, testosterone was administered to control or hypophysectomized female rats. The administration of testosterone to female rats induced CYP4A2 in the kidneys (Table III). The levels of CYP4A2 in female rats treated with testosterone was close to the male level. The hepatic male-specific form, CYP2C11 was not induced in female livers by treatment with testosterone (data not shown). Lauric acid hydroxylation activity was also induced in female kidney by treatment with testosterone and the pattern of lauric acid hydroxylation profile was masculinized; the ratio of ω/ω–1 was increased to the level of male rats. Treatment of the hypophysectomized female rats with testosterone also induced CYP4A2 in the kidneys. These results suggested that the expression of CYP4A2 was regulated directly by androgen. CYP4A8 and 2C23 were not induced by treatment with testosterone. Level of CYP2C23 was decreased by hypophysectomy of female rats plus treatment with testosterone. Level of CYP4A8 was also decreased by hypophysectomy of female rats.

Table III Effects of testosterone on renal cytochrome P450s in female rats

		Lauric acid hydroxylation			P450 forms		
	n	ω	ω-1	ω/ω–1	CYP2C23	CYP4A2	CYP4A8
Cont, female	5	0.372 ± 0.043	0.208 ± 0.019	1.79	13.4 ± 4.6	18.1 ± 2.7	2.2 ± 0.38
Female + T	6	0.771 ± 0.147**	0.267 ± 0.045 *	2.89	12.0 ± 3.2	58.5 ± 7.4**	2.1 ± 0.24
Hyp, female	5	0.290 ± 0.087	0.141 ± 0.028**	2.06	16.7 ± 2.0	21.6 ± 4.1	1.4 ± 0.19**
Hyp + T	6	0.685 ± 0.180††	0.215 ± 0.067†	2.36	9.6 ± 2.9††	45.2 ± 17.0†	1.3 ± 0.24
Cont, male	5	0.752 ± 0.176**	0.222 ± 0.021	3.39	7.7 ± 2.2*	74.5 ± 8.4**	1.5 ± 0.27*

Catalytic activities are expressed as nmol of product/min/mg of microsomal protein. The levels of cytochrome P450 measured by immunoblotting are expressed as pmol of P450/mg of microsomal protein. Values are shown as means ± SD. Female + T, female rats treated with testosterone; Hyp, female, hypophysectomized female rats; Hyp + T, hypophysectomized female rats treated with testosterone.

* Significantly different from cont, femal, at $P < 0.05$.

** Significantly different from cont, female, at $P < 0.01$.

† Significantly different from Hyp, female, at $P < 0.05$.

†† Significantly different from Hyp, female, at $P < 0.01$.

2.4 DISCUSSION

Many experiments have suggested that the effects of androgen on the expression of hepatic cytochrome P450s such as CYP2C11, major form in male rats, are indirect and are mediated by pituitary growth hormone secretion (1-5, 22). In rat kidneys, the levels of CYP4A2 was decreased by hypophysectomy of male rats, and administration of growth hormone in a pulsatile pattern prevented the decrease in CYP4A2, but administration by infusion did not. The expression of CYP4A2 in rat kidneys in response to the level of pituitary growth hormone seemed to be similar to that of the male-specific form in rat liver. However, in a study that used mice deficient in growth hormone, Henderson *et al.* (10, 13) found that the expression of renal cytochrome P450 in mice independent of the growth hormone level and that the androgen receptor mediates the sex difference in the expression of renal cytochrome P450 in mice. Sundseth and Waxman (14) reported that levels of CYP4A2 mRNA in rat kidneys are not affected by treatment of rats with growth hormone but are increased by treatment of hypophysectomized rats with testosterone. We found that CYP4A2 was induced in female rat kidneys by treatment of females with testosterone, although CYP2C11, a major hepatic male-specific form under the regulatory control of growth hormone, was not induced in female rat livers. Treatment of hypophysectomized female rats with testosterone also induced CYP4A2 in the kidneys. These results suggested that CYP4A2 was regulated directly by androgen as well as pituitary growth hormone. On the contrary, CYP2C23 was abundant in female rats. Hypophysectomy of male rats

increased the level of CYP2C23, and treatment with testosterone decreased its levels, suggesting that expression of CYP2C23 was suppressively regulated by androgen and pituitary growth hormone. CYP4A8 was a minor form in rat kidneys. Expression of CYP4A8 was suppressed by castration and hypophysectomy. Decreased levels of CYP4A8 were recovered by administration of testosterone to castrated rats but not by administration of growth hormone to hypophysectomized rats. The regulation of CYP4A8 was complicated. Hormonal regulation of CYP4A2 was different from those of CYP2C23 or CYP4A8.

CYP4A2 is a major lauric acid hydroxylase in the male rat kidney because CYP4A2 was a major form in male rat kidneys and because anti-CYP4A2 antibody completely inhibited lauric acid hydroxylation by renal microsomes of male rats. In female rats, lauric acid ω-hydroxylation activity was half that of male rats; the levels of CYP4A2 in female rats were one-fifth those in male rats. These results suggested that another lauric acid hydroxylase is present in female rat kidneys. It may be CYP4A3 (originally P450 DM-3) (23). The CYP4A3 was purified from the diabetic rats and was induced in rats by diabetes (23). CYP4A3 is a homologous cytochrome P450 to CYP4A2 and has similar catalytic activity to CYP4A2 but its molecular weight calculated on SDS-polyacrylamide gel elctrophoresis is higher than that of CYP4A2 and the ratios of ω/ω–1 hydroxylation for lauric acid by purified CYP4A2 and CYP4A3 are 5.17 and 2.93, respectively (23). These results well explain that ω/ω–1 ratio of lauric acid hydroxylation of female rats is lower that that of male rats.

Hormonal regulation of cytochrome P450 in the kidney could explain why there are many findings of sex differences in susceptibility to toxins and carcinogens (24, 25). There is a sex difference in the nephrotoxicity and carcinogenesity of some chemicals such as nitrosoamine in mice and humans; the difference seems to be related to different rates of matabolic activation of these toxins and carcinogens in the kidneys (24, 25). Many toxins and carcinogens are activated by cytochrome P450s. Steroid hormones have been implicated in the etiology of renal cancer, which occurs in men more often than in woman (26). We found that there are sex differences in the levels of CYP4A2, a major renal cytochrome P450, and lauric acid hydroxylation activity of rat renal microsomes, and that the expression of CYP4A2 was directly regulated by androgen as well as pituitary growth hormone. A major renal cytochrome P450 in humans, CYP4A11, is similar to CYP4A2 (6). These forms may contribute to the sex difference in the nephrotoxicity and carcinogenesity of some chemicals in rats and humans.

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CHAPTER 3
REGULATION OF RAT RENAL CYTOCHROME P450S BY THYROID HORMONE IN DIABETES

3.1 INTRODUCTION

Diabetes is a pathophysiological state that is a useful probe of cytochrome P450 regulation. Diabetes increases serum glucose and ketone bodies and decreases the serum levels of pituitary growth hormone, androgen, and thyroid hormone (1, 2). Cytochrome P450 expression is affected by the status of pituitary, gonadal, and thyroidal hormone secretion (3). Diabetes induces CYP2C7, 2A1, and a 4A form, and especially CYP2E1 in rat livers (4-6). On the contrary, it suppresses the expression of CYP2C11, and 2C13, male-specific forms (4-6). CYP2E1 is induced by ketones such as acetone and its expression is suppressively regulated by growth hormone (7-9). Increased levels of ketone bodies and decreased level of pituitary growth hormone seem to induce CYP2E1. However, treatment with testosterone and growth hormone only partly reverses these changes, although treatment of diabetic rats with insulin reverses changes in the levels of these cytochrome P450s (1, 10). Insulin-treatment reverses many factors such as serum glucose and ketone body levels in diabetic rats. Therefore, which factor mainly contributes to pathophysiological changes in cytochrome P450s in the diabetic state is not clear.

Rat kidney has the next highest amount of cytochrome P450 after the livers. Renal cytochrome P450s have different properties from hepatic cytochrome P450s. Fatty acids such as lauric acid and arachidonic acid are better substrates for renal cytochrome P450s than drugs such as aminopyrine and 7-ethoxycoumarin, which are often used for the characterization of hepatic cytochrome P450s such as CYP2B1 and 1A1 (11), and the induction of cytochrome P450 by phenobarbital is low in rat kidney (12). Therefore, the regulation of renal cytochrome P450 is probably different from that of hepatic cytochrome P450. Recently, Sandseth and Waxman reported that renal cytochrome P450 is directly regulated by testosterone (13). We also obtained similar results (14). However, the regulation of renal cytochrome P450 has not been studied as much as that of hepatic cytochrome P450.

In this study, we investigated the changes in the levels of cytochrome P450 in rat kidney by diabetes and the effects of thyroid hormone on the regulation of cytochrome P450 expression in diabetic rats.

3.2 MATERIALS AND METHODS

3.2.1 Treatment of animals

Sprague-Dawley rats were obtained from Japan Clea (Tokyo). Diabetes was induced with a single injection of streptozotocin (STZ, 65 mg/kg) intravenously at 7 weeks of

age. Insulin (6 IU/rat/day, two injections a day) was given subcutaneously to diabetic rats. Thyroidectomy was done at 7 weeks of age. Thyroid hormone (T_3, 5 μg/kg or 50 μg /kg) was given subcutaneously to rats. The treatments with insulin or T_3 were initiated at 8 weeks of age and continued for two weeks. Rats were killed at 10 weeks of age. Serum glucose was measured by a glucose oxidase method (15). Serum T_3 was assayed by a radioimmunoassay (RIA) using a SPAC T_3 RIA kit (Daiichi Radioisotope Laboratory, Tokyo).

3.2.2 Immunochemical assay of cytochrome P450s

CYP2C23 (P450 K-2), CYP4A8 (P450 K-4), and CYP4A2 (P450 K-5) were purified from renal microsomes of untreated male rats (16-20). Antibodies against purified P450s were prepared with Japanese White rabbits (Biotech, Saga, Japan). Characterization of antibodies has been described elsewhere (21). Each form of cytochrome P450 was assayed by a quantitative immunoblotting described previously (21). SDS-polyacrylamide gel electrophoresis was done with 7.5% gels for analysis of microsomal cytochrome P450. Microsomal proteins (5-30 μg) were electrophoresed and electrophoretically transferred to a nitrocellulose membrane. The transferred cytochrome P450 was reacted with a specific antibody from a rabbit and was stained with a Vectastain ABC kit with horseradish peroxidase (Vector Laboratory). Quantitation was done by densitometry of the stained band. The microsomal levels of individual cytochrome P450 forms were estimated relative to purified cytochrome P450 as a standard. Assay of lauric acid hydroxylation activity was described elsewhere (16).

3.3 RESULTS

3.3.1 Effects of thyroid hormone on renal P450 in diabetic rats

Induction of diabetes caused by STZ was evident from the elevated blood glucose levels seen in these rats (Table I). Insulin treatment lowered the blood glucose level. As previously reported (2), diabetes caused by STZ decreased the serum T_3 level. Insulin treatment reversed the decrease in T_3 level. The content of total cytochrome P450 measured photometrically was increased by diabetes. Lauric acid hydroxylation activity was also induced by diabetes. Changes in the three forms of cytochrome P450 caused by diabetes in the rat kidney were measured by immunoblotting (Table I). Diabetes did not induce CYP4A8, which is a minor renal form, but induced significantly CYP4A2, a major renal form, and CYP2C23. The increase was reversed by treatment of diabetic rats with insulin. Thummel and Schenkman (1) found that diabetes decreased the levels of serum testosterone and growth hormone and that the changes in the levels of cytochrome P450 caused by diabetes in rat hepatic microsomes were not recovered by treatment with growth hormone and testosterone although they were recovered completely by insulin treatment. Sundseth and Waxman (13) found that the CYP4A2 mRNA level in rat kidney was not affected by growth hormone treatment and

Table I Effects of diabetes on levels of rat renal cytochrome P450s

	Control	DM	DM + Insulin	DM + T_3
n	5	7	6	6
Glucose (mg/dl)	112 ± 7.7	487 ± 19.0 *	80 ± 23.4 ††	463 ± 22.9
T_3 (ng/ml)	0.876 ± 0.079	0.624 ± 0.065 *	0.885 ± 0.145 ††	1.154 ± 0.256 ††
Total P450 (nmol/mg)	0.085 ± 0.011	0.115 ± 0.016 *	0.078 ± 0.010 ††	0.080 ± 0.017 ††
Lauric acid (nmol/min/mg)				
(ω-1)-hydroxylation	0.264 ± 0.053	0.564 ± 0.115 *	0.317 ± 0.070 ††	0.313 ± 0.074 ††
ω-hydroxylation	0.600 ± 0.143	1.087 ± 0.218 *	0.739 ± 0.086 ††	0.611 ± 0.184 ††
P450 forms (pmol/mg)				
CYP2C23	5.2 ± 0.6	7.6 ± 0.5 *	6.1 ± 0.5 ††	5.0 ± 1.0 ††
CYP4A8	2.3 ± 0.6	2.7 ± 0.6	2.0 ± 0.1	1.8 ± 0.5 †
CYP4A2	77.7 ± 7.6	130.1 ± 9.8*	101.4 ± 10.5 ††	66.0 ± 7.7 ††

Total content of cytochrome P450 was assayed photometrically. Levels of each form of cytochrome P450 were measured by immunoblotting. Values are expressed as means ± SD. DM, diabetic rats induced with STZ; DM + insulin, diabetic rats treated with insulin; DM + T_3, diabetic rats treated with T_3 (50 μg/kg).
* Significantly different from control rats, $P < 0.01$.
† Significantly different from diabetic rats, $P < 0.05$.
††Significantly different from diabetic rats, $P < 0.01$.

was decreased by the decreased level of serum testosterone. If the decreased levels of testosterone and growth hormone affect the level of renal cytochrome P450 in the diabetic state, the level of CYP4A2 will be decreased. However, the level of CYP4A2 was increased by diabetes. Other factors affect the CYP4A2 level in diabetic rats. Thus, the thyroid hormone was given to diabetic rats, because diabetes decreased the serum T_3 level as well as testosterone and growth hormone levels (1). The increase in the levels of CYP4A2 was prevented by treatment of diabetic rats with T3 and the increase in lauric acid hydroxylation activity was also reversed (Table I). These results suggest that induction of a major renal cytochrome P450 caused by diabetes was due to the decrease in serum thyroid hormone level. The increased level of CYP2C23 was also reversed by T_3 treatment.

3.3.2 Effects of thyroidectomy on the levels of renal cytochrome P450

We further investigated the effects of thyroid hormone on the renal cytochrome P450 by thyroidectomy and hormone replacement study (Table II). Thyroidectomy of male rats significantly increased the content of total cytochrome P450 and lauric acid ω- and (ω-1)-hydroxylation activities, indicating an increase in the levels of P450 forms. In fact, the levels of CYP2C23 and CYP4A2 measured by immunoblotting were increased by thyroidectomy. Treatment of thyroidectomized rats with T_3 prevented the increases

in the levels of these forms. These findings suggest that these forms are suppressively regulated by thyroid hormone. The level of CYP4A8 was not affected by diabetes or thyroidectomy. Regulation of CYP4A8 in diabetes was different from that of CYP2C23 and CYP4A2.

Table II Effects of thyroidectomy on levels of renal cytochrome P450s

	Sham	Thydx	Thy + T_3	Cont + T_3
n	5	5	5	5
Total P450 (nmol/mg)	0.075 ± 0.014	0.107 ± 0.026 *	0.053 ± 0.008 ††	0.074 ± 0.010
Lauric acid (nmol/min/mg)				
(ω-1)-hydroxylation	0.362 ± 0.032	0.556 ± 0.157 *	0.311 ± 0.062 †	0.347 ± 0.119
ω-hydroxylation	0.743 ± 0.085	1.325 ± 0.380 *	0.653 ± 0.148 †	0.778 ± 0.230
P450 forms (pmol/mg)				
CYP2C23	5.7 ± 1.7	10.9 ± 0.4 **	5.9 ± 1.7 ††	6.1 ± 2.3
CYP4A8	2.0 ± 0.3	2.3 ± 0.3	1.4 ± 0.3 ††	2.3 ± 0.4
CYP4A2	84.3 ± 20.1	127.8 ± 8.9 **	74.8 ± 23.0 ††	83.2 ± 13.2

Total content of cytochrome P450 was assayed photometrically. Levels of each form of cytochrome P450 were measured by immunoblotting. Values are expressed as means ± SD. Sham, sham-operated rats; thydx, thyroidectomized rats; Thy + T_3, thyroidectomized rate treated with T_3 (50 μg/kg); Cont + T_3, control rats treated with T_3 (50 μg/kg).
* Significantly different from control rats, $P < 0.05$.
**Significantly different from control rats, $P < 0.01$.
† Significantly different from diabetic rats, $P < 0.05$.
††Significantly different from diabetic rats, $P < 0.01$.

3.4 DISCUSSION

The present study establishes that diabetes caused by STZ induced CYP4A2 and CYP2C23 in the rat kidney but not CYP4A8. CYP4A2 is a major male-predominant form in the rat kidney and its level is decreased by the decrease in serum level of testosterone or growth hormone (14). Diabetes decreases the levels of serum testosterone and growth hormone (1) but induced CYP4A2. Expression of CYP2C11, a major male-specific form in rat liver, is suppressed by diabetes (4-6). The major renal cytochrome P450 and the major hepatic cytochrome P450 are regulated differently, although both are male-predominant forms. In rat liver, increased levels of ketone bodies as well as changes in the hormone levels caused by diabetes induced cytochrome P450s such as CYP2E1 (8, 9, 22). However, acetone-treatment does not induce CYP4A2 in rat kidney (23). Factors other than acetone, testosterone, and growth hormone regulate the expression of CYP4A2. In this study, we found that thyroid hormone (T_3) as well as insulin reversed the level of CYP4A2 in diabetic rats. Expression of CYP4A2 and CYP2C23 in the rat kidney was suppressively regulated by

thyroid hormone. In rat liver, CYP2B1, 2B2 and 2A1 are suppressively regulated by thyroid hormone (24, 25). The hepatic content of these forms in rat liver is increased by thyroidectomy and the increase is reversed by treatment with thyroid hormone. The response of these forms to the thyroid hormone seems similar to that of CYP4A2 and CYP2C23. Low dose of T_3 (5 μg/kg) reversed the increased level of CYP2C23 but not that of CYP4A2 in diabetic and thyroidectomized rats although high dose (50 μg/kg) suppressed expression of both forms. Regulation of CYP4A2 was different from that of CYP2C23. Hypophysectomy decreases the level of CYP4A2 but induces CYP2C23 in rat kidney (14). Thyroid hormone affects the level of other hormones such as growth hormone (25) and it remains possible that expression of these forms was regulated indirectly by thyroid hormone. The thyroid hormones have catabolic effects on the metabolism of carbohydrates and lipids. Cytochrome P450 in the CYP4A subfamily can metabolize fatty acids (16). Increased urinary excretion of dicarboxylic acids which may be derived from ω-oxidation of medium chain fatty acid in diabetic condition is reported (26). The induction of CYP4A2 may alter the metabolism of fatty acids in diabetes.

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CHAPTER 4
COMPLETE cDNA SEQUENCE AND cDNA-DIRECTED EXPRESSION OF HUMAN CYP4A11

4.1 INTRODUCTION

Cytochrome P450 are a superfamily of hemoproteins that participate in metabolism of endogenous and foreign compounds. Certain cytochrome P450s involved in steroidogenic pathways are well conserved in mammals, whereas the foreign compound-metabolizing enzymes exhibit a large degree of species specificity (1). Over the last 10 years, many laboratories have been focused on the direct study of human cytochrome P450s. Most of this work has been carried out on cytochrome P450 forms expressed in hepatic tissue (2, 3). Only limited information exist on cytochrome P450s expressed in extrahepatic tissues due, in large part, to the difficulty in obtaining fresh tissues of sufficient quantity to purify these enzymes. This is particularly a problem with kidney, which is used almost exclusively for transplantation. However, direct cDNA cloning has yield information on cytochrome P450s expressed in pulmonary tissue (4, 5).

Renal cytochrome P450s have been studied in rodents but, to date, analysis of their human counterparts have been limited to a few purification studies (6, 7). In general, renal cytochrome P450s appear to favor hydroxylation of substrates such as fatty acids, prostaglandins, and arachidonic acid rather than compounds represented by drugs, steroids and carcinogens (8). Since rodent cytochrome P450s within the CYP4A subfamily have shown to carry out reactions on fatty acid and to be expressed in kidney, a rat CYP4A3 cDNA (9) was used to clone human P450 cDNAs within the same subfamily from a human kidney cDNA library. Analysis of human renal cytochrome P450s will allow a species comparison of catalytic activities of CYP4A enzymes to define more precisely their roles in kidney physiology. The sequence and some catalytic activities of a new cDNA designated CYP4A11 (10) and an allelic variant, CYP4A11v, were determined in the present report, and the biological significance of renal cytochrome P450s is discussed.

4.2 MATERIALS AND METHODS

4.2.1 Materials

A human renal cDNA library was obtained from Clontech Laboratories (Palo Alto, CA). This library was produced in the vector λgt10 using mRNA isolated from a 20-year-old Caucasian female. All enzymes were purchased from Bethesda Research Laboratories (Bethesda, MA), New England Biolabs (Beverly, MA), and International Biotechnologies (New Haven, CT). The pCMV4 vector was provided by Dr. David W. Russell (University of Texas Southwestern Medical Center). Baculovirus and a

baculovirus expression system were obtained from the Invitrogen Corp. (San Diego, CA). [1-^{14}C]lauric acid, [1-^{14}C]arachidonic acid, and [5, 6-^{3}H]prostaglandin E_1 were purchased from the Amersham Corp. (Arlington Heights, IL).

4.2.2 Cloning and sequencing

The cDNA library was screened by plaque hybridization using the full-length CYP4A3 cDNA isolated by Kimura *et al.* (9) as a probe. The filters were hybridized at 65 °C in 6 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate), 10 x Denhardt's, 0.5% sodium dodesyl sulfate, and 200 µg/ml denatured double-stranded salmon sperm DNA, and washed three times in 2 x SSC at 65 °C. The clones having the largest inserts were processed further by removing the inserts using *Eco* RI partial digestion followed by subcloning into the *Eco* RI site of the vector pUC9. Sequencing was carried out by the plasmid walking method using synthetic oligonucleotide primers produced with an Applied Biosystems Model 380B synthesizer. New primers were synthesized from additional sequence obtained after each extension of the cDNA. Sequencing was performed by using fluorescent label-tagged dideoxynucleotides and Taq polymerase, and products were analyzed with an Applied Biosystems model 373A DNA sequencer. Each base was determined at least once in both directions. Data were analyzed using Mac Vector software (International Biotechnologies).

4.2.3 Expression of the CYP4A cDNA

The CYP4A11 and CYP4A11v enzymes were produced with the baculovirus cDNA expression system. cDNAs were inserted into the pBlueBac-2 vector and the recombinant baculovirus was constructed with the baculovirus expression kit using Sf9 cells. Details of the expression method for cytochrome P450 production were reported previously (11). Purified recombinant baculovirus was used to infect Sf9 cells and a solution of hemin (hemin/BSA, 1:1) was added to the media at a final concentration of 1 mg/ml, 24 hr after infection (12). The cells were harvested 48 hr after addition of hemin, lysed by sonication in 0.1 M sodium phosphate buffer pH 7.4, containing 20% glycerol, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonylfluoride (PMSF), and 1mM EDTA at 4 °C and then analyzed by Western blotting using antibody generated against rat CYP4A2 (6). The lysates were also used for measurement of cytochrome P450 CO-reduced spectra and for assay of enzymatic activity. Lauric acid, arachidonic acid, and prostaglandin metabolic activities were measured by the methods previously described (6, 8). NADPH-cytochrome P450 oxidoreductase was also expressed using baculovirus and purified as described by Tamura *et al.* (13). Purified oxidoreductase (100 pmoles) was added to 500 µg of cell lysate protein prior to assay. This amount of enzyme was found to produce saturation of maximal lauric acid hydroxylation activity catalyzed by CYP4A11.

4.2.4 Detection of the CYP4A11v allele by PCR-mediated allele-specific oligonucleotide hybridization

High-molecular weight DNA was prepared from peripheral blood leukocytes (14). For analysis of mutations in the CYP4A11 gene by polymerase chain reaction (PCR), two primers, 5'-CAATTTGCCATGAACGAGCT-3' and 5'-AGACAGGTAGACAAGCAGGTA-3', were synthesized. Two probes, normal (5'-TGAAATCCAAAAA-TGGAATCC-3') and mutated (5'-TGAAATCCAAAATGGAATCC-3'), were also synthesized. Human genomic DNA samples (ca. 1 μg) were amplified by 35 cycles using the annealing and extension temperature, 55 °C and 72 °C, respectively. Resulting fragments were applied to a nylon membrane filter and the filters were treated by the method of Yoshioka *et al.* (15). After treatments, the filters were hybridized with normal and mutated oligonucleotide probes and washed with a solution of 0.9 M sodium chloride, 0.09 M sodium citrate, pH 7.0 and 0.1% sodium dodecylsulfate at 46 °C and 48 °C (mutated and normal, respectively). In some cases, the PCR products were directly sequenced using a nested oligonucleotide primer.

4.3 RESULTS

4.3.1 Sequence of the CYP4A11 cDNAs

A human renal cDNA library was screened with the rat CYP4A3 cDNA probe and clones having the largest inserts were isolated and sequenced. The renal cytochrome P450 cDNA isolated was designated CYP4A11, and contained an open-reading frame of 519 amino acids encoding a protein having a calculated molecular weight of 59,295 daltons (Fig. 1). Amino-terminal amino acid sequence derived from the CYP4A11 cDNA agreed with that of renal cytochrome P450 purified from human renal microsomes previously (6). This cytochrome P450 is a major form in human kidneys. Another cDNA clone, isolated from the same library, had a deletion of an adenine (A) at position 1,540 in the coding region. The A deletion causes a change of amino acid sequence due to a shift in the reading frame of codons from nucleotide position 1,601 to 1,818 in the cDNA. The variant cDNA, designated CYP4A11v, contains 591 amino acids, which is considerably larger than typical cytochrome P450s, which range from about 480 to 520 amino acids.

The CYP4A11 amino acid sequence was compared with other members of the CYP4A subfamily. Overall amino acid sequence similarities of 71%, 72%, 74%, and 80% were calculated for rat CYP4A2 (9), rat CYP4A3 (9), rat CYP4A8 (16), and rabbit CYP4A6 (17), respectively. The previously identified region between residues 310-330, which is highly conserved among all P450s in the CYP4 family (4, 16), is also completely conserved in CYP4A11. The functional significance of this sequence conservation is presently unknown. However, it is noteworthy that Thr-301 has been shown to be involved in binding and metabolism of fatty acids by cytochrome P450s in CYP2 family (18).

```
        10         20         30         40         50         60         70         80         90
ATTCCGGGGGGCACTCAGAGATCCAGCAGGTGCTGCACCATGAGTGTCTCTGTGCTGAGCCCCAGCAGACTCCTGGGTGATGTCTCTGGA
                                                  MetSerValSerValLeuSerProSerArgLeuLeuGlyAspValSerGly
       100        110        120        130        140        150        160        170        180
ATCCTCCAAGCGGCCTCCCTGCTCATTCTGCTTCTGCTGCTGATCAAGGCAGTTCAGCTCTACCTGCACAGGCAGTGGCTGCTCAAAGCC
IleLeuGlnAlaAlaSerLeuLeuIleLeuLeuLeuLeuIleLysAlaValGlnLeuTyrLeuHisArgGlnTrpLeuLeuLysAla
       190        200        210        220        230        240        250        260        270
CTCCAGCAGTTCCCGTGCCCTCCCTCCCACTGGCTCTTCGGGCACATCCAGGAGCTCCAACAGGACCAGGAGCTACAACGGATTCAGAAA
LeuGlnGlnPheProCysProProSerHisTrpLeuPheGlyHisIleGlnGluLeuGlnGlnAspGlnGluLeuGlnArgIleGlnLys
       280        290        300        310        320        330        340        350        360
TGGGTGGAGACATTCCCAAGTGCCTGTCCTCATTGGCTATGGGGAGGCAAAGTTCGTGTCCAGCTCTATGACCCTGACTATATGAAGGTG
TrpValGluThrPheProSerAlaCysProHisTrpLeuTrpGlyGlyLysValArgValGlnLeuTyrAspProAspTyrMetLysVal
       370        380        390        400        410        420        430        440        450
ATTCTGGGGAGATCAGACCCGAAATCCCATGGTTCCTACAGATTCCTGGCTCCATGGATTGGGTACGGCTTGCTCCTGTTGAATGGGCAG
IleLeuGlyArgSerAspProLysSerHisGlySerTyrArgPheLeuAlaProTrpIleGlyTyrGlyLeuLeuLeuAsnGlyGln
       460        470        480        490        500        510        520        530        540
ACATGGTTCCAGCATCGACGGATGCTGACCCCAGCCTTCCACTATGACATCCTGAAGCCCTATGTGGGGCTCATGGCAGACTCTGTACGA
ThrTrpPheGlnHisArgArgMetLeuThrProAlaPheHisTyrAspIleLeuLysProTyrValGlyLeuMetAlaAspSerValArg
       550        560        570        580        590        600        610        620        630
GTGATGCTGGACAAATGGGAAGAGCTCCTTGGCCAGGATTCCCCTCTGGAGGTCTTTCAGCACGTCTCCTTGATGACCCTGGACACCATC
ValMetLeuAspLysTrpGluGluLeuLeuGlyGlnAspSerProLeuGluValPheGlnHisValSerLeuMetThrLeuAspThrIle
       640        650        660        670        680        690        700        710        720
ATGAAGTGTGCCTTCAGCCATCAGGGCAGCATCCAGGTGGACAGGAATTCTCAGTCCTACATACAGGCCATTAGTGACCTGAACAACCTG
MetLysCysAlaPheSerHisGlnGlySerIleGlnValAspArgAsnSerGlnSerTyrIleGlnAlaIleSerAspLeuAsnAsnLeu
       730        740        750        760        770        780        790        800        810
GTTTTTTCCCGTGTGAGGAATGCCTTTCACCAGAATGACACCATCTACAGCCTGACCTCTGCTGGCCGCTGGACACACCGCGCCTGCCAG
ValPheSerArgValArgAsnAlaPheHisGlnAsnAspThrIleTyrSerLeuThrSerAlaGlyArgTrpThrHisArgAlaCysGln
       820        830        840        850        860        870        880        890        900
CTGGCCCATCAGCACACAGACCAAGTGATCCAACTGAGGAAGGCTCAACTACAGAAGGAGGGGGAGCTGGAGAAGATCAAGAGGAAGAGG
LeuAlaHisGlnHisThrAspGlnValIleGlnLeuArgLysAlaGlnLeuGlnLysGluGlyGluLeuGluLysIleLysArgLysArg
       910        920        930        940        950        960        970        980        990
CATTTGGATTTTCTGGATATCCTCCTCTTGGCCAAAATGGAGAATGGGAGCATCTTGTCAGACAAGGACCTCCGTGCTGAGGTGGACACG
HisLeuAspPheLeuAspIleLeuLeuLeuAlaLysMetGluAsnGlySerIleLeuSerAspLysAspLeuArgAlaGluValAspThr
      1000       1010       1020       1030       1040       1050       1060       1070       1080
TTCATGTTTGAGGGCCACGACACCACAGCCAGTGGGATCTCCTGGATCCTCTATGCTCTGGCCACACACCCCAAGCATCAGGAGAGGTGC
PheMetPheGluGlyHisAspThrThrAlaSerGlyIleSerTrpIleLeuTyrAlaLeuAlaThrHisProLysHisGlnGluArgCys
      1090       1100       1110       1120       1130       1140       1150       1160       1170
CGGGAGGAGATCCACAGCCTCCTGGGTGATGGAGCCTCCATCACCTGGAACCACCTGGACCAGATGCCCTACACCACCATGTGCATTAAG
ArgGluGluIleHisSerLeuLeuGlyAspGlyAlaSerIleThrTrpAsnHisLeuAspGlnMetProTyrThrThrMetCysIleLys
      1180       1190       1200       1210       1220       1230       1240       1250       1260
GAGGCACTGAGGCTCTACCCACCGGTGCCAGGCATTGGCAGAGAGCTCAGCACTCCCGTCACCTTCCCTGATGGGCGCTCCTTGCCCAAA
GluAlaLeuArgLeuTyrProProValProGlyIleGlyArgGluLeuSerThrProValThrPheProAspGlyArgSerLeuProLys
      1270       1280       1290       1300       1310       1320       1330       1340       1350
GGTATCATGGTCCTCCTCTCCATTTATGGCCTTCACCACAACCCAAAAGTGTGGCCCAACCCAGAGGTGTTTGACCCTTCCCGTTTTGCA
GlyIleMetValLeuLeuSerIleTyrGlyLeuHisHisAsnProLysValTrpProAsnProGluValPheAspProSerArgPheAla
      1360       1370       1380       1390       1400       1410       1420       1430       1440
CCGGGTTCTGCTCAACACAGCCACGCTTTCCTGCCCTTCTCAGGAGGATCAAGGAACTGCATCGGGAAACAATTTGCCATGAACGAGCTG
ProGlySerAlaGlnHisSerHisAlaPheLeuProPheSerGlyGlySerArgAsnCysIleGlyLysGlnPheAlaMetAsnGluLeu
      1450       1460       1470       1480       1490       1500       1510       1520       1530
AAGGTGGCCACGGCCCTGACCCTGCTCCGCTTTGAGCTGCTGCCTGATCCCACCAGGATCCCCATCCCCATTGCACGACTTGTGTTGAAA
LysValAlaThrAlaLeuThrLeuLeuArgPheGluLeuLeuProAspProThrArgIleProIleProIleAlaArgLeuValLeuLys
   AAAA*       1550       1560       1570       1580       1590       1600       1610       1620
TCCAAAAATGGAATCCACCTGCGTCTCAGGAGGCTCCCTAACCCTTGTGAAGACAAGGACCAGCTTTGAGGGCCTCCACCTGCCGTCCTG
SerLysAsnGlyIleHisLeuArgLeuArgArgLeuProAsnProCysGluAspLysAspGlnLeuEND
        MetGluSerThrCysValSerGlyGlySerLeuThrLeuValLysThrArgThrSerPheGluGlyLeuHisLeuProSerCys
      1630       1640       1650       1660       1670       1680       1690       1700       1710
TCTTCCTGACCCCCGCTTCTGTCCCCTTCCTGTCTGCCCATATCCTGTTTTCTGTCTGCCCACCTTCCCTTCTTCCCACCTGCCTGCTGT
 LeuProAspProArgPheCysProLeuProValCysProTyrProValPheCysLeuProThrPheProSerSerHisLeuProAlaVal
      1720       1730       1740       1750       1760       1770       1780       1790       1800
CCCCCAGTCTGCCTGCCCTTCTCTCTCTCACCTTTCTCCAGGGCTCCCTACCTGCTTGTCTACCTGTCTCCTACCCACCTGTATCTCTTG
 ProGlnSerAlaCysProSerLeuSerHisLeuSerProGlyLeuProThrCysLeuSerThrCysLeuLeuProThrCysIleSerCys
      1810       1820       1830       1840       1850       1860       1870       1880       1890
TTGGGAGAAAAGCTGAGTGTTGGGAGAAGCTGAGGCCGAGCTTGCATGTCTGACATAATGTAAAAGAGTCTTGAATCATGTCCAGGATCCA
 TrpGluLysSerEND
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Fig.1 cDNA and deduced amino acid sequences of human CYP4A11 and CYP4A11v. The differences between CYP4A11v and CYP4A11 are displayed above and below the latter nucleotide and amino acid sequences. The asterisk denotes the A deleted in CYP4A11v.

4.3.2 Spectral and catalytic properties of cDNA-expressed cytochrome P450s

CYP4A11 and CYP4A11v were expressed in Sf9 cells infected with recombinant baculoviruses. Expressed proteins were subjected to SDS-polyacrylamide gel electrophoresis and detected by immunoblotting with antibody against rat CYP4A2 (Fig. 2). We previously reported that this antibody cross-reacted with human renal cytochrome P450 and that human renal microsomes yielded an immnoreactive band having a similar mobility with rat CYP4A2 on SDS-polyacrylamide gel electrophoresis (6). This result agreed with that of a previous report comparing purified rat CYP4A2 with purified human CYP4A11 (6). As expected from the cDNA-deduced amino acid sequences, CYP4A11v had a lower mobility than CYP4A11.

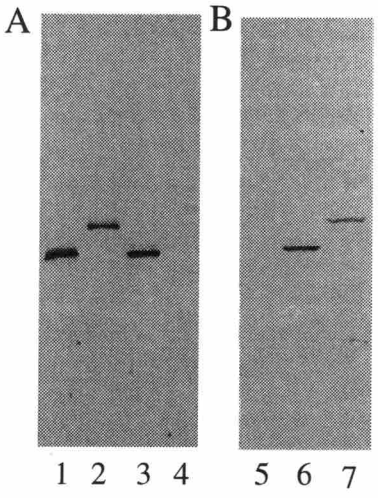

Fig. 2 Immunoblot of cDNA-expressed CYP4A11 and CYP4A11v in Sf9 cells with baculovirus (A) and COS cells (B). Cell lysate containing 10 μg of protein for Sf9 cells, 100 μg for COS cells, and 5 μg of rat renal microsomal protein were subjected to SDS-polyacrylamide gel electrophoresis. Proteins were electroblotted to nitocellulose membranes and were reacted with CYP4A2 antibody. Lanes 1, 2, 3, and 4 contain rat renal microsomes, CYP4A11v, CYP4A11, and cell lysate infected by wild-type baculovirus, respectively. Lanes 5, 6, and 7 contain cell lysate transfected by control vector, CYP4A11, and CYP4A11v, respectively.

CO-reduced absorption spectra revealed that CYP4A11 had a maximum absorbance at 452 nm (Fig. 3). CYP4A11v had only low absorption around 450 nm; almost all CYP4A11v absorbed around 420 nm. In the experiment described in Fig. 3, specific contents of expressed CYP4A11 and CYP4A11v were estimated at 0.091 and 0.014 nmol of P450/mg of cell lysate, respectively. The degree of the difference between expression of the variant and normal holoenzymes is about seven-fold; however, proteins detected by immunoblots differ in content by only about two-fold. These data suggest that CYP4A11v is synthesized but is unstable, reverting to P420, an enzyme having the thiolate of cysteine displaced from the 5 th position to the heme. P420 is generally enzymatically inactive.

Since rodent CYP4A forms tend to favor fatty acids as substrates, lauric acid was analyzed as a substrate for the expressed P450s (Table I). CYP4A11, produced in Sf9 cells, had lauric acid hydroxylation activity with turnover numbers of 10.11 and 0.99 nmol/min/nmol of P450. Catalytic properties of CYP4A11 agreed with those of cytochrome P450 purified from human renal microsomes (6), although its activity was a

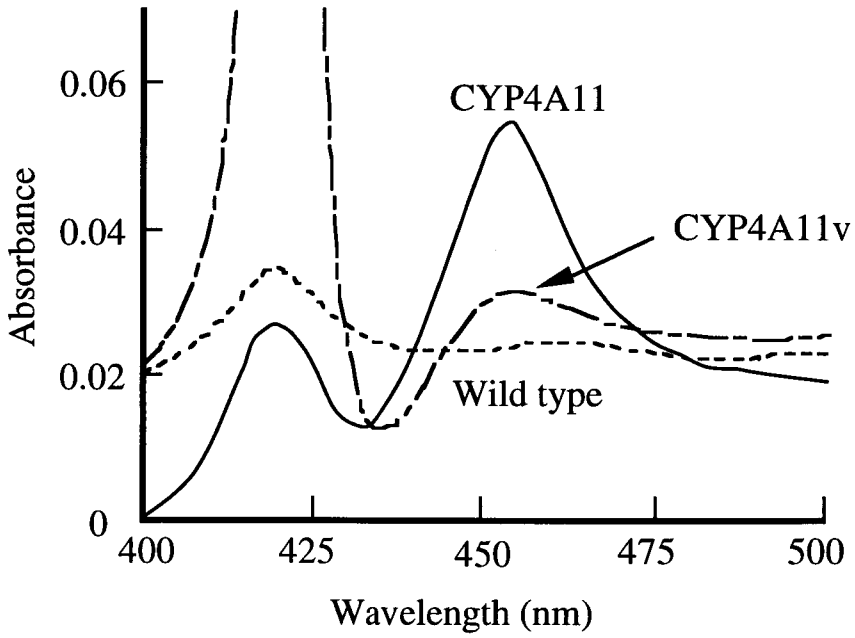

Fig.3 Spectra of cDNA-expressed CYP4A11 and CYP4A11v. Spectra were measured in 0.1 M sodium phosphate buffer, pH 7.4, containing 20% glycerol and 0.2% Emulgen 913 using 400 μg of cell lysate from Sf9 cells infected with a recombinant baculovirus having the CYP4A11 cDNA, or the CYP4A11v cDNA, and Sf9 cells infected with wild-type baculovirus.

Table I Lauric acid hydroxylation activity of cDNA-expressed CYP4A11 and CYP4A11v

	Sf9 cell				COS cell			
	CYP4A11		CYP4A11v		CYP4A11		CYP4A11v	
	$-fp_2$	$+fp_2$	$-fp_2$	$+fp_2$	$-fp_2$	$+fp_2$	$-fp_2$	$+fp_2$
ω–1	0.02 (0.22)	0.09 (0.99)	–	0.01 (0.71)	–	0.02	–	–
ω	0.18 (2.09)	0.92 (10.11)	–	–	0.02	0.09	–	–

Sf9 cells were infected with recombinant baculoviruses having CYP4A11 or CYP4A11v cDNAs. COS cells were transfected with CYP4A11or CYP4A11v in pCMV4 vector. Lauric acid hydroxylation activity was determined with cell lysate (500-800 μg) in the presence ($+fp_2$) and absence ($-fp_2$) of NADPH-cytochrome P450 oxidoreductase (fp_2, 100 pmol). Activities are expressed as nmol of product/min/mg of protein. Values in parentheses are the activities expressed as nmol of product/min/nmol of P450 measured photometrically. Results are the average of triple determinations with <10% variance between measurements. – , not detected (less than 0.01 nmol/min/mg).

little lower that that of purified CYP4A11. It may be due to the absence of cytochrome b_5 in assay system. Usually cytochrome P450s in CYP4 family require cytochrome b_5 to express sufficient activity. In previous study (6), addition of cytochrome b_5 to a reconstituted system including purified CYP4A11 increased the lauric acid

hydroxylation activity two-fold. In this study, cytochrome b_5 was added to assay system but it did not work; it may be due to the difference of lipid composition around cytochrome P450 from Sf9 cells (19). Also, NADPH-cytochrome P450 reductase was added to the reaction systems. Lower activities were obtained in the absence of NADPH-cytochrome P450 reductase as expected from an insufficient level of the endogenous reductase in Sf9 cells. CYP4A11v had low activity, although measurable (ω-1)-product was formed (table I). The activities of CYP4A11 towards arachidonic acid and prostaglandin E_1 were low (data not shown).

cDNA expression using the vector pCMV4 and COS cells further confirmed that CYP4A11 was an active enzyme whereas expressed CYP4A11v failed to metabolize lauric acid efficiently, even though it was synthesized and readily detectable by immunoblot analysis (Fig. 3 and Table I). This result rules out the possibility that the instability and low activity of the variant is due solely to the expression system used.

4.3.3 Investigation of the nature of the CYP4A11v

The possibility exists that CYP4A11v is the result of a cloning artifact produced during construction of the cDNA library. However, five independent cDNA clones of different length were sequenced and found to have the A deletion. This result suggests that the variant mRNA transcript was expressed in the mRNA preparation used to prepare the library. A PCR assay was developed for the A deletion in the CYP4A11v gene. Screening of DNAs from 9 Caucasians and 6 Asians (a total of 30 alleles) failed to reveal the presence of CYP4A11v. This result suggests that CYP4A11v is not produced from a gene that is distinct from CYP4A11 and was from a rare allelic variant.

4.4 DISCUSSION

CYP4A11 is probably the major cytochrome P450 in human kidney and may be the orthologous counterpart of rat CYP4A2. Indeed, purification studies revealed that most of the spectrally measured cytochrome P450 in human renal microsomes corresponded to a single cytochrome P450 form having an amino terminus almost identical CYP4A11 (6, 7). CYP4A11 appears to have properties similar to CYP4A2 including CO-reduced absorption maximum of 452 nm, lauric acid ω- and (ω-1)-hydroxylation activity, and a preference for production of the ω-hydroxy metabolite. CYP4A11v was unstable in the Sf9 cells but was able to carry out lauric acid (ω-1)-hydroxylation at a substrate turnover not too dissimilar to the normal enzyme. The variant cytochrome P450 form contains an extension of 72 residues at its carboxyl end, yielding the largest cytochrome P450 ever identified in eukaryotes (10). This additional sequence must render the enzyme an altered substrate recognition capacity in addition to a stability difference as compared to CYP4A11. Indeed, residues in the carboxy-terminal region of cytochrome P450s have been known to alter substrate specificity (20). It is unknown whether the variant cytochrome P450 is

unstable in the intact human kidney.

Fatty acids and eicosanoids play important roles in renal function, including regulating vasoconstriction and the Na^+-K^+-ATPase pump (21, 22). Thus, renal cytochrome P450s by their abilities to metabolize these compounds could modulate kidney physiology. It is noteworthy that CYP4A2 is expressed at high levels in kidneys of spontaneously hypertensive rats (23, 24). In the present study, we uncovered a mutation in a renal cytochrome P450 that might cause deficient or altered catalytic activity. The variant allele was not detected in DNA from 15 normal individuals in our laboratory (a total of 30 alleles), indicating that it may be rare. Unfortunately, tissue or DNA from the subject used prepare the library from which the cDNA was isolated is no longer available to determine more precisely the nature and consequence of the CYP4A11v allele. Although the precise physiological role of CYP4A enzymes have yet to be firmly established, it should be interesting to determine whether altered expression of CYP4A11 and, in particular, CYP4A11v is involved in human kidney diseases.

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PART II
THE CYP4B SUBFAMILY

CHAPTER 5
cDNA CLONING AND CHARACTERIZATION OF MOUSE Cyp4b1 INVOLVED IN MUTAGENIC ACTIVATION OF 3-METHOXY-4-AMINOAZOBENZENE

5.1 INTRODUCTION

3-methoxy-4-aminoazobenzene (3-MeO-AAB) is a potent procarcinogen that is metabolitically activated by cytochrome P450s (1) and causes hepatic tumors in rats (2). Its activation is efficiently catalyzed by CYP1A and 2B forms in rat liver as assessed by the *umu* gene expression assay which is an indirect measure of mutagenic activation by DNA damage (3). CYP1A forms are known to activate many carcinogens such as aromatic amines and polycyclic aromatic hydrocarbons and contribution of these forms to hepatic carcinoma has been reported (4). However, there are few reports concerning the metabolic activation of procarcinogens by cytochrome P450s in extrahepatic tissues such as kidney which has the highest content of cytochrome P450 next to the liver. Degawa *et al.* (5) found that mouse renal microsomes had high 3-MeO-AAB activation and that there exists a sex-difference in this activity. Renal microsomes of female mice have no activity but this activity is induced in kidneys of female mice by androgen treatment (5). P450 15α (a CYP2A form) and CYP2E1 are known to be present in renal microsomes of male mice and are induced in kidneys of female mice by androgen (6, 7). However, based on studies with chemical inducers and inhibitors of cytochrome P450s by Degawa *et al.* (5), it seems that the cytochrome P450 which activates 3-MeO-AAB is different from those cytochrome P450s. Thus, unique cytochrome P450 which bioactivate procarcinogens may be present in extrahepatic tissues.

In a preliminary study, antibodies against CYP1A1/2, 2A1, 2B1/2, 2C11, 2D1, 2E1, 3A2, and 4A2 failed to inhibit mutagenic activation of 3-MeO-AAB in the *umu* gene expression system with mouse renal microsomes and 3-MeO-AAB (8). These findings suggest the presence of a novel cytochrome P450 other than CYP1A or 2B forms that contributes to this activation in renal microsomes of male mice.

In the present study, we purified and characterized cytochrome P450s which activated 3-MeO-AAB from renal microsomes of male mice using an index of *umu* gene expression. A cDNA of a cytochrome P450 responsible for mutagenic activation of 3-MeO-AAB was cloned and found to correspond to be a new mouse cytochrome P450, CYP4B1.

5.2 MATERIALS AND METHODS

5.2.1 Chemicals

3-MeO-AAB was synthesized by the method described previously (9). 2-Acetylaminofluorene (2-AAF), 2-aminofluorene (2-AF), and 2-aminoanthracene (2-AA) were obtained from Sigma Chemical Co. (St. Louis, MO). 2-Amino-3,5-dimethylimidazo[4,5-f]quinoline (MeIQ) was the gift from Dr. M. Nagao of the National Cancer Center Research Institute (Tokyo). $NADP^+$, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase were obtained from Oriental Yeast Co. (Tokyo). Emulgen 911 was a gift from Kao Corp. (Tokyo). Cholic acid was obtained from Nacalai Tesque (Kyoto). All other chemicals and reagents were purchased from Wako Pure Chemical Industries (Osaka).

5.2.2 Purification of cytochrome P450 from renal microsomes of male mice

Male ddy mice 9 weeks of age were obtained from Kiwa Experimental Animals (Wakayama, Japan). The renal microsomes were prepared as described elsewhere (10). Cytochrome P450s were purified by use of a method previously reported (10). Renal microsomes containing 1.26 g of protein (specific content, 0.19 nmol/mg of protein) were solubilized with sodium cholate, fractionated with polyethylene glycol (7-15%), and then applied to an octylamino-Sepharose 4B column using the same method described for a purification of rat renal cytochrome P450 (10). The cytochrome P450 fraction eluted with 0.1 M potassium phosphate buffer (pH 7.2) containing 1 mM EDTA, 1 mM DTT, 20% glycerol, 0.5% sodium cholate, and 0.2% Emulgen 911 was further purified by anion-exchange HPLC with a DEAE-5PW column (7.6 x 100 mm, Tosoh Corp., Japan). Chromatography was done at a flow rate of 1 ml/min with a linear gradient of sodium acetate from 0 to 0.2 M for 30 min in 0.02 M Tris-acetate buffer (pH 7.2) containing 20% glycerol and 0.4% Emulgen 911. Mutagenic activation of 3-MeO-AAB for each fraction was assayed by *umu* gene expression. Three fractions which had high activity were further purified with hydroxylapatite HPLC with KB-column (7.6 x 100 mm, Koken, Tokyo). Chromatography was done at a flow rate of 0.7 ml/min with a linear gradient of sodium phosphate (pH 7.2) from 0.01 to 0.35 M. This phosphate buffer contained 20% glycerol, 0.2% sodium cholate, and 0.2% Emulgen 911. Purified cytochrome P450 (20-50 μg) was digested with cyanogen bromide (10% solution in formic acid: H_20, 80:20) at room temperature over night. The digested peptides were separated by HPLC with a reversed phase column (Ultrasphere, 2.0 x 150 mm, Beckman, Fullerton, CA). The column was developed at a flow rate of 0.5 ml/min with a linear gradient of acetonitrile from 0 to 70% in 0.1% TFA for 30 min at room temperature. The NH_2-terminal amino acid sequence of purified cytochrome P450 or BrCN-digested peptides were determined with a 477A peptide sequencer (Applied Biosystems, Foster City, CA).

5.2.3 Preparation of antibodies

Antibodies against rat CYP1A1, 1A2, 2A1, 2B1, 2C11, 2D1, 2E1, 3A2, 4A1, and 4A2 were prepared as described previously (11). Antibodies against cytochrome P450s purified from mouse renal microsomes were prepared by the same method. Purified renal cytochrome P450 (50 μg) in Freund's complete adjuvant (Calbiochem, San Diego, CA) was injected on the back of a female Japanese White rabbit obtained from Bioteck (Saga, Japan) and the rabbit was given boosters intravenously at 2, 5, and 9 week with 25 μg each of purified cytochrome P450 in saline.

5.2.4 Isolation of mouse kidney poly $(A)^+$RNA, synthesis and screening of the cDNA library

Poly $(A)^+$RNA was prepared from mouse kidney by using guanidinium thiosulfate and oligo(dT)-Sepharose (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden). An oligo (dT)-primed Uni-Zap cDNA library was synthesized using a Lambda-Zap cDNA synthesis kit (Stratagene, La Jolla, CA) following the manufacturer's instructions. For screening, the phage was plated, using XL-1 Blue *Escherichia coli* as host, at a density of $5x10^4$ plaque/plate. Ten plates were screened with the probe generated by polymerase chain reaction (PCR) using synthesized oligo probes of 5'-CCAGTACCATAATGACTTCA (rat CYP4B1 689-708) and 5'-TAGAGGCGGA AGCACTCCTT (rat CYP4B1 1113-1132). These oligo probes were selected from the rat CYP4B1 cDNA sequence which was conserved in CYP4B1 subfamily between rat and human (12, 13). The mouse cDNA library was amplified by 35 cycles using the annealing and extension temperature, 55 and 72 °C, respectively. The amplified products were purified by electrophoresis on a 1.0% agarose gel and labeled by a random primed cDNA labeling kit (Takara, Kyoto, Japan) with [^{32}P]dCTP. Nucleic acid hybridizations were done at 65 °C in 0.9 M NaCl and 0.09 M sodium citrate (pH 7.0), containing heat-denaturated salmon sperm DNA (50 μg/ml) and 0.5% SDS. Nitrocellulose membranes were washed with 0.3 M NaCl and 0.03 M sodium citrate (pH 7.0) containing 0.5% SDS. Ninety positive clones were obtained. The clones which had the largest inserts were isolated and rescued into pBluescript SR(+) (Stratagene). Plasmid DNA was purified for DNA sequencing by polyethylene glycol precipitation after lysis of cells with 0.2N NaOH and 1.0% SDS, following the manufacturer's instruction. The pBluescript cDNA insert (ca. 1.9 kb) was sequenced by a 373A DNA sequencer (ABI) with fluorescent label-tagged dideoxynucleotides and Taq polymerase. Sequencing was done by the plasmid walking method, using synthetic oligonucleotide primers.

5.2.5 Analytical and assay methods

Metabolic activation of 3-MeO-AAB, 2-AAF, 2-AF, 2-AA, and MeIQ in microsomes and in a reconstituted monooxygenase system was assayed with *umu* gene expression system by the method described elsewhere (3). The standard reaction mixture (final

volume 1.0 ml) containing 50 mM potassium phosphate buffer (pH 7.25), 10 μM substrate such as 3-MeO-AAB, NADPH-generating system (a mixture of 0.25 mM $NADP^+$, 5 mM glucose 6-phosphate, and 0.5 unit of glucose 6-phosphate dehydrogenase), suspension of *S. typhimurium* TA 1535/pSK 1002, and microsomes (20 - 100 μg). In a reconstituted system, the reaction mixture contained purified cytochrome P450 (2 pmol), NADPH-cytochrome P450 reductase (0.3 unit), cytochrome b_5 (4 pmol), and dilauroylphosphatidylcholine (5 μg) instead of microsomes. Incubation was at 37 °C for 120 min and *umu* gene expression was measured as the specific β-galactosidase activity per minute per nanomole of cytochrome P450.

Activities of lauric acid hydroxylation, testosterone hydroxylation, and aniline hydroxylation were assayed as described elsewhere (11). Cytochrome b_5 and NADPH-cytochrome P450 reductase were purified as described previously (11). Peptide mapping was done by the method reported previously (10).

5.3 RESULTS

5.3.1 Inhibition of 3-MeO-AAB activation by male mouse renal microsomes using chemical inhibitors and antibodies against rat cytochrome P450s

To characterize the cytochrome P450s which bioactivate 3-MeO-AAB, inhibition was done with chemical inhibitors and antibodies (Table I and II). As previously found by Degawa *et al* (5), α-naphthoflavone was an efficient inhibitor of *umu* gene expression

Table I Effects of inhibitor for *umu* gene expression of renal microsomes of male mice on the activation of 3-MeO-AAB

Inhibitor	Concentration (μM)	*umu* gene expression (*umu* units/min/mg protein)	
None (control)		265	(100 %)
α-Naphthoflavone	50	61	(23)
	10	156	(59)
Lauric acid	50	60	(23)
	10	158	(60)
Testosterone	50	204	(77)
	10	262	(99)
Quinine	50	271	(102)
	10	277	(105)
Metyrapone	50	242	(91)
	10	259	(98)

3-MeO-AAB (10 μM) was reacted with mouse renal microsomes in the presence of each inhibitor.

Table II Effects of antibodies against rat cytochrome P450s on the activation of 3-MeO-AAB in mouse renal microsomes

IgG		mg IgG / mg Ms	*umu* gene expression (% of control)
Anti-P450	1A1	0.5	127
		1.0	118
	1A2	0.5	106
		1.0	110
	2A1	0.5	120
		1.0	136
	2B1	0.5	143
		1.0	86
	2C11	0.5	110
		1.0	92
	2D1	0.5	103
		1.0	82
	2E1	0.5	100
		1.0	118
	3A2	0.5	109
		1.0	83
	4A2	0.5	109
		1.0	104

3-MeO-AAB (10 μM) was reacted with mouse renal microsomes (20 μg) in the presence of each antibody.

induced by 3-MeO-AAB in renal microsomes of male mice. Lauric acid was also an effective inhibitor. In contrast, testosterone, quinine, and metyrapone had little effect on the activation. Antibodies against rat CYP1A1, 1A2, 2B1, 2C11, 2D1, 2E1, 3A2, and 4A2 had little effect on 3-MeO-AAB bioactivation. α-Naphthoflavone is an effective inhibitor for CYP1A2 and lauric acid is a substrate for cytochrome P450s in CYP4A subfamily. However, antibodies against these cytochrome P450s did not inhibit activity. These results suggest that a cytochrome P450 belonging to another subfamily contributes bioactivation of 3-MeO-AAB in renal microsomes of male mice.

5.3.2 Purification of cytochrome P450s from renal microsomes of male mice

The inhibition study suggested that a novel cytochrome P450 contributes mutagenic activation of 3-MeO-AAB in mouse kidney, the cytochrome P450 having this activity was directly purified. Renal microsomes of male mice was fractionated with polyethylene glycol (PEG) and 7-15% fraction of PEG was applied to an octylamino-Sepharose 4B column. About 40% of cytochrome P450 was recovered from microsomes and the specific content of cytochrome P450 increased 10-fold in this step (Table III). The fraction eluted from the octylamino-Sepharose column was applied to an anion-exchange HPLC with a DEAE-5PW column and separated into

Table III Purification of cytochrome P450s from renal microsomes of male mice

	P450 (nmol)	Protein (mg)	Specific content (nmol/mg)	Recovery of P450 (%)
Microsomes	238	1255	0.19	100
PEG	165	225	0.73	69.3
OA-Sepharose	103	44.1	2.34	43.2
DEAE-5PW HPLC				
PT	34.7	11.4	3.04	14.6
MK-2	10.7	2.65	4.04	4.5
MK-3	6.17	2.40	2.57	2.6
Hydroxylapatite HPLC				
MK-1	9.56	0.84	11.4	4.0
MK-2	5.19	0.39	13.2	2.2
MK-3	3.35	0.25	13.6	1.4

PEG, polyethylene glycol fractionation (7-15%); OA, octylamino;
PT, pass-through fraction.

four peaks (Fig. 1A). The first peak was the non-adsorbed fraction (PT), which contained the highest amount of cytochrome P450 and the last small peak contained mainly cytochrome b_5. The second and third peaks also contained cytochrome P450 and were designated MK-2 and MK-3, respectively. *umu* Gene expression induced by 3-MeO-AAB was assayed for each fraction and the first three peaks had highest activity. PT-fraction was further applied to hydroxylapatite HPLC and separated to one major and some small peaks (Fig. 1B). The Major peak contained cytochrome P450 and was designated MK-1. MK-2 and MK-3 fractions were also purified by hydroxylapatite HPLC. Purified P450 MK-1, MK-2, and MK-3 had specific contents of 11-13 nmol/mg and total recovery of these forms from renal microsomes were 4.0, 2.2, and 1.4%, respectively (Table III). These forms gave a single protein-staining band on SDS-polyacrylamide gel electrophoresis (Fig 2). The apparent molecular weight of these forms was identical and calculated to 54,000.

5.3.3 Characterization of purified renal cytochrome P450s

Catalytic properties of purified cytochrome P450s were investigated (Table IV). These forms metabolized lauric acid efficiently but not testosterone and aniline, suggesting these forms were different from P450 15α or CYP2E1 which are male-dominant form in mouse kidney (6, 7). These results explained that lauric acid efficiently inhibited 3-MeO-AAB bioactivation in renal microsomes. NH_2-terminal sequence of these forms was analyzed (Fig. 3). NH_2-terminal sequences of MK-1, MK-2, and MK-3 were identical through 16 amino acid residues. In addition, trypsin proteolytic digestion pattern of these forms was also similar (Fig. 4), suggesting that they are closely related

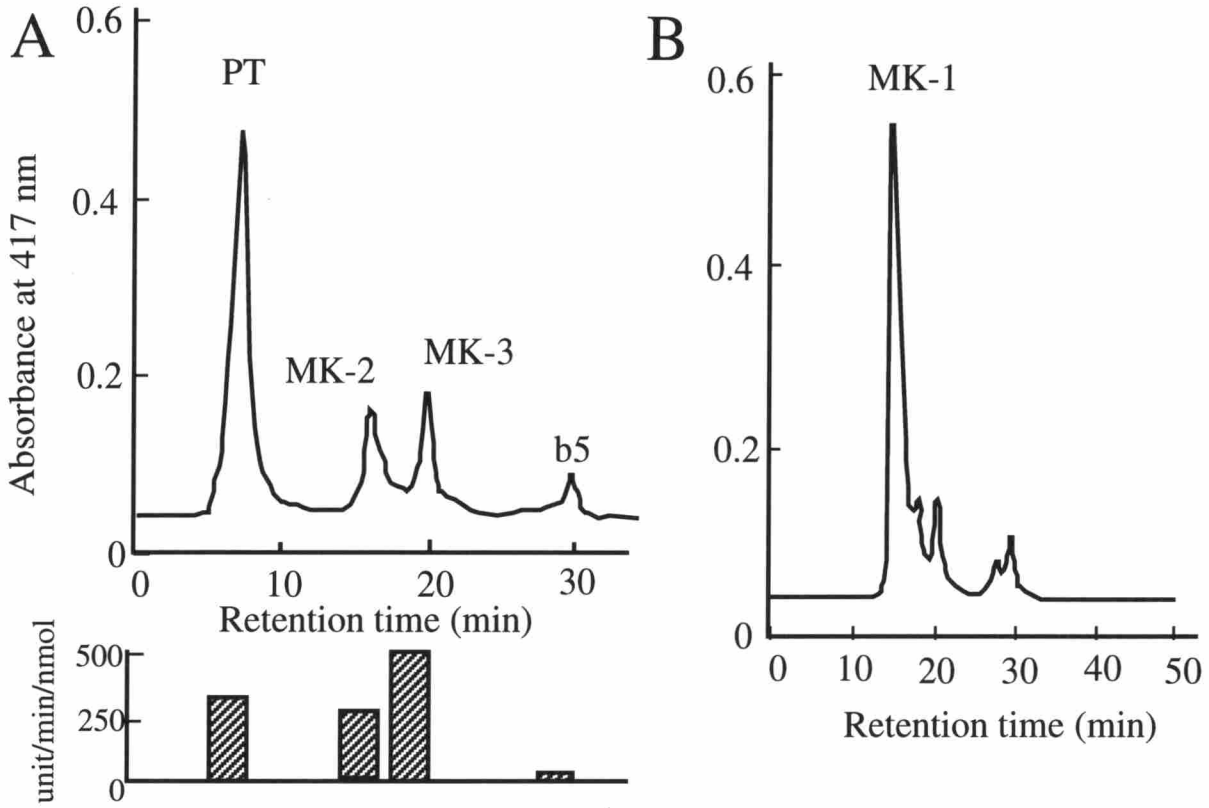

Fig. 1 Chromatographic profiles of cytochrome P450 in mouse renal microsomes. Renal microsomes of male mice were solubilized, fractionated with polyethylene glycol, and applied onto an octylamino-Sepharose 4B column as described in Materials and methods. The eluted fraction was applied onto HPLC with a DEAE-5PW anion-exchange column (A) and the pass-through fraction (PT) from this column was further purified with hydroxylapatite (KB-column) HPLC (B). A: Chromatography was done at a flow rate of 1 ml/min with a linear gradient of sodium acetate from 0 to 0.2 M over 30 min in 0.02 M Tris-acetate buffer (pH 7.2) containing 0.4% Emulgen 911 and 20% glycerol. Each fraction was collected and activation of 3-MeO-AAB by these fractions was assayed with *umu* gene expression. The activity is shown under the chromatogram A. B: Chromatography was done at a flow rate of 0.7 ml/min with a linear gradient of sodium phosphate buffer (pH 7.2) from 0.01 to 0.35 M over 50 min. This buffer contained 0.2% Emulgen 911, 0.2% sodium cholate, and 20% glycerol.

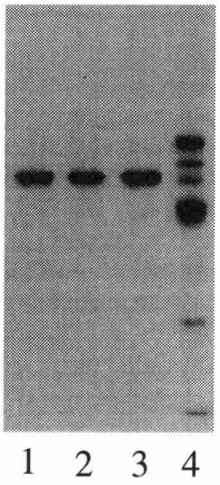

Fig. 2 SDS-polyacrylamide gel electrophoresis of purified cytochrome P450. Electrophoresis was done with 10% acrylamide gel. Lanes 1, 2, and 3 contained 0.5 μg of P450 MK-1, MK-2, and MK-3, respectively. Lane 4 contained standard proteins: bovine serum albumin (68,000), catalase (57,000), glutamate dehydrogenase (53,000), ovalbumin (45,000), and chymotrypsinogen (25,000).

Table IV Monooxygenase activity of P450, MK-1, MK-2 and MK-3, purified from mouse renal microsomes

Substrate	MK-1	MK-2	MK-3
Aniline	—	—	—
Lauric acid			
ω	1.75	2.63	1.92
ω–1	1.01	1.52	1.10
Testosterone			
2α	—	—	—
2β	—	—	—
6β	—	—	—
15α	—	—	—
16α	—	—	—
16β	—	—	—

Purified cytochrome P450s (30 pmol) were reacted with aniline (4 mM), lauric acid (0.2 mM), and testosterone (1 mM). Values are expressed as nmol products/min/nmol of P450. ω, ω–1, 2α, 2β, 6β, 15α, 16α, and 16β indicate hydroxylation site of each substrate. — : < 0.01

P450	1	5	10	15
MK-1	Ala-Leu-Ser-Phe-Leu-Ser-Pro-Ser-Leu-Ser-Arg-Leu-Gly-Leu-Trp-Ala			
MK-2	Ala-Leu-Ser-Phe-Leu-Ser-Pro-Ser-Leu-Ser-Arg-Leu-Gly-Leu-Trp-Ala			
MK-3	Ala-Leu-Ser-Phe-Leu-Ser-Pro-Ser-Leu-Ser-Arg-Leu-Gly-Leu-Trp-Ala			
Rat 4B1	Met-Val-Leu-Asn-Phe-Leu-Ser-Pro-Ser-Leu-Ser-Arg-Leu-Gly-Leu-Trp-Ala			

Fig. 3 NH_2-terminal amino acid sequence of purified cytochrome P450.
Purified cytochrome P450 (200-300 pmol) was used for NH_2-terminal amino acid sequence analysis. Rat CYP4B1 was purified from rat pulmonary microsomes previously (18).

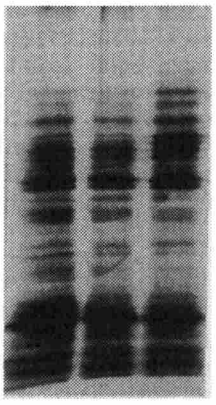

Fig. 4 Peptide mapping of purified cytochrome P450. Purified cytochrome P450s (about 5 μg per lane) were digested with trypsin (1 μg) at 37 °C for 15 min and put on an SDS-polyacrylamide gel (15% acrylamide). The gel was stained with silver staining. Lanes 1, 2, and, 3 contained digested MK-1, MK-2, and MK-3, respectively.

cytochrome P450. Further, their NH_2-terminal sequences were very similar with that of rat CYP4B1; only the first and third residue of mouse cytochrome P450 are different from those of rat CYP4B1 through 16 amino acid residues although methionine in mouse cytochrome P450 appears to have been lost by proteolytic digestion. Taken together, these results suggest that these mouse cytochrome P450s belong to CYP4B subfamily.

5.3.4 Activation of procarcinogen by mouse renal microsomes and purified renal cytochrome P450

Metabolic activation of some procarcinogens was assayed with *umu* gene expression (Table V). Renal microsomes of male mice efficiently bioactivated 2-AF which is known to be activated by CYP4B1 in rat and rabbit (14, 15). 3-MeO-AAB and 2-AAF were also activated by renal microsomes of male mice. These three procarcinogens were activated by male mice more efficiently than by female mice. In contrast, 2-AA was activated by both male and female mice. MeIQ which is known to be activated by CYP1A2 (4) was not efficiently activated by renal microsomes. Purified MK-1, MK-2, and MK-3 had high activity toward 3-MeO-AAB and also 2-AF and 2-AA.

Table V Activations of procarcinogens to genotoxic metabolites by renal microsomes and purified renal cytochrome P450s

	3-MeO-AAB	2-AF	2-AAF	2-AA	MeIQ
Renal microsomes		units/min/mg protein			
Male mouse	281±21	3896±554	210±17	172±2	2±1
Female mouse	72±3	497±37	13±2	224±24	8±2
Purified P450		units/min/nmol P450			
MK-1	802	4568	71	1490	129
MK-2	641	7264	24	1660	96
MK-3	812	3891	5	1457	105

Renal microsomes (20 μg) or purified cytochrome P450s (2 pmol) were reacted with each substrate (10 μM). Activities of renal microsomes are expressed as means ± SD of five different animals. 3-MeO-AAB, 3-methoxy-4-aminoazobenzene; 2-AF, 2-aminofluorene; 2-AAF, 2-acetylaminofluorene; 2-AA, 2-aminoanthracene; MeIQ, 2-amino-3,5-dimethylimidazo[4,5-f]quinoline.

5.3.5 Effects of antibody against mouse renal cytochrome P450 on 3-MeO-AAB bioactivation of mouse renal microsomes

Antibodies against MK-1, MK-2, and MK-3 were prepared. These antibodies did not cross-reacted with rat CYP1A1, 1A2, 2B1, 2C11, 2D1, 2E1, 3A2, and 4A2 but

cross-reacted with MK-1, MK-2, and MK-3 each other (data not shown). Renal, pulmonary, and hepatic microsomes of male and female mice were analyzed with immunoblotting (Fig. 5). Renal microsomes of male mice had the highest content of MK-1, MK-2, and MK-3. Pulmonary microsomes of both male and female mice also had these forms but hepatic microsomes did not. To assess the role of mouse renal cytochrome P450s in 3-MeO-AAB activation, these antibodies were added to an assay system with *umu* gene expression of male renal microsomes toward 3-MeO-AAB (Fig. 6). The antibody efficiently inhibited 3-MeO-AAB bioactivation of renal microsomes of male mice, suggesting that MK-1, MK-2, and MK-3 play a major role in bioactivation of 3-MeO-AAB in mouse renal microsomes. The antibody also inhibited bioactivation of 2-AF and 2-AA in mouse renal microsomes.

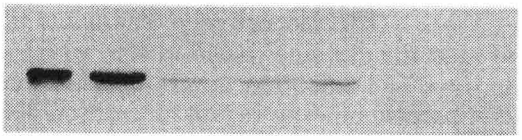

Fig. 5 Immunoblots of renal, pulmonary, and hepatic microsomes of male and female mice. Lane 1 contains purified P450 MK-1 (0.7 pmol). Lanes 2, 4, and 6 contain renal, pulmonary, and hepatic microsomes of male mice (20 μg), respectively. Lanes 3, 5, and 7 contain renal, pulmonary, and hepatic microsomes of female mice (20 μg), respectively. Electrophoresis was done with 7.5% polyacrylamide gel and proteins were transferred to a nitrocellulose membrane. Proteins were stained with anti-MK-1 antibody which reacted with P450 MK-2 and MK-3 as well as P450 MK-1.

5.3.6 Sequencing of mouse renal cytochrome P450 cDNA

Results of NH_2-terminal amino acid sequence suggested that the mouse renal cytochrome P450s purified in this study may belong to CYP4B subfamily. Therefore, oligonucleotides for PCR were selected from the rat CYP4B1 cDNA sequence of the conserved region in CYP4B subfamily and used to prepare a probe for cDNA library screening. Mouse renal cDNA library was screened with this 0.4 Kb probe and six clones having the largest inserts were isolated and sequenced (Fig. 7). Six clones obtained in this study yielded identical nucleotide sequence. The cDNA isolated contained an open reading frame of 511 amino acids encoding a protein having a calculated molecular weight of 58,854 daltons. The cDNA deduced amino acid sequence of this cytochrome P450 shared 90%, 86%, and 84% identity with rat (12), rabbit (12), and human CYP4B1 (13), respectively. The heme binding region around the cysteine (position 453) which is conserved in the CYP4 and other families of this hemoprotein and substrate binding region at position 305-320 which is conserved in

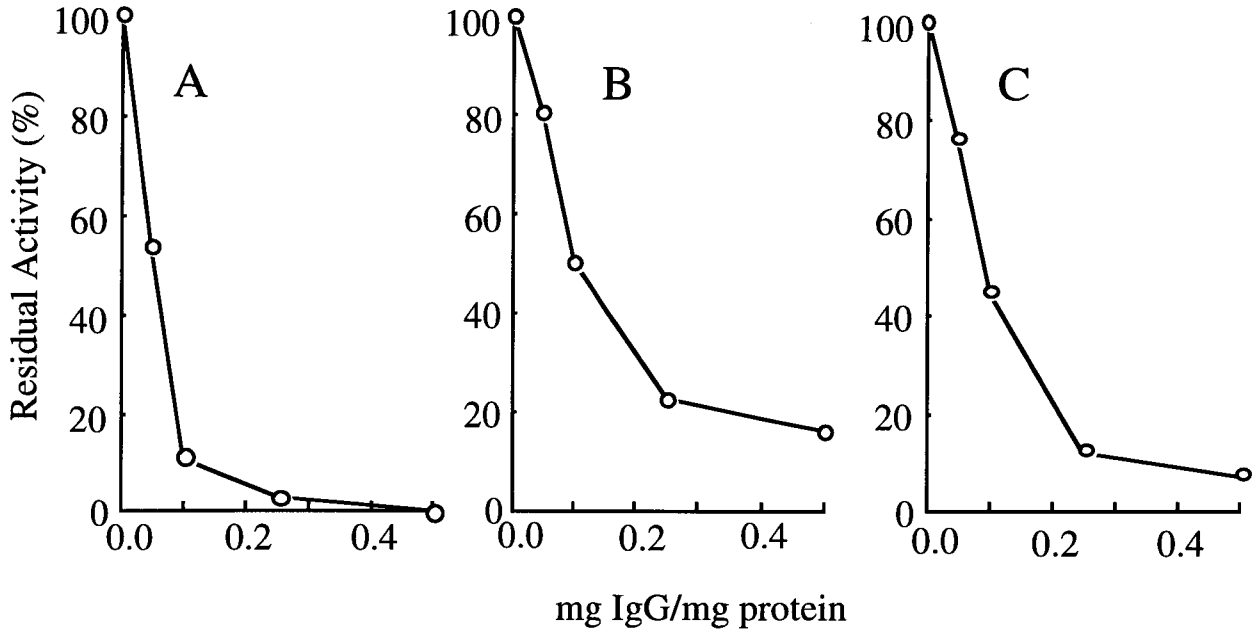

Fig. 6 Effects of anti-MK-1 antibody on the bioactivation of 3-methoxy-4-aminoazobenzene (A), 2-aminofluorene (B) and 2-aminoanthracene (C) with *umu* gene expression. The catalytic activity in the presence of control immunoglobulin G (IgG) was set at 100%.

the CYP4A and CYP4B subfamilies were conserved. cDNA-deduced amino acid sequences agreed with the NH_2-terminal amino acid sequence and amino acid sequences of peptides from cyanogen bromide digestion of purified mouse renal P450 MK-1. Based on these results, renal cytochrome P450 purified in this study is a mouse CYP4B1. We obtained three forms of P450 MK-1, MK-2, and MK-3 from mouse renal microsomes but only single cDNA was obtained in this study. NH_2-terminal amino acid sequences of these forms were identical and peptide digestive pattern and partial amino acid sequence of these peptides were also identical. The reason that these migrated differently during purification by HPLC is currently not clear but may be due to modification of cytochrome P450 protein by processing or due to different aggregation levels on HPLC.

5.4 DISCUSSION

We found that a CYP4B form plays a major role in mutagenic activation of 3-MeO-AAB in mouse renal microsomes. Data from cDNA cloning indicate that cytochrome P450s purified from mouse renal microsomes is mouse Cyp4b1. In extrahepatic tissue, this form may contribute to bioactivation of procarcinogens. In renal microsomes of male mice, the Cyp4b1 was a major cytochrome P450. A sex-difference in renal content of this form was found accounts for differences in bioactivation of 3-MeO-AAB. The CYP4B1 was purified first from rabbit pulmonary microsomes by Guengerich (16) and by Philpot and coworkers (17). We also purified CYP4B1 from rat pulmonary microsomes (18). cDNAs coding these cytochrome P450s

```
        10         20         30         40         50         60         70         80         90
GGTCCTGCGAGTTTAGCCATGGCGCTCAGCTTTCTCTCTCCAAGCCTTTCCCGCCTCGGCCTGTGGGCTTCTGTAGTGATCCTGATGGTA
                  MetAlaLeuSerPheLeuSerProSerLeuSerArgLeuGlyLeuTrpAlaSerValValIleLeuMetVal
       100        110        120        130        140        150        160        170        180
ACCGTCCTGAAGCTCCTCAGCCTGCTGTTTCGGAGGCAGAAGCTGGCCAGGGCTTTGGACAGCTTCCCAGGGCCCCCCAAGCACTGGCTT
ThrValLeuLysLeuLeuSerLeuLeuPheArgArgGlnLysLeuAlaArgAlaLeuAspSerPheProGlyProProLysHisTrpLeu
       190        200        210        220        230        240        250        260        270
TTTGGTCATGCCCTTGAGATCCAGAAGACAGGAGGCCTGGACAAGGTGGTAACTTGGACCGAACAGTTCCCCTATGCCCACCCACTCTGG
PheGlyHisAlaLeuGluIleGlnLysThrGlyGlyLeuAspLysValValThrTrpThrGluGlnPheProTyrAlaHisProLeuTrp
       280        290        300        310        320        330        340        350        360
CTTGGACAATTTATTGTTTTCCTGAACATCTATGAGCCTGACTATGCTAAAGCTGTATACAGCCGAGGGGACCCGAAGGCTGCATATGTG
LeuGlyGlnPheIleValPheLeuAsnIleTyrGluProAspTyrAlaLysAlaValTyrSerArgGlyAspProLysAlaAlaTyrVal
       370        380        390        400        410        420        430        440        450
TATGACTTCTTCCTCCAGTGGATCGGAAAAGGCCTACTGGTTCTGGAAGGGCCAAAATGGTTCCAGCACCGCAAGCTGCTCACACCTGGC
TyrAspPhePheLeuGlnTrpIleGlyLysGlyLeuLeuValLeuGluGlyProLysTrpPheGlnHisArgLysLeuLeuThrProGly
       460        470        480        490        500        510        520        530        540
TTCCATTATGATGTGCTGAAGCCCTATGTGGCCATATTTGCTGAGTCCACACGGGTGATGCTGGACAAGTGGGAGAAAAAGGCTAGTGAG
PheHisTyrAspValLeuLysProTyrValAlaIlePheAlaGluSerThrArgValMetLeuAspLysTrpGluLysLysAlaSerGlu
       550        560        570        580        590        600        610        620        630
AATAAGAGCTTTGACATCTTCTGTGACGTGGGCCACATGGCACTGGACACCCTCATGAAGTGCACCTTTGGCAAAGGAGACAGCGGCCTA
AsnLysSerPheAspIlePheCysAspValGlyHisMetAlaLeuAspThrLeuMetLysCysThrPheGlyLysGlyAspSerGlyLeu
       640        650        660        670        680        690        700        710        720
AGCCACAGTGACAACAGCTACTACCTGGCAGTTAGTGACCTCACACTGCTGATGCAGCAGCGCATCGACTCCTTCCAGTACCATAATGAC
SerHisSerAspAsnSerTyrTyrLeuAlaValSerAspLeuThrLeuLeuMetGlnGlnArgIleAspSerPheGlnTyrHisAsnAsp
       730        740        750        760        770        780        790        800        810
TTCATTTACTGGCTCACACCACATGGCCGCCGTTTCTTGCGGGCCTGCCAGATAGCCCATGACCATACAGATCATGTCATCAGGCAGCGG
PheIleTyrTrpLeuThrProHisGlyArgArgPheLeuArgAlaCysGlnIleAlaHisAspHisThrAspHisValIleArgGlnArg
       820        830        840        850        860        870        880        890        900
AAGGCAGCTCTGCAGGATGAGAAGGAGCAGAAAAAGCTTCAGGAGCGGAGACACCTGGACTTCCTCGACATTCTCCTGGGTGCCCGGGAT
LysAlaAlaLeuGlnAspGluLysGluGlnLysLysLeuGlnGluArgArgHisLeuAspPheLeuAspIleLeuLeuGlyAlaArgAsp
       910        920        930        940        950        960        970        980        990
GAAAGTGGGATCAAGTTGTCAGATGCAGACCTCCGGGCTGAAGTGGACACATTCATGTTCGAAGGCCACGACACCACCACTAGTGGTATC
GluSerGlyIleLysLeuSerAspAlaAspLeuArgAlaGluValAspThrPheMetPheGluGlyHisAspThrThrThrSerGlyIle
      1000       1010       1020       1030       1040       1050       1060       1070       1080
TCTTGGTTTCTCTACTGCATGGCCCTTTATCCTATGCACCAGCAGCGATGTAGGGAGGAGGTCCGTGAGATCCTAGGGGACCGGGACTCC
SerTrpPheLeuTyrCysMetAlaLeuTyrProMetHisGlnGlnArgCysArgGluGluValArgGluIleLeuGlyAspArgAspSer
      1090       1100       1110       1120       1130       1140       1150       1160       1170
TTCCAGTGGGATGATCTGGCCCAGATGACCTACCTGACCATGTGCATGAAGGAGTGCTTCCGCCTCTACCCACCTGTACCCCAAGTGTAC
PheGlnTrpAspAspLeuAlaGlnMetThrTyrLeuThrMetCysMetLysGluCysPheArgLeuTyrProProValProGlnValTyr
      1180       1190       1200       1210       1220       1230       1240       1250       1260
CGCCAGCTCAGCAAGCCAGTAACCTTTGTGGATGGCCGCTCTCTACCTGCAGGCAGCCTGATCTCTCTGCACATCTATGCCCTCCATCGG
ArgGlnLeuSerLysProValThrPheValAspGlyArgSerLeuProAlaGlySerLeuIleSerLeuHisIleTyrAlaLeuHisArg
      1270       1280       1290       1300       1310       1320       1330       1340       1350
AACAGTGCTGTGTGGCCTGACCCAGAGGTCTTTGACCCACTGCGCTTTTCTCCTGAGAATATGACAGGACGGCATCCCTTTGCCTTCATG
AsnSerAlaValTrpProAspProGluValPheAspProLeuArgPheSerProGluAsnMetThrGlyArgHisProPheAlaPheMet
      1360       1370       1380       1390       1400       1410       1420       1430       1440
CCTTTTTCTGCAGGGCCCAGGAATTGCATTGGGCAACAGTTTGCCATGAACGAGATGAAGGTGGTCACAGCCCTCTGTTTGCTGCGCTTT
ProPheSerAlaGlyProArgAsnCysIleGlyGlnGlnPheAlaMetAsnGluMetLysValValThrAlaLeuCysLeuLeuArgPhe
      1450       1460       1470       1480       1490       1500       1510       1520       1530
GAATTCTCTCCAGATCCCTCAAAGATCCCCATTAAGGTCCCCCAGCTGATCTTGCGCTCCAAAAATGGCATCCACCTCTACCTGAAGCCA
GluPheSerProAspProSerLysIleProIleLysValProGlnLeuIleLeuArgSerLysAsnGlyIleHisLeuTyrLeuLysPro
      1540       1550       1560       1570       1580       1590       1600       1610       1620
CTGGGCCCTGGGTCTGGAAAGTAGGTCTTAGGAGAGCAAGGATATGGAGTCATTGTGGATCCCTGCCTGTGGGGGGTTTGTGAGATAAAA
LeuGlyProGlySerGlyLysEND
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Fig. 7 cDNA and deduced amino acid sequences of mouse renal cytochrome P450. The amino acid residues underlined agreed with NH_2-terminal amino acid sequence of P450 MK-1 or amino acid sequences of BrCN-digested peptides from P450 MK-1.

of rat and rabbit have been isolated and sequenced (12). Rat CYP4B1 had similar catalytic properties with mouse Cyp4b1; rat CYP4B1 also had high bioactivation of 3-MeO-AAB (data not shown). CYP4B1 is present in lung and a major form in pulmonary microsomes in rabbits and rats (12, 17, 18). Philpot and coworkers found that rabbit CYP4B1 can activate 2-AF and 4-ipomeanol (14, 19). In this study, we demonstrated that Cyp4b1 in mice exhibits high activation toward 3-MeO-AAB as well as 2-AF. CYP4B form was a major cytochrome P450 in renal microsomes of male mice as well as in pulmonary microsomes. However, this form was not in rat renal microsomes, suggesting an important species difference in tissue-specific expression of CYP4B1 (data not shown). Mouse Cyp4b1 was present in renal microsomes of male and in pulmonary microsomes of male and female. These results are consistent with the previous findings that 4-ipomeanol is activated by rabbit CYP4B1 and 4-ipomeanol activation presents in both mouse renal and pulmonary microsomes with rabbit CYP4B1 antibody (19, 20). They also found that this activation exists in rat pulmonary microsomes but not in hepatic and renal microsomes (20). Rat hepatic microsomes have activity toward 3-MeO-AAB but different cytochrome P450s contribute this reaction (3). Recently human CYP4B1 cDNA was cloned (21, 23). Gonzalez and co-workers expressed human CYP4B1 with human cells (21). Unlike in CYP4B1 in other species such as rabbit, human CYP4B1 had no activity toward 2-AF and 4-ipomeanol (21). Thus the human counterpart has different catalytic properties from that found in other species. Philpot *et al.* (15, 22) reported that rabbit CYP4B1 is in bladder and it bioactivates dibutylnitrosoamine to mutagenic products and suggested the possibility of contribution of CYP4B1 to bladder carcinoma. CYP1A forms can activate many procarcinogens and contribution of these forms to carcinogenesis is extensively studied (4). However, this study shows that not only CYP1A forms but also CYP4B form may contribute to carcinogenesis especially in extrahepatic organs.

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CHAPTER 6
MUTAGENIC ACTIVATION OF URINARY BLADDER CARCINOGENS BY CYP4B1 AND THE PRESENCE OF CYP4B1 IN BLADDER MUCOSA

6.1 INTRODUCTION

Primary aromatic amines such as benzidine and 2-naphthylamine have been identified as human bladder carcinogens and also produce bladder cancer in animals (1, 2). Carcinogenic aromatic amines are thought to require activation to electrophilic species before exerting carcinogenic effects (3). Benzidine has been extensively studied and several different metabolic pathways involving N-oxidation leading to DNA-binding have been described (4). After benzidine is N-acetylated, it can then be N'-hydroxylated and this appears to be the major activation pathway in humans (5-7). On the contrary, biphenyl amine which is also a bladder carcinogen is inactivated by N-acetylation (8). Benzidine is strongly activated by prostaglandin H synthetase and it is suggested that this enzyme is involved in initiation of bladder cancer by benzidine (9). 3,3'-dichlorobenzidine (DCB) is one of benzidine derivative and also a potent bladder carcinogen in rat and human although benzidine is a weak carcinogen for rat bladder (10). DCB is not activated by prostaglandin H synthetase and DCB as well as acetylated DCB has high mutagenic activity unlike benzidine (10). Activation pathway of DCB may be different from that of benzidine. Iba suggested that contribution of cytochrome P450 to mutagenic activation of DCB (10).

Several carcinogenic aromatic amines are metabolized to toxic and carcinogenic compounds by cytochrome P450s (11). The liver has the highest content of cytochrome P450 and most studies of aromatic amine metabolism have used the liver as the target (12). Little information is available regarding the isoforms of cytochrome P450 in extrahepatic organs or tissues. Studies of aromatic amine activation in target tissues have demonstrated that bovine bladder mucosa microsomes contain cytochrome P450 and mediate the N-hydroxylation of 4-aminobiphenyl (13). Vanderslice *et al.* (14) have found that a cytochrome P450 in rabbit bladder mucosa activates 2-aminofluorene to a mutagenic product. These findings suggest that cytochrome P450s contribute to the bladder carcinogenesis by chemical carcinogens. However, there are little data for metabolism of biphenyl amine or benzidine derivatives by cytochrome P450s.

In this study, we investigated the mutagenic activation of the aromatic amine bladder carcinogens, 2-naphthylamine (2-NA), 3,2'-dimethyl-4-aminobiphenyl (DMAB), and DCB by cytochrome P450s using the *umu* test that detects DNA damage. 2-NA is a potent bladder carcinogen in humans but weak in rodents (2). DMAB and DCB induce bladder carcinoma in the rat (10, 15-17). Furthermore, the presence and localization of CYP4B1 in rat bladder were investigated with immunostaining and

Northern blotting to evaluate the role of CYP4B1 in the induction of bladder cancer.

6.2 MATERIALS AND METHODS

6.2.1 Chemicals

Salmonella typhimurium NM2009 for *umu* test was gift from Dr. T. Shimada of Osaka Prefectural Institute of Public Health. DCB were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). 3-MeO-AAB was synthesized by the method described previously (18). 2-NA, DMAB, $NADP^+$, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, dilauroylphosphatidylcholine (DLPC) were obtained from Sigma Chemical Co. (St. Louis, MO). Purities of 3-MeO-AAB, 2-NA, DMAB, and DCB were more than 95%. Other chemicals and reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan).

6.2.2 Animals and treatment

Male F344 rats (6 weeks old) were obtained from Charles River (Shiga, Japan) and housed in an air-conditioned room at 23 ± 1 °C under a relative humidity of 36 ± 6% and with a 12 h light-12 h dark cycle. Rat bladders were fixed in Carnoy's fixative, embedded in paraffin, sectioned at 4 mm, and stained immunohistochemically to investigate the localization of CYP4B1.

6.2.3 Purification of cytochrome P450 and preparation of antibody

Male BALB/c mice (7 weeks old) were obtained from Japan Clea (Tokyo). Kidney and urinary bladder microsomes of rats and mice were prepared as described (19). CYP1A1, 1A2, 2A1, 2B1, 2C11, 2D1, 2E1, 3A2, 4A2, and 4B1 were purified from hepatic, renal, and pulmonary microsomes of male rats and characterized previously (19-22). Cyp4b1 was purified from renal microsomes of male mice. This cytochrome P450 was characterized and its cDNA was cloned as described (23). Originally we purified three cytochrome P450s from renal microsomes of male mice and designated them MK-1, MK-2 and MK-3 (23). These forms could not be distinguished by peptide mapping, N-terminal amino acid sequence, catalytic activity or mobility on SDS-polyacrylamide gel electrophoresis (23). Also, mouse Cyp4b1 expressed in yeast cells had similar properties with those of MK-1, MK-2, and MK-3 (data not shown). In this study, we used MK-2 as mouse Cyp4b1. Cytochrome b_5 and NADPH-cytochrome P450 reductase were purified as described (24). Antibodies against cytochrome P450 were raised in a rabbit and immunoglobulin G (IgG) was prepared as reported (24).

6.2.4 Immunochemical study

Immunoblotting was done by the method reported previously (24). Detection of protein on a nitrocellulose membrane blotted from acrylamide gel was done by means of chemiluminescence (ECL, Amersham, Buckinghamshire, England) following the manufacturer's instructions. Immunohistochemistry proceeded as follows. The

avidin-biotin-peroxidase complex (ABC) method described by Hsu *et al.* (25) was used to demonstrate CYP4B1 immunohistostaining. After deparaffinization, tissue sections were sequentially immersed in normal serum (as a control) or anti-Cyp4b1 antibody (1:750, reaction time: 2 hrs, room temperature), biotin-labeled goat anti-rabbit IgG (1:400), and ABC. The sites of peroxidase binding were detected with diaminobenzidine. The sections were then counterstained with hematoxylin for microscopic examination.

6.2.5 Preparation of RNA and Northern blotting

The bladders were rapidly removed from rats and immediately frozen in liquid nitrogen. The total RNAs were isolated as described by Chomczynski and Sacchi (26). The total RNA was analyzed by electrophoresis with 1% agarose gel and blotted onto a nylon membrane (Genescreen, NEN Research Products, Boston, MA), which was hybridized with CYP4B1 cDNA probe (23). Nucleic acid hybridization were done at 65 °C in 0.9 M NaCl and 0.09 M sodium citrate (pH 7.0), containing heat-denaturated salmon sperm DNA (50 μg/ml) and 0.5% SDS. Membranes were washed with 0.3 M NaCl and 0.03 M sodium citrate (pH 7.0) containing 0.5% SDS.

6.2.6 RT-PCR

cDNA was synthesized from total RNA (1 μg) by means of the reverse transcriptase reaction (total 20 μl) using an RNA PCR kit (Perkin-Elmer, Norwalk, CT) according to the manufacturer's instructions. PCR reactions were done in a 100-μl final volume consisting of 1 x Taq DNA polymerase buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, Triton X-100, 1.25 mM MgCl2), 0.2 mM of each deoxyribonucleotide triphosphate, 10 pmol of each forward and reverse primers, 1 unit of Taq DNA polymerase (Perkin-Elmer), and 20 μl of the cDNA reaction mixture. The PCR was done for 30 cycles by sequence temperature; 1 min denaturation at 94 °C, 1 min annealing at 54 °C, and 2 min 30 sec extension at 72 °C. The forward and reverse primers for rat CYP4B1 were 5 ' -CCAGTACCATAATGACTTCA and 5 ' -TAGAGGCGGAAGCACTCCTT, respectively, and predicted to produce a 444-bp fragment (nucleotide positions 689-1132 of the reported rat CYP4B1 cDNA (27)). PCR products (10 μl) were directly analyzed by electrophoresis on a 1.0% agarose gel and stained with ethidium bromide.

6.2.7 Analytical and assay methods

The activation of *umu* gene expression by activated metabolites of procarcinogen in the microsomes and in a reconstituted monooxygenase system was measured as described (28). In brief, the standard reaction mixture (final volume, 1.0 ml) contained 50 mM potassium phosphate buffer (pH 7.25); substrate (10 μM) such as DCB dissolved in dimethyl sulfoxide (10 μl); a mixture (NADPH-generating system) of $NADP^+$ (0.25 mM), glucose-6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (0.5 unit), and $MgCl_2$ (1.5 mM); either microsomes (20-50 μg) or a reconstituted system

containing purified cytochrome P450 (10-30 pmol), NADPH-cytochrome P450 reductase (0.3 unit), cytochrome b_5 (10-30 pmol) and DLPC (5 μg); and a suspension of *Salmonella typhimurium* (0.75 ml). The reaction at 37 °C for 120 min was stopped by rapidly cooling the mixture in an ice-water bath. A portion (0.2 ml) of the reaction mixture was assayed for β-galactosidase activity, and *umu* gene expression was measured as the specific β-galactosidase activity per minute per nanomole of P450 (28). Individual cytochrome P450 (CYP) forms are designated by the systematic nomenclature of Nelson *et al.* (29).

6.3 RESULTS

6.3.1 Mutagenic activation of procarcinogens by mouse renal microsomes and purified mouse Cyp4b1

We have found that mouse Cyp4b1 activates 3-MeO-AAB, 2-aminoanthracene, and 2-aminofluorene (23). In this study, we further investigated the mutagenic activation of aromatic amines, 3-MeO-AAB, 2-NA, and DCB by mouse Cyp4b1 using *umu* test (Table I). 2-NA induces bladder tumor in humans but not in rodents (2). Mouse Cyp4b1 and renal microsomes had low activity towards 2-NA. However, Cyp4b1 had high mutagenic activation towards not only 3-MeO-AAB but also DCB which induces bladder tumors in rats (15, 16). Mouse renal microsomes contain Cyp4b1 as the major form and it had also high activity towards DCB. Mouse bladder microsomes significantly activated DCB, although to a lesser extent than that of mouse renal microsomes.

Table I Mutagenic activation of procarcinogens by microsomes and Cyp4b1 from the mouse.

	3-MeO-AAB	2-NA	DCB
Microsomes			
Kidney	241 ± 16	23 ± 3	269 ± 14
Bladder	–	–	16 ± 1
Purified Cyp4b1	1240	90	1620

Values of microsomes (means ± SD for 3 samples) are expressed as units/min/mg. Values of purified cytochrome P450 were expressed as units/min/nmol. Renal microsomes (20 μg), bladder microsomes (50 μg), or purified Cyp4b1 (10 pmol) were reacted with procarcinogens (10 μM) under the conditions described in Materials and methods. 3-MeO-AAB, 3-methoxy-4-aminoazobenzene; 2-NA, 2-naphthylamine; DCB, 3,3'-dichlorobenzidine, -; not assayed.

6.3.2 Effects of Cyp4b1 antibody on *umu* gene expression of DCB by mouse renal microsomes

Mouse renal microsomes and purified Cyp4b1 had high activity towards DCB. We investigated the effects of Cyp4b1 antibody on bioactivation of DCB by renal microsomes (Fig.1). Anti-Cyp4b1 antibody completely inhibited the mutagenic

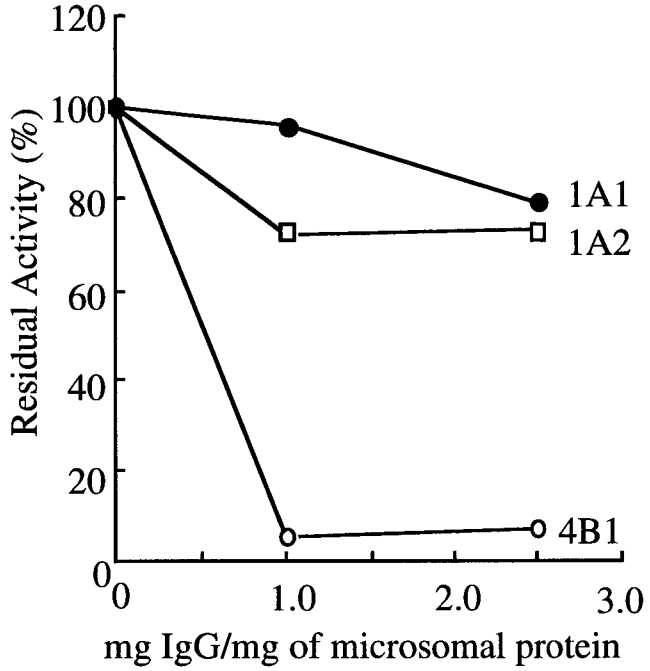

Fig. 1. The effects of antibodies on *umu* gene expression of 3,3'-dichlorobenzidine by mouse renal microsomes. Various amounts of immunoglobulin G (IgG) were incubated with renal microsomes then the assay proceeded as described in the footnote to Table I. 1A1, 1A2, and 4B1 indicate antibodies against rat CYP1A1, rat CYP1A2, and mouse Cyp4b1, respectively.

activation of DCB by mouse renal microsomes. CYP1A1 and 1A2 antibodies were also used for comparison. CYP1A1 and 1A2 activate many procarcinogens, including aromatic or heterocyclic amines (28). The effects of these antibodies on DCB bioactivation were slight. These results indicated that Cyp4b1 plays a major role in the bioactivation of DCB in the mouse renal microsomes. We also investigated the effects of CYP1A1, 1A2, and 4B1 antibodies on the bioactivation of DCB in bladder microsomes. Unfortunately inhibition of *umu* activity by antibodies was undetectable because the activity of bladder microsomes was too low and the increased amount of microsomal protein might interfere with the inhibition by the antibody. However, the Cyp4b1 substrate, lauric acid (23) efficiently inhibited the umu activation of DCB by both mouse renal and bladder microsomes, suggesting that Cyp4b1 activates DCB in mouse bladder microsomes (table II).

6.3.3 Immunoblotting of mouse kidney and bladder

Renal and bladder microsomes of the male mouse were subjected to immunoblotting with Cyp4b1 antibody (Fig. 2). Both microsomes gave a single staining band, indicating the presence of Cyp4b1 in the mouse bladder. The level of CYP4B1 in bladder microsomes was calculated to be 3.5 pmol/mg of the microsomal protein. The level was 2.5% that of mouse renal microsomes.

Table II Effects of Lauric Acid on *umu* Gene Expression of DCB.

Sodium laurate (mM)	Liver microsomes	Kidney microsomes	Bladder microsomes	Purified Cyp4b1
0	100 (146)	100 (309)	100 (15)	100 (1439)
1	93	103	91	100
10	90	96	74	88
100	80	42	24	34

Hepatic microsomes (20 μg), renal microsomes (20 μg), bladder microsomes (50 μg) and purified cytochrome P450 (10 pmol) were used in the assay. The activities without sodium laurate were set at 100% and values are expressed as a percentage of the residual activity. The values in parentheses indicate absolute activities and expressed as unit/min/mg of protein for microsomes and unit/min/nmol of P450 for purified cytochrome P450.

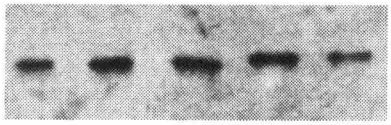

Fig. 2 Immunoblotting of mouse kidney and bladder microsomes. The renal microsomes (0.5 μg), bladder microsomes (20 μg), and purified Cyp4b1 (0.04 pmol) were resolved by SDS-polyacrylamide gel electrophoresis. Lanes 1 and 2 are individual renal microsomes. Lanes 3 and 4 are individual bladder microsomes. Lane 5 is purified mouse Cyp4b1.

6.3.4 *umu* Gene expression of DCB by purified rat cytochrome P450s

We found that mouse Cyp4b1 had high activity toward DCB, a benzidine derivative. Little is understood about the bioactivation of benzidine and biphenyl amine derivatives by the multiple forms of cytochrome P450. It is established that DMAB and DCB induce bladder carcinoma in rats (15, 17) and we have purified several forms of cytochrome P450 from the rat liver (20). Thus, we performed the *umu* test for DCB, DMAB, and 2-NA bioactivation using ten forms of purified rat cytochrome P450s, including rat CYP4B1 (Fig. 3). Rat CYP4B1 had extremely high activity towards DCB and DMAB. Isoform-specificity towards these chemicals was high. Its activity is similar to that of mouse Cyp4b1. CYP1A1 and 1A2 had the activity towards DCB and DMAB but their activity was less than 20% that of CYP4B1. 2-NA was also activated by CYP4B1 although its activity toward 2-NA was 10% that towards DMAB and DCB.

6.3.5 Immunostaining of rat bladder

We investigated localization of CYP4B1 in the rat bladder by immunohistochemistry. CYP4B1 was immunohistochemically demonstrated in the bladder epithelium,

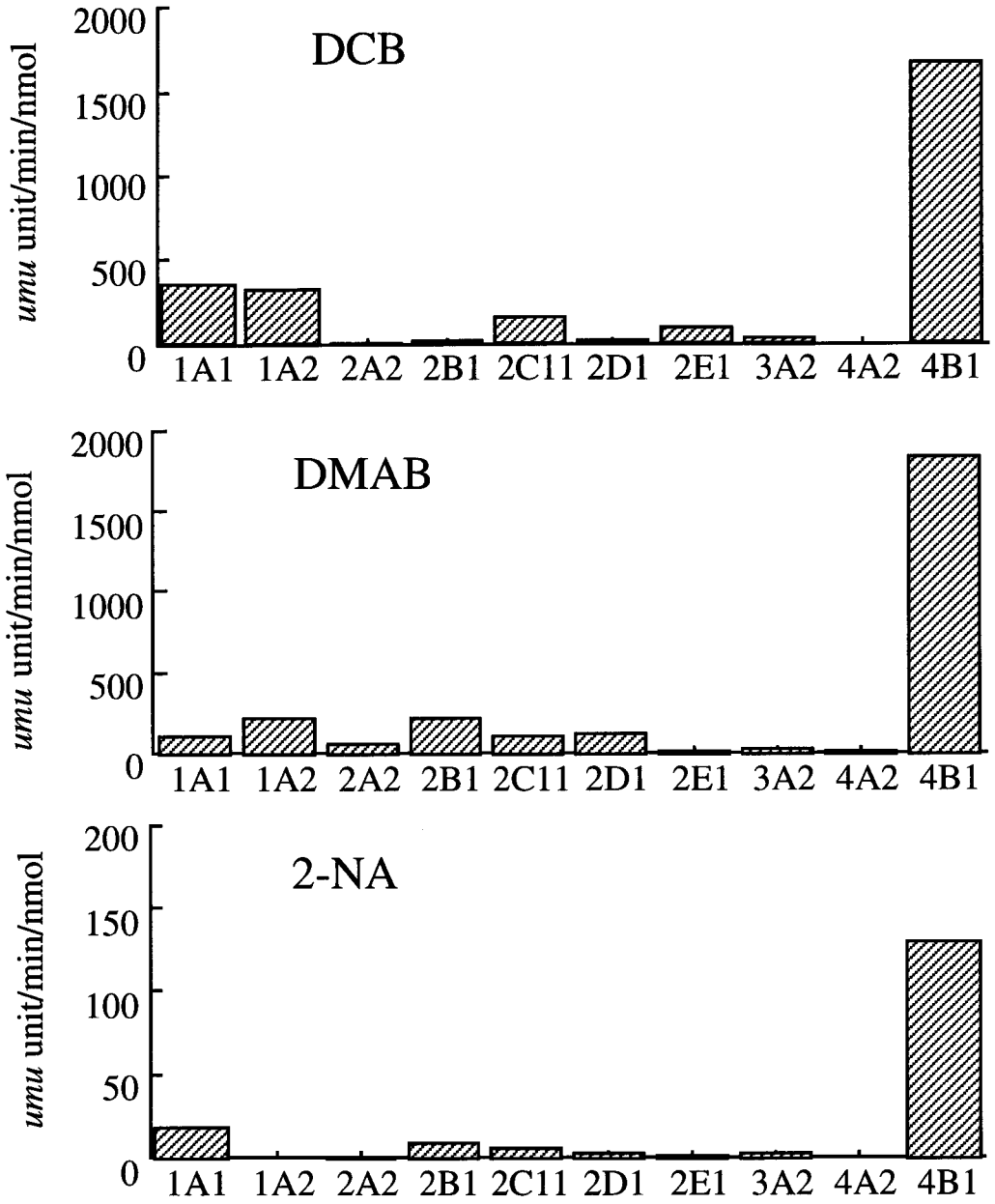

Fig. 3. *umu* Gene expression of 2-naphthylamine (2-NA), 3,2'-dimethyl-4-aminobiphenyl (DMAB), and 3,3'-dichlorobenzidine (DCB) by purified rat cytochrome P450s. Purified rat cytochrome P450s (10 pmol) were reacted with procarcinogens (10 μM) under the conditions described in Materials and methods.

especially in the surface epithelium of the normal rat bladder (Fig. 4A). Procarcinogens in urine can be activated on the surface of bladder epithelium. Non-immune serum did not give any staining in rat bladder (Fig. 4B).

6.3.6 Northern blotting and RT-PCR of rat bladder RNA

Total RNA was isolated from the rat bladder and subjected to agarose gel electrophoresis (Fig. 5A). RNA was blotted onto a membrane and hybridized with a CYP4B1 cDNA probe (Fig. 5B). A single band appeared, indicating the presence of

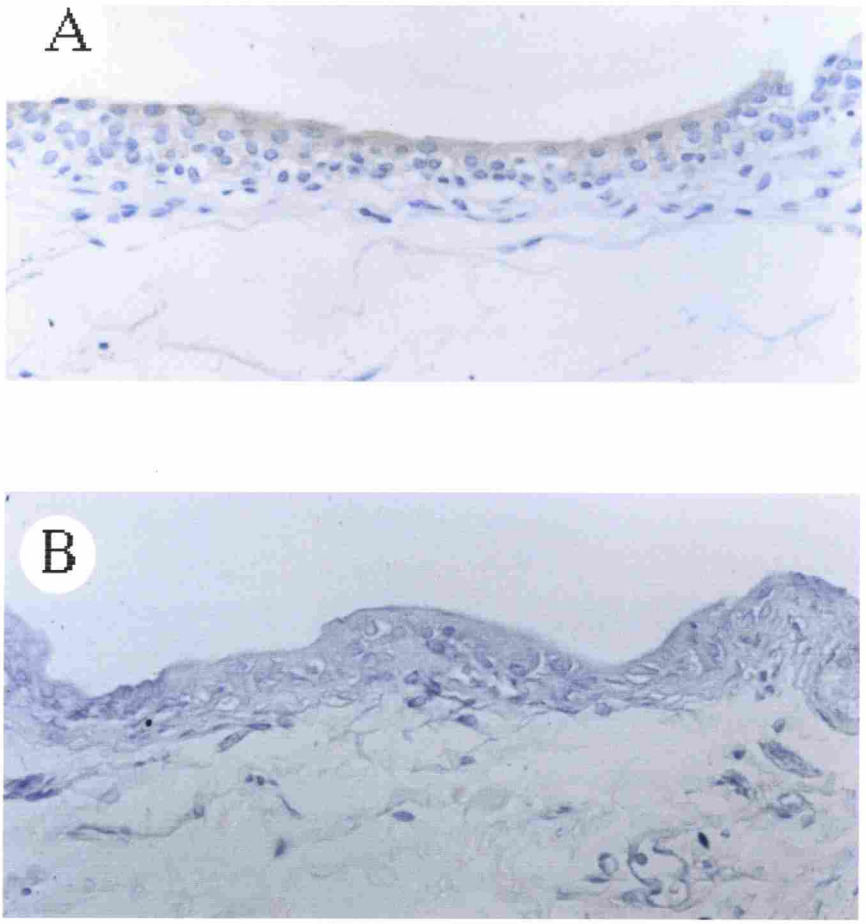

Fig. 4 Localization of CYP4B1 in rat bladder. The rat bladder was immunohistochemically stained with anti-Cyp4b1 antibody (A) and with non-immune serum (B). CYP4B1 was visualized with the avidin-biotin-peroxidase complex (ABC) and diaminobenzidine.

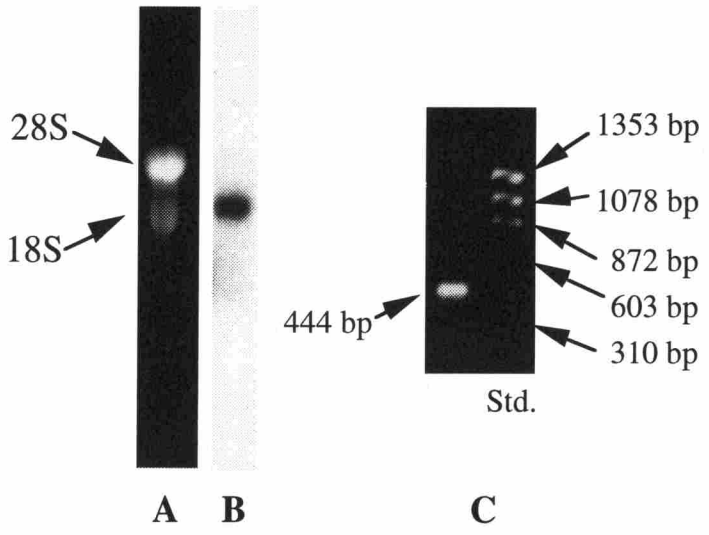

Fig. 5 Northern blots and RT-PCR analysis of CYP4B1 mRNA. Total cellular RNA was isolated from whole rat bladder. Total RNA (10 μg/well) was separated on 1% agarose gel (A) and subjected to Northern blot analysis using a CYP4B1 cDNA probe (B). RT-PCR was done using total RNA with the CYP4B1 primers described in Materials and methods. Amplified fragment oligonucleotide was resolved by electrophoresis with 1.0% agarose gel stained with ethidium bromide (C).

CYP4B1 mRNA in the rat bladder. Furthermore, RNA was converted to cDNA and amplified by PCR. The predicted band (444 bp) migrated in an agarose gel (Fig. 5C). These results are further evidence for the presence of CYP4B1 in the rat bladder.

6.4 DISCUSSION

This study provided direct evidence that cytochrome P450 can activate the biphenyl derivative, DMAB and the benzidine derivative, DCB to mutagenic substances. Vanderslice *et al.* (14) found that the bladder mucosa of the rabbit contained CYP4B1, which plays a major role in the metabolism of 2-aminofluorene, a carcinogenic aromatic amine and that cytochrome P450 is important enzyme in carcinogenesis of the bladder as well as the liver, which has the highest cytochrome P450 content. CYP4B1 also metabolizes 2-aminoanthracene and aflatoxin B1 (30). CYP4B1 genes of rabbit and rat are first established by Gasser and Philpot (27). Poupko *et al.* (13) have found that the N-hydroxylation activity of 4-aminobiphenyl (a carcinogenic amine in the rat bladder) is inhibited by a chemical inhibitor of cytochrome P450. Although the bladder has a low content of cytochrome P450, it may be sufficient to initiate bladder cancer upon long exposure to carcinogens in the urine. Human CYP4B1 cDNA was isolated by Nhamburo *et al.* (31). They and Czerwinski *et al.* (31, 32) reported that the cDNA-expressed human CYP4B1 had no activity towards 2-aminofluorene and lauric acid which are typical substrates for CYP4B1 and

concluded that substrate specificity of human CYP4B1 is different from that in other species. However, human renal microsomes had lauric acid ω-hydroxylation activity like mouse renal microsomes and was efficiently inhibited by anti-CYP4B1 antibody which did not cross-react with CYP4A forms (data not shown). CYP4B1 forms of rabbit, rat, mouse, and hamster have activity towards 2-aminofluorene (33). Nhamburo *et al.* (31) reported the possibility of genetic polymorphism in human CYP4B1 gene. There may be human CYP4B1 variants which have different catalytic specificity.

Aromatic amines are first metabolized in the liver by N-acetylation, N-oxidation, and other modification. N-Oxidation is activation pathway of aromatic amines and activated amines bind to protein or DNA (7, 34). 4-Aminobiphenyl-hemoglobin adducts and DNA adducts in urothelial cell are detected in humans and adduct levels are correlated with bladder cancer risk (35). Part of amines activated in the liver may reach the bladder through blood stream (34) and amines are also activates directly on the bladder surface (36-38). N-Acetylation is important on the activation of aromatic amines. N-acetylation of 4-aminobiphenyl seems inactivation of its mutagenesity because slow acetylators have high risk of bladder cancer (6). On the contrary, benzidine may be activated after N-acetylation because almost DNA adducts of benzidine in human is acetylated derivatives and activity of acetylation of benzidine in human individuals seems to have no effect on levels of urothelial DNA adducts (6). Whereas, DCB as well as N-acetylated DCB has high mutagenic activity and DCB is also important in initiation of bladder cancer (10). Activation pathway of DCB may be different from that of benzidine. Benzidine is strongly activated by prostaglandin H synthetase but not by hepatic cytochrome P450s (9, 10). However, DCB is not activated by prostaglandin H synthetase (10).

Cytochrome P450, especially CYP4B1, can bioactivate DMAB and DCB, bladder carcinogens. CYP4B1 was revealed in the rat and mouse bladder microsomes by means of immunoblotting and immunohistochemistry. Tissue-staining indicated that CYP4B1 was present in epithelial cells of the bladder. This is the first direct evidence of CYP4B1 in the bladder mucosa. These findings, together with results of a metabolic study, indicated that DMAB and DCB are activated by CYP4B1 in the mucosa of the rat and mouse bladder. These results suggested that CYP4B1 could contribute to the initiation of carcinogenesis in rat and mouse bladder by activation of aromatic amines.

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CHAPTER 7
ANDROGEN REGULATION OF CYP4B1 RESPONSIBLE FOR MUTAGENIC ACTIVATION OF BLADDER CARCINOGENS IN THE RAT BLADDER

7.1 INTRODUCTION

Significant sex differences have been observed in comparative bladder cancer studies; males are several times more at risk than females (1). In experimental animals such as rats and mice, bladder cancer is induced by chemical carcinogens more easily in males than in females (2). Aromatic amines such as 2-naphthylamine and benzidine can induce bladder carcinomas (3,4). These amines are included in cigarette smoke and in industrial chemicals. A large percentage of such carcinogens are activated by cytochrome P450 to nucleophilic chemicals which cause mutagenesis (5,6).

Degawa *et al.* (7) found that mutagenic activation of 3-methoxy-4-aminoazobenzene in the renal microsomes of male mice is much higher than in those of female mice. Furthermore, activity in female mice can be induced by treatment with testosterone. We have purified cytochrome P450 responsible for activation of 3-methoxy- 4-aminoazobenzene from renal microsomes of male mice and found that it is mouse Cyp4b1 by cDNA cloning (8). We also found that rat and mouse CYP4B1 specifically activates 2-naphthyamine, 3,4 ' -dimethylaminobiphenyl, and 3,3 ' -dichlorobenzidine, all of which can cause bladder carcinoma in rats and in humans (9).

Expression of cytochrome P450 as a sex-linked factor was first discovered in rat liver models; it was shown that the metabolism of several xenobiotics differs between male and female rats (10, 11). The mechanism of the sex-dependent expression of cytochrome P450 has been extensively studied in rat liver (10, 11). However, regulation of cytochrome P450 in extrahepatic tissues is not as well understood. In a previous study, we found that CYP4A2 in the male rat kidney is more highly expressed than in the female kidney; moreover, the expression is regulated by androgen and thyroid hormone (12, 13). Immunochemical study and Northern blot analysis revealed that CYP4B1 is present in the mucosa of the rat bladder; this suggests that CYP4B1 contributes to carcinogenesis in the rat bladder (9). It is possible that the sex-dependent expression of cytochrome P450 contributes to sex differences observed in carcinogenesis.

In this study, we developed a method of competitive reverse transcriptase-polymerase chain reaction (RT-PCR) in order to measure CYP4B1 mRNA and to investigate expression levels of CYP4B1 mRNA in the bladder of male and female rats at various ages. We found that male rats had higher levels of expression than female rats and that CYP4B1 in the bladder was regulated by androgen.

7.2 MATERIAL AND METHODS

7.2.1 Preparation of total RNA and RT-PCR of CYP4B1 mRNA

Sprague-Dawley rats at various ages were obtained from Japan Clea (Tokyo, Japan). The castration of male rats and treatment with testosterone have been described elsewhere (14). The male rats at 8 weeks old were castrated and recovered for 1 week. Testosterone (10 mg/kg) was subcutaneously injected every other day for 2 weeks. Castrated and testosterone-treated rats were sacrified at 11 weeks old. Rat bladders were removed and were immediately frozen in liquid nitrogen. Total RNA was isolated from a single bladder with Isogen (Nippon Gene, Toyama, Japan). The isolated RNA was treated at 37 °C for 60 min with DNase (Nippon Gene) to remove contamination of genomic DNA. An RNA PCR Kit (Takara, Shiga, Japan), including AMV RNA reverse transcriptase, was used for RT-PCR of CYP4B1 mRNA. The reaction was performed in 20 µl solution, according to the manufacturer's instructions; 2.0 µg of total RNA from a single rat bladder were used. The mixture was preincubated for 10 min at 30°C, incubated for 30 min at 55°C for the mRNA conversion to cDNA, and then the enzyme was inactivated for 5 min at 99°C. The synthesized cDNA were stored at -20°C until the PCR reaction was performed. PCR reactions for CYP4B1 and β-actin contained 0.1 µg and 0.01 µg of cDNA, respectively. The PCR primers for rat CYP4B1 and β-actin were designed according to previous reports (15, 16). The forward and reverse primers for CYP4B1 were 5 ' -CCAGTACCATAATGACTTCA-3' and 5'-TAGAGGCGGAAGCACTCCTT-3', respectively; the primers were predicted to produce 444-bp fragments (15). The forward and reverse primers for β-actin were 5'-CAAGAGATGGCCACTGCCGCA-3' and 5'- TCCTTCTGCATCCTGTCAGCG-3', respectively, and were predicted to produce 275-bp fragments (16). The fragment sizes of the competitors were 363 bp for CYP4B1 and 363 bp for β-actin. The competitors for CYP4B1 and β-actin were prepared with a competitive DNA construction kit (Takara). Various amounts of competitor were added to the reaction mixtures with cDNA. The reaction was carried out in a 50 µl final volume that consisted of 1x Taq DNA polymerase buffer (50mM KCl,10mM Tris-HCl, pH 9.0, Triton X-100, 1.25 mM $MgCl_2$), 0.2 ml of each of deoxyribonucleotide, 10 pmol each of the forward and reverse primers, 1 unit of Taq DNA polymerase (Takara). The PCR for the bladder tissue was performed for 35 cycles at the sequence temperature; denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, extension at 72 °C for 2.5 min.

7.2.2 Quantification of CYP4B1 mRNA

Ten µl of PCR products were loaded on 2% agarose gel and separated by electrophoresis. The gel was stained with ethidium bromide. Images of the gel were taken by a gel imaging system (ATTO, Tokyo, Japan). The density of the bands of the PCR products was analyzed with an NIH program.

The CYP4B1 to β-actin ratio was calculated with the density of the bands. The

significances of intergroup differences were assessed using the two-sided Student's *t*-test.

7.3 RESULTS

7.3.1 Expression of CYP4B1 mRNA in the bladder of male and female rats at various ages

Total RNA was isolated from the bladders of 3 - 34 week-old male rats and 3 - 40 week-old female rats. The RNA was converted to cDNA by reverse-transcriptase. The resulting cDNA was amplified with various amounts of competitor by PCR (Fig. 1). As

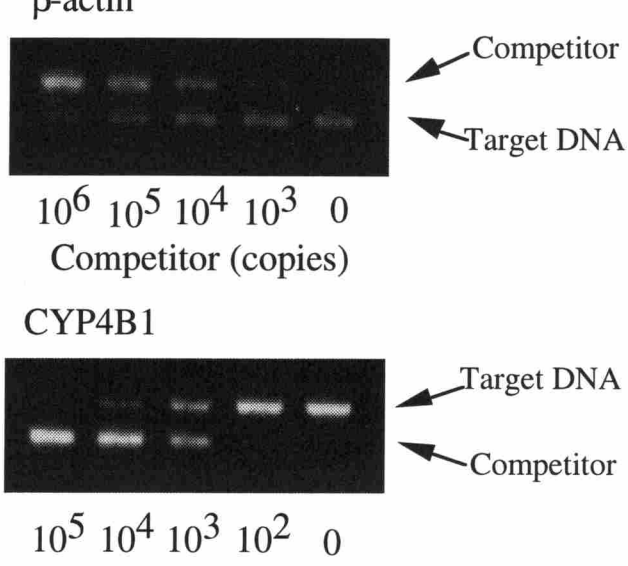

Fig.1 Competitive RT-PCR of rat bladder RNA. Total RNA from rat bladder was isolated and converted to cDNA by reverse-transcriptase. Various amounts of competitor (10^2-10^6 copies) were added to the reaction mixtures. PCR was then performed. Predicted sizes of the oligo-nucleotides amplified from the cDNA are 444 bp (CYP4B1), 363 bp (CYP4B1 competitor), 275 bp (β-actin), and 363 bp (β-actin competitor).

Western and Northern blot analyses require large quantities of tissue, we developed a microassay for CYP4B1 mRNA with competitive PCR. The band intensity of the target DNA was decreased by increasing the amount of competitor. The levels of CYP4B1 mRNA were calculated by comparing the known amounts of competitors. Furthermore, these values were normalized by levels of β-actin mRNA. In the bladders of the immature rats, no sex difference in CYP4B1 mRNA expression was observed (Fig. 2). In the bladders of the male rats, expression of CYP4B1 gradually increased with development; clear sex differences appeared at 14 weeks of development. Expression levels of CYP4B1 did not change in the female rat bladders at any age.

7.3.2 Regulation of CYP4B1 by androgen

As CYP4B1 mRNA was expressed in a male-dominant pattern, we investigated the effects of castration on the expression of CYP4B1 (Fig. 3). Castration of male rats drastically decreased CYP4B1 levels in the bladder. The levels of CYP4B1 were partly recovered by treatment with testosterone, suggesting that the expression of CYP4B1 isregulated by androgens. The treatment of female rats with testosterone did not induce CYP4B1.

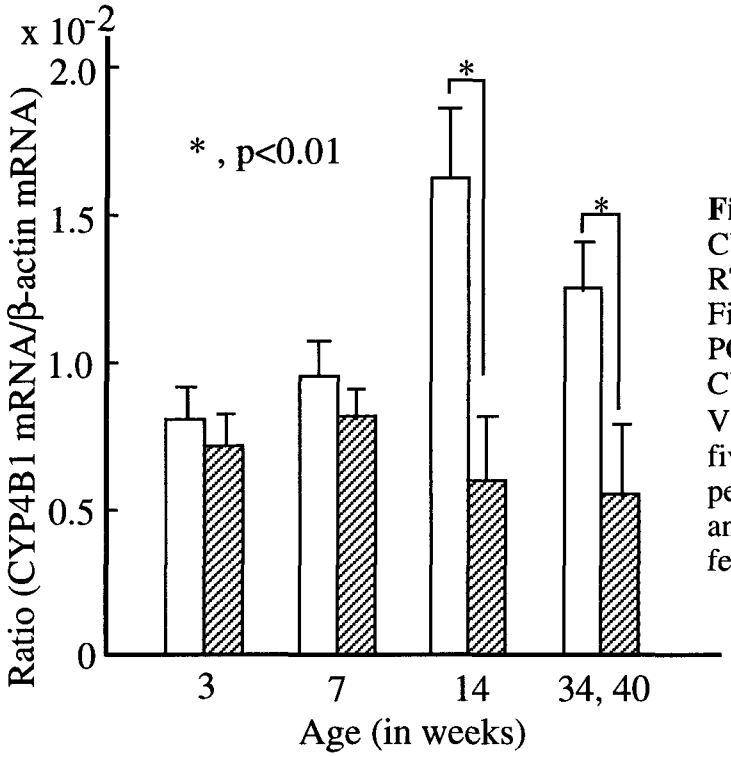

Fig. 2 Age-dependent expression of CYP4B1 in rat bladder. Competitive RT-PCR was performed as described for Fig. 1. The density of the bands of the PCR products was measured and the CYP4B1 to β-actin ratio was calculated. Values are expressed as mean ± SD of five different rats at each age. PCR was performed in duplicate. The open bar and the hatched bar indicate male and female rats, respectively.

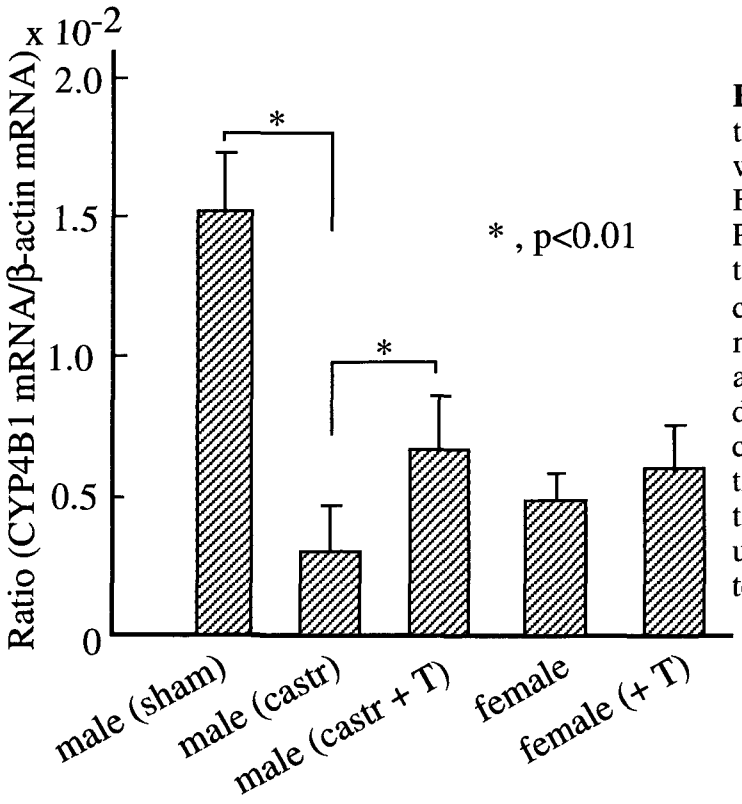

Fig.3 Regulation of CYP4B1 by testosterone. Competitive RT-PCR was performed as described for Fig. 1. The density of the band of PCR products was measured and the CYP4B1 to β-actin ratio was calculated. Values are expressed as mean ± SD of five different animals. PCR was performed in duplicate. sham, sham operation; castr, castration, castr+T; treatment of castrated rats with testosterone; +T, treatment of untreated females with testosterone.

7.4 DISCUSSION

Androgen plays an important role in bladder carcinogenesis (17-19). In animal models such as a rat, blocking of testosterone production by castration suppresses bladder carcinogenesis and administration of testosterone to the castrated male rats induces bladder cancer caused by chemical carcinogens (17, 18). However, exact mechanism how testosterone acts on bladder carcinogenesis is not clear. The androgen receptor of rats is expressed in the bladder urothelium (19). CYP4B1 is also expressed in similar regions and activates procarcinogens such as 2-naphthylamine and benzidine which induce bladder cancer in human and experimental animals (9). In this study, we found that CYP4B1 expression was sex-dependent in the rat bladder; expression of CYP4B1 in male rats was higher than that in female rats. Castration of male rats decreased the levels of CYP4B1 and treatment of castrated male rats with testosterone recovered the CYP4B1 levels. The profile of CYP4B1 expression in the rat bladder agrees with those of bladder carcinogenesis. Levels of several cytochrome P450s are sex- or age-dependent in the rat liver (10). For example, CYP2C11 and CYP2C12 are typical male and female specific forms, respectively. Expression of these forms is regulated by the growth hormone as well as sex-hormones (10, 11). CYP2C11 level is decreased by castration of male rats and is partly recovered by testosterone treatment (11). Furthermore, treatment of female rats with testosterone does not induce CYP2C11 (10, 11). In the rat bladder, castration decreased CYP4B1 expression. However, treatment of female rats with testosterone did not induce CYP4B1. The expression profile of CYP4B1 in the rat bladder resembled that of CYP2C11 in the liver. Expression of CYP4B1 in the rat bladder may be also regulated by growth hormone as well as by androgens. It is reported that some growth factors have a promoting action on rat bladder carcinogenesis (20).

We found that CYP4B1 in the rat bladder activates amines that are carcinogenic in the bladder (8,9). It is not clear whether androgen receptor contributes to the expression of CYP4B1. Androgens induce mutagenic activation of chemical carcinogens catalyzed by CYP4B1 (7). Together with findings of this study, it is possible that sex-specific expression of CYP4B1 in bladder may contribute to the male-dominant expression of bladder carcinoma.

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CHAPTER 8
CYP4B1 IS A POSSIBLE RISK FACTOR FOR BLADDER CANCER IN HUMANS

8.1 INTRODUCTION

Bladder cancer has become a major health hazard in older populations of industrialized societies. Understanding the etiology of and mechanism for the development of this carcinoma is of high priority for the reduction of human sufferings. Bladder cancer is clearly associated with cigarette smoke and environmental chemicals such as 2-naphthylamine, which is one of the first pure chemicals to be identified as a human carcinogen (1). Most chemical carcinogens are not able to cause genetic damage by themselves but require metabolic activation to electrophilic chemicals that bind genomic DNA and proteins. Cytochrome P450 is the primary catalyst involved in such activation (2). There is a large inter-individual variation in the enzymatic activity of cytochrome P450s. Variations in the expression and activity of cytochrome P450s have been associated with variations in risk of developing some cancers. A positive correlation has been observed between both CYP1A1 expression and CYP2E1 expression and lung cancer risk (3, 4). However, there is limited information available regarding the metabolic activation of carcinogens by cytochrome P450s in the human bladder. Organ culture of the human bladder in the presence of 2-acetylaminofluorene, which is a substrate for CYP4B1, causes DNA binding of this chemical (5). Bovine (6) and rabbit (7) bladder mucosa have CYP4B1, which activates 2-aminofluorene (2-AF) and 4-aminobiphenyl, which are also bladder carcinogens. We have found that mouse and rat bladders have CYP4B1, which can activate benzidine and 2-naphthylamine, suggesting the contribution of the CYP4B1 isoform to the initiation of bladder carcinoma (8).

In this study, CYP4B1 was detected in the human bladder by Western blot analysis. In order to clarify the function of CYP4B1, a microassay of CYP4B1 expression levels was developed and the variation of CYP4B1 expression was investigated in bladders of human with or without bladder tumors. Until recently, studies of the genetic polymorphism of cytochrome P450s have focused on drug metabolism and carcinogenesis. In the present study, we found evidence indicating that inter-individual variations in the level of expression of CYP4B1 are also important factors in bladder carcinogenesis.

8.2 MATERIALS AND METHODS

8.2.1 Chemicals

Salmonella typhimurium NM2009 for the *umu* test was a gift from Dr. T. Shimada of the Osaka Prefectural Institute of Public Health. 2-AF was obtained from Sigma Chemical

Co. (St. Louis, MO). Other chemicals and reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan).

8.2.2 Preparation of microsomes and measurement of catalytic activity

Human bladder and kidney microsomes were prepared as described (8). The activation of *umu* gene expression by activated metabolites of 2-AF was measured as described (9). CYP4B1 antibody was prepared with purified mouse Cyp4b1 (9). The expression of human CYP4B1 in yeast cells was carried out by a previously described method (10). A cDNA clone containing the entire coding region of human CYP4B1 (11) was cloned from human lung cDNA library (Clontech, Palo Alto, CA); mouse CYP4B1 cDNA was used as a probe (9). The CYP4B1 cDNA was subcloned into a pGYR1 vector for the transfection of yeast. The expression plasmid pGYR1, including CYP4B1, was transfected into the *Saccharomyces cerevisiae* AH22 strain (10).

8.2.3 Immunochemical study

Immunoblotting was carried out by a previously reported method (8). The bladder microsomes were analyzed by SDS-polyacrylamide gel electrophoresis with 7.5% acrylamide gel. Proteins were electrically blotted to a nitrocellulose membrane. Proteins on the nitrocellulose membrane were detected by means of chemiluminescence (ECL, Amersham-Pharmacia, Buckinghamshire, England) following the manufacturer's instructions.

8.2.4 Tissue characteristics

Human bladder tissue samples were obtained by total cystectomy or transurethal resection at the Osaka City General Hospital and Osaka City University Hospital. Twenty-five clinical specimens of bladder carcinoma and of normal bladder mucosa remote from the tumor were collected from the same individual. An additional 13 specimens of normal bladder mucosa from the patients with bladder tumors were collected. Twenty-one specimens of normal bladder mucosa were obtained from patients undergoing surgery for something other than a bladder tumor; this group included patients with prostatic cancer and those with benign prostatic hypertrophy. The patients with bladder tumors included 33 men and 5 women (mean age at diagnosis, 66 years; range 45-86). The non-bladder tumor patients included 18 men and 3 women (mean age, 63 years; range 38-72). None of these patients had received any type of therapy for bladder tumors before surgery. From the resected bladders, small pieces of tissue, including the bladder mucosa, were rapidly extracted and frozen in liquid nitrogen. All tissue was taken after informed consent had been obtained from the patients and their families.

8.2.5 Preparation of total RNA from bladder and quantitative RT-PCR

Total RNA was isolated from 10-20 mg of homogenized human bladder by Isogen (Nippon Gene, Toyama, Japan) and treated with DNase (Nippon gene) in order to remove the contamination of the genomic DNA. Total RNA was converted to cDNA by

an RNA PCR Kit (Takara, Kyoto, Japan) that included the use of AMV RNA reverse transcriptase. This reaction was carried out according to the manufacturer's instructions with 20 μl samples containing 2.0 μg of total RNA from the bladder. The mixture was preincubated for 10 min at 30 °C, incubated for 30 min at 55 °C for the conversion of mRNA to cDNA. The synthesized cDNA was stored at –20 °C until the PCR reaction was performed. The primers of CYP4B1, CYP2D6, and β-actin, used for the PCR, were designed according to nucleotide sequences that had been reported previously (11-13). The quantitation of CYP4B1 mRNA was perfomed by real-time RT-PCR with a fluorogenic probe (14). The fluorogenic probe consisted of an oligonucleotide with a 5'-reporter dye (6-carboxyfluorescein, Fam) and a downstream, 3'-quencher dye (6-carboxy-tetramethylrhodamine, Tamra). The forward and reverse primers of human CYP4B1 for real-time PCR were 5'-TGGGCTGATCTTGGTCTTAGG-3' and 5'-GTCCATAGCCTTAGCCAACGT-3', respectively, and the fluorogenic probe was 5'-Fam-TTCTCAAGCTCATCCACCTGCTGCT-Tamra-3'. The forward and reverse primers of CYP2D6 for real-time PCR were 5'-TAGTGGTGGCTGACCTGTTCTCT-3' and 5'-TCGTCGATCTCCTGTTGGACA-3', respectively; the fluorogenic probe was 5'-Fam-CTCCTGCTCATGATCCTACATCCGGA-Tamra-3'. The forward and reverse primers of human β-actin for real-time PCR were 5'-GGTCATCACCATTGGCAATG-3' and 5'- CGTCACACTTCATGATGGAGTTG-3', respectively; the fluorogenic probe was 5'-Fam-ATGGAGTCCTGTGGCATCCACGAAACTAC-Tamra-3'. The PCR reactions were carried out with 0.1 μg of cDNA for CYP4B1 or CYP2D6 and 0.01 μg for β-actin in a 50 μl final volume consisting of 0.2 mM of each deoxyribonucleotide, 10 pmol of each forward and reverse primer, 5 pmol of the fluorogenic probe, and 1.25 units of Amplitaq DNA polymerase (Perkin-Elmer, Foster City, CA). The thermal cycling was performed by monitoring fluorescence intensity using Prism 7700 (Perkin-Elmer), initiated with a denaturation step of 10 min at 95 °C, and then 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. Known amounts of full-length CYP4B1, CYP2D6, and β-actin cDNA were used as the standard, and the number of copies of the cDNA in the samples were calculated from the intensity of the fluorescence. The CYP4B1 mRNA levels were normalized by β-actin mRNA levels.

8.3 RESULTS

8.3.1 Detection of CYP4B1 protein and activity in human bladder

In the present study, we investigated the presence of CYP4B1 in the human bladder by Western blotting with an antibody against mouse Cyp4b1. A clear band appeared with the same mobility as that of human CYP4B1 expressed in yeast cells (Fig. 1A). There were large inter-individual variations in the expression of CYP4B1 in these four samples. The sample in lane 4 was from a non-bladder tumor patient, whereas the samples in the other lanes were from bladder-tumor patients. The expression of

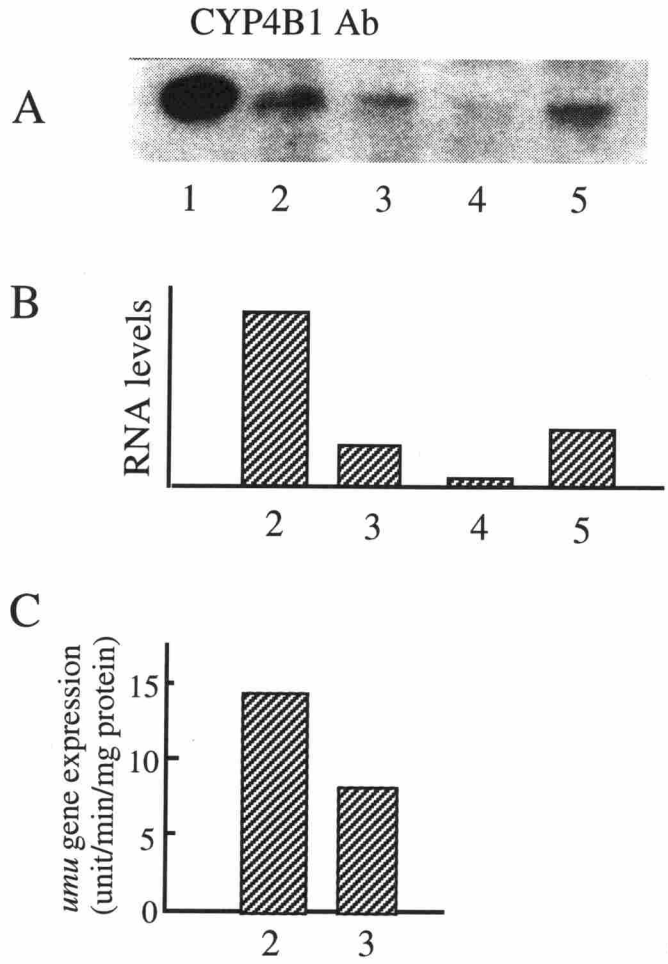

Fig. 1 Western blotting of human bladder microsomes (A), CYP4B1 mRNA levels by quantitative RT-PCR (B) and *umu* gene expression of 2-aminofluorene by human bladder microsomes (C). Human bladder microsomes (20 μg) and yeast microsomes (1 μg) expressing human CYP4B1 were applied to SDS-polyacrylamide gel electrophoresis and the nitrocellulose blotted from the acrylamide gel was reacted with anti-CYP4B1 antibody. Lane 1 is human CYP4B1 expressed in the yeast and lanes 2-5 are human bladder microsomes. The samples in lanes 2,3, and 5 were from bladder tumor patients and the sample in lane 4 was from a non-bladder tumor patient. CYP4B1 mRNA levels in the human bladder were measured by quantitative RT-PCR. The levels are expressed as relative intensity. Human bladders (No. 2 and 3) were reacted with 2-aminofluorene in the presence of *umu* gene expression system.

CYP4B1 in the bladder of the non-bladder tumor patient appeared to be low. Furthermore, Northern blotting and RT-PCR with CYP4B1-specific primers were carried out to confirm the expression of CYP4B1 mRNA. The CYP4B1 mRNA levels correlated well with those of the CYP4B1 protein detected by the antibody (Fig. 1B). Because of the difficulty involved in obtaining large amount of human bladder samples, only four samples of human bladder were analyzed by Western blotting in this study. 2-AF is a typical substrate for CYP4B1 (11). Two samples of bladder microsomes (No.2 and 3) were reacted with 2-AF in the presence of *umu* gene expression system (Fig. 1C). The bladder microsomes (No. 2) had higher *umu* gene expression toward 2-AF than the bladder microsomes (No. 3). The former had higher expression of CYP4B1 than the latter as shown in Fig. 1A. The level of CYP4B1 responded to the activity. Human kidney microsomes had higher activity toward 2-AF than bladder microsomes (53 *umu* units/min/mg protein). The activity was inhibited efficiently by CYP4B1 antibody. The results suggest that human CYP4B1 had metabolic activity toward 2-AF like other animal's CYP4B1 (8).

8.3.2 Levels of CYP4B1 mRNA in human bladders with or without bladder tumors

As Western and Northern blottings require large quantities of tissue, we developed a

microassay for CYP4B1 mRNA with real-time detection PCR. The real-time PCR provides a powerful tool to evaluate cytochrome P450 mRNA expression and gives a new information about the role of cytochrome P450s. This method is high-throughput and has a wide dynamic range of quantitation (over 1000). Moreover, only 10 mg of

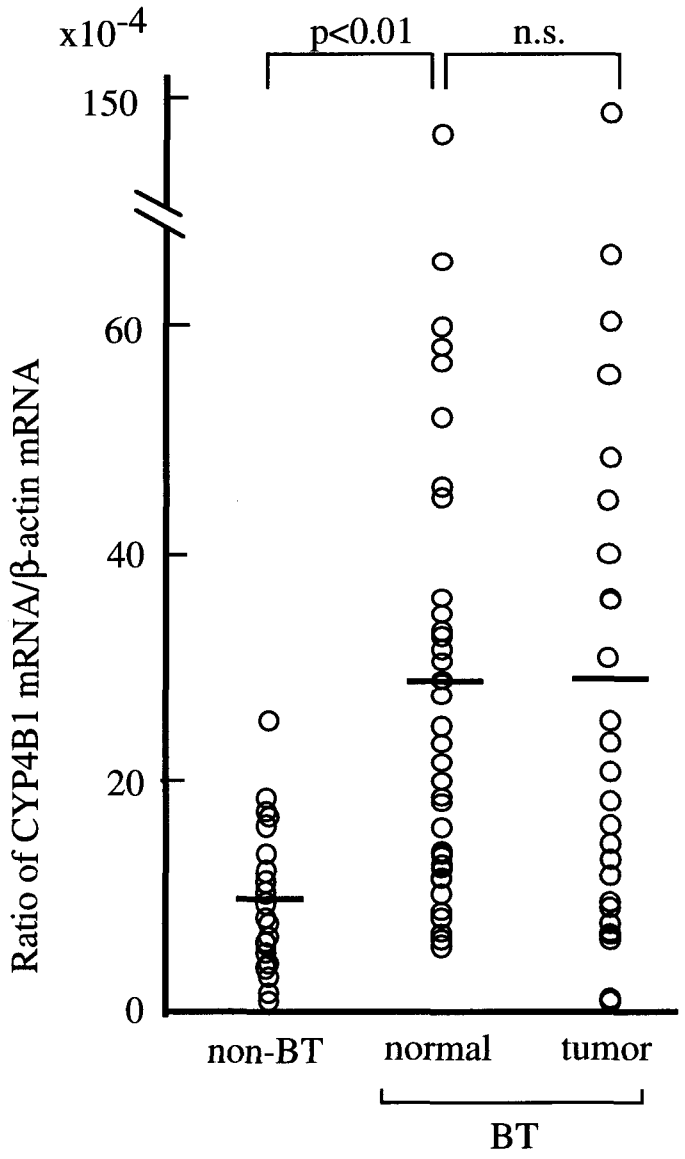

Fig. 2 CYP4B1 mRNA levels in the bladders from non-bladder tumor patients (non-BT) and from bladder tumor patients (BT). Tissues from bladder tumor patients were separated pathologically into normal and tumor regions. Total RNA was isolated from both regions and CYP4B1 mRNA levels were measured by quantitative RT-PCR. CYP4B1 levels were expressed as the ratio of CYP4B1 mRNA/β-actin mRNA. The bladder tissues from non-BT (n=21), from the normal region of BT (n=38), and from the tumor region of BT (n=25) were used for the assay, and the CYP4B1 expression levels were 10.15±0.97, 29.64±4.17, and 29.73±6.18 (mean±SE), respectively. The mean levels are indicated by the horizontal bar.

tissue is needed for the assay. The expression of CYP4B1 mRNA in the human bladder from non-bladder tumor and bladder-tumor patients was measured by this method in the present study. Normal tissues from the bladder-tumor patients had a significantly higher expression of CYP4B1 than those from the non-bladder tumor patients (Fig. 2). In the bladder-tumor patients, CYP4B1 expression in the normal region of the bladder was not significantly different from that of the tumor region. Romkes-Sparks *et al.* (15) have reported an association between CYP2D6 mRNA levels and human bladder cancer. CYP2D6 levels were also assayed by real-time PCR. In the present study, there was no correlation observed between CYP4B1 mRNA and CYP2D6 mRNA levels (Fig. 3). The results suggest that CYP2D6 is not a risk factor common to CYP4B1 for bladder carcinogenesis. At the least, CYP4B1 activates several bladder carcinogens but not CYP2D6 (8). CYP2D6 may contribute to bladder carcinogenesis by different mechanism from CYP4B1. It has also been reported that

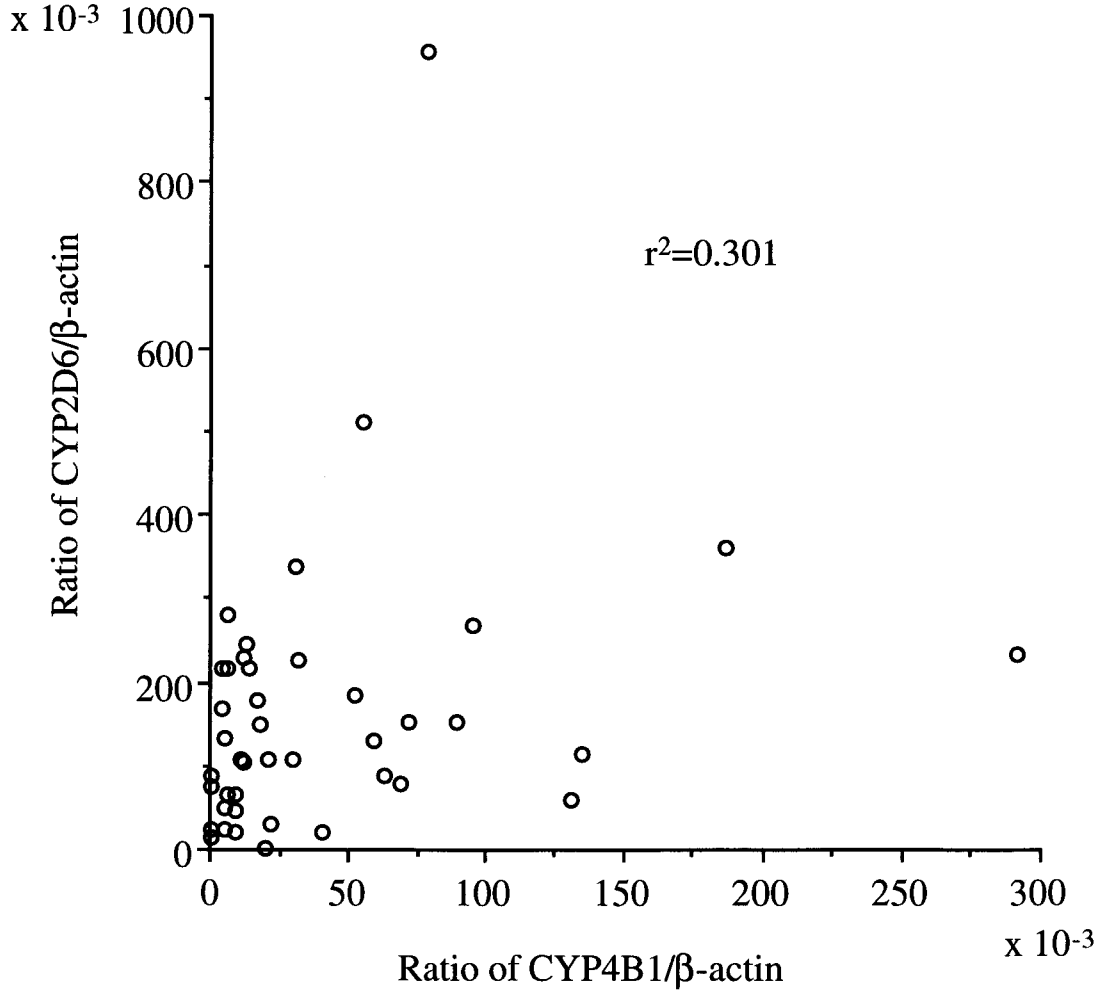

Fig. 3 Measurement of CYP4B1 and 2D6 mRNA in human bladder tissue. CYP4B1 and 2D6 mRNA levels were measured by quantitative RT- PCR. No correlation (r^2=0.301) was observed.

CYP4B1 is expressed in tumor tissues, although almost all other cytochrome P450s are known to disappear in tumor tissues (11). In this study, the expression of CYP4B1 in normal region was not significantly different from that of the tumor region. That is, CYP4B1 levels in the bladder-tumor patients must have been high even before the tumor developed. An expression of CYP4B1 may increase the conversion of chemical carcinogens into mutagenic compounds in the urine and may as a result initiate the development of a bladder tumor.

8.4 DISCUSSION

It is unclear whether aromatic amines induce bladder cancer as a result of their activation at the target site, the bladder mucosa, or *via* activation by other organs such as the liver. However, some evidence that aromatic amines are activated by cytochrome P450 at the bladder has been obtained using bladder microsomes and urothelial cells (6, 16). In this study, we found that human bladder and kidney had metabolic activity toward 2-AF like mice and rats (8, 9). The activity was inhibited by CYP4B1 antibody, indicating human CYP4B1 also had such activities. The bladder is a storage organ for urine, which contains the excreted metabolites of many compounds and is exposed to these compounds over a long period. The metabolic activities of the bladder may be lower than those of the liver or kidney but long exposure is important in activating these chemicals.

In this study, we found that bladder-tumor patients have a high expression of CYP4B1 in the bladder. Inter-individual differences in the expression of CYP4B1 seem to be a factor in the susceptibility to bladder cancer. Genetic polymorphisms of the human CYP2D6 gene are known to be associated with bladder cancer risk (17). Expression levels of the CYP2D6 enzyme or its catalytic activity are linked to the activation of carcinogens, but it is unclear which carcinogens are activated by CYP2D6 in the human bladder. In this regard, CYP4B1 efficiently converts bladder carcinogens, including benzidine, naphthylamine, and aminobiphenyl derivatives, to mutagenic compounds (8). At the least, CYP2D6 did not activate these carcinogens (unpublished data). The nucleotide sequences of CYP4B1 cDNA (coding region) from human bladders used in this study were analyzed (3 samples from non-bladder tumor patients and 7 samples from bladder tumor patients) and they had same nucleotide sequences with that reported previously (11, 18), suggesting contribution of genetic polymorphism of CYP4B1 to bladder cancer is low (data not shown).

Cytochrome P450s are induced by many chemicals such as TCDD in the environment or foods, and the induction of cytochrome P450 increases the risk in carcinogenesis by activating procarcinogens (19). In addition, sex hormones such as androgens regulate the expression of cytochrome P450s. Some chemicals might induce CYP4B1 in bladder tumor patients. It has been found that there is a sex

difference in the incidence of bladder tumors with males have a much higher risk than females (20). We have found that CYP4B1 is expressed male-dominantly in the kidney and bladder of the mouse or rat, and that androgens induce its expression (9). Bladder cancer is also more easily induced in male mice than in females (21). In bladder-tumor patients, androgens or endocrine disrupting chemicals might therefore induce CYP4B1 that might increase the incidence of bladder tumors by increase in activation of procarcinogens.

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CHAPTER 9
DEVELOPMENT OF TRANSGENIC MOUSE WITH HUMAN CYP4B1

9.1 INTRODUCTION

Cytochrome P450s are the principal enzymes responsible for mediating the toxicity of chemicals through metabolic activation of pro-toxin and procarcinogens. Studies of these enzymes are critical in order to achieve an understanding of toxic mechanisms and chemical carcinogenesis. Cytochrome P450s consist of a large superfamily of proteins that includes four families: CYP1, CYP2, CYP3, and CYP4; these proteins are primarily responsible for metabolizing foreign compounds (1). There are many reports that deal with the activation of chemical carcinogens by cytochrome P450s obtained from experimental animals (2). However, there are marked species differences in the catalytic activities of cytochrome P450s when using animal models in research and testing chemicals for safety in humans. Transgenic animals, into which human genes have been introduced, can be used in a variety of ways in order to gain a better understanding of the mechanisms of chemical toxicity to humans.

Cytochrome P450s in the CYP4 family metabolize fatty acids, prostaglandins, and leukotriene (1). CYP4B1 is an especially interesting cytochrome P450 that activates 2-aminofluorene (2-AF), procarcinogen, and 4-ipomeanol, a pulmonary toxin, as well as fatty acids (3). CYP4B1 was first isolated from rabbit lung (4). This protein is a major cytochrome P450 in rabbit lung tissue and its level is low in livers (4, 5). In addition, CYP4B1 is present in bovine and rabbit bladder mucosa, and activates carcinogenic aromatic amines (6, 7). We have purified CYP4B1 from mouse renal micorosomes and found that CYP4B1 is present in mouse and rat bladders and can activate benzidine and 2-naphthylamine, which are bladder carcinogens (8, 9). Human CYP4B1 cDNA was first isolated by Nhamburo *et al.* (10). CYP4B1 was expressed in HepG2 cells and revealed only testosterone 6β-hydroxylation activity. However, testosterone 6β-hydroxylation activity appeared to be achieved by CYP3A5 contamination (11). Recently, human CYP4B1 was expressed in insect cells with a baculovirus system; however, this was a form of P420 that had no catalytic activity (11). The artificial mutant CYP4B1 (Ser427-Pro) has been successfully expressed by this system, revealing lauric acid hydroxylation activity (11). However, there is to date no information about the activity of human native CYP4B1. Recently, we detected CYP4B1 in human bladders and kidneys and found that microsomes in these tissue types had lauric hydroxylation activity and showed mutagenic activation of 2-AF (12).

In this study, we established transgenic mice expressing human CYP4B1 and also established a heterologous expression system of CYP4B1 in order to clarify the activity of CYP4B1 and to begin understanding its biological role in humans. CYP4B1 was expressed in mouse liver using the promoter of the apolipoprotein E (apo E) gene.

Mouse liver has a very low detectable level of constitutively expressed mouse Cyp4b1, but mouse liver tissue includes sufficient amounts of NADPH-cytochrome P450 reductase and cytochrome b_5 for CYP4 family cytochrome P450 to express activity. By introducing human CYP4B1 into a null background, we were able to establish a mouse system in which metabolism by human CYP4B1 could be studied *in vivo*. Furthermore, the fusion protein of human CYP4B1 and NADPH-cytochrome P450 reductase was successfully expressed in *Saccharomyces cerevisiae*. This latter model rendered it possible to investigate the activities of human CYP4B1 *in vitro*.

9.2 MATERIALS AND METHODS

9.2.1 Chemicals

Salmonella typhimurium NM2009, used for the *umu* test, was a gift from Dr. T. Shimada of the Osaka Prefectural Institute of Public Health. 2-AF was obtained from Sigma Chemical Co. (St. Louis, MO). Other chemicals and reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan).

9.2.2 Construction of CYP4B1 transgene for microinjection

A cDNA clone containing the entire coding region of human CYP4B1 was cloned from the human lung cDNA library (Clontech, Palo Alto, CA); mouse Cyp4b1 cDNA was used as a probe (8). Except for a three-nucleotide insertion (Ser207) that has already been discussed in the literature (13), the nucleotide sequence of human CYP4B1 was identical with that reported previously (10). The *Mlu*I and *Cla*I linkers were connected with CYP4B1 by PCR. The cDNA was ligated with a pLiv 11 vector with *Mlu*I and *Cla*I sites. The pLiv 11 was the kind gift of Dr. John M. Taylor of the Gladstone Institute of Cardiovascular Disease (USA). The pLiv 11 included a 5'-flanking sequence and a hepatic control region of the human apo E gene; the plasmid is able to express CYP4B1 in the liver (14). The resulting construct was commercially microinjected into mouse oocytes from Slc:BDF1xSlc:BDF1 mice at the Japan SLC, Inc. (Hamamatsu, Japan). The genomic DNA was extracted from the tail tips of 4-week-old mice. Integration of the transgene into the mouse genomic DNA was confirmed by PCR. The primers used for the PCR were as follows: a sense primer, 5 ' -GGCTG ATCTTGGTCTTAGGC-3' and an antisense primer, 5'-AGCCAATGAACTGTCCGAAC-3', both of which corresponded to human CYP4B1 (10). The predicted size of the PCR product was 215 bp. Expression of CYP4B1 was also confirmed by immunoblot analysis of hepatic microsomes from the mice.

9.2.3 Expression of human CYP4B1 and the fusion protein CYP4B1/NADPH-cytochrome P450 reductase

The expression of human CYP4B1 was carried out by a previously described method (15). The CYP4B1 cDNA was subcloned into a pGYR1 vector for the transfection of yeast cells. The expression plasmid pGYR1, including CYP4B1, was transfected into

the *Saccharomyces cerevisiae* AH22 strain (15). The fused enzyme consisted of CYP4B1 and the NADPH-cytochrome P450 reductase was prepared by a previously described method (16, 17). The 3'-end of the CYP4B1 cDNA was connected to the reductase cDNA lacking the 56 amino-terminal amino acids by a linker including CGAGCC (Arg-Ala). The expression plasmid encoding the fusion enzyme was transfected into *Saccharomyces serevisiae* AH22. The yeast cells were cultured and microsomes of the yeast cells were prepared as described previously (15). The expression of CYP4B1 and the fusion enzyme, CYP4B1/reductase, was confirmed by Western blot analysis.

9.2.4 Measurement of catalytic activity

The activation of *umu* gene expression by activated metabolites of 2-AF in the yeast microsomes and hepatic microsomes of transgenic mice was measured as previously described (8). Measurement of lauric acid hydroxylation activity has been described elsewhere (18). The immunoblot analysis was carried out by a previously reported method (19). Hepatic microsomes of transgenic mice were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 7.5% acrylamide gels. Proteins were electrically blotted onto a nitrocellulose membrane. Proteins on the nitrocellulose membrane were detected by means of peroxidase and 4-chloro-1-naphthol.

9.3 RESULTS

9.3.1 Establishment of transgenic mice expressing human CYP4B1

Hepatic microsomes of mice which were found to contain human CYP4B1 in chromosomal genes were prepared and an immnoblot analysis of these samples was performed (Fig.1). The promoter of the apo E is able to efficiently express the protein in the liver. The mouse liver did not express mouse Cyp4b1 (8). Mouse Cyp4b1 is dominantly expressed in the kidneys of male mice (8). To avoid contamination by

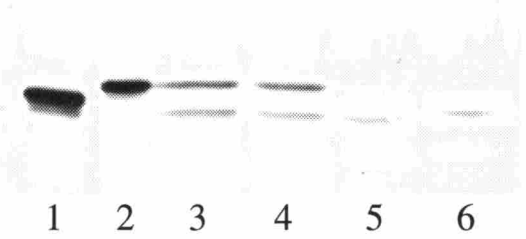

Fig. 1 Immunoblot analysis of hepatic microsomes from transgenic and control mice with anti-Cyp4b1 antibody. Lane 1, renal microsomes from control male mice (0.1 μg); lane 2, yeast microsomes expressing human CYP4B1 (2 μg); lanes 3 and 4, hepatic microsomes from transgenic female mice (10 μg); lanes 5 and 6, hepatic microsomes from control female mice (10 μg)

mouse Cyp4b1, female mice were used in this study. Hepatic microsomes from transgenic mice revealed a clear band of the same mobility as that of human CYP4B1 expressed in yeast cells. Samples from control mice did not display a band with the

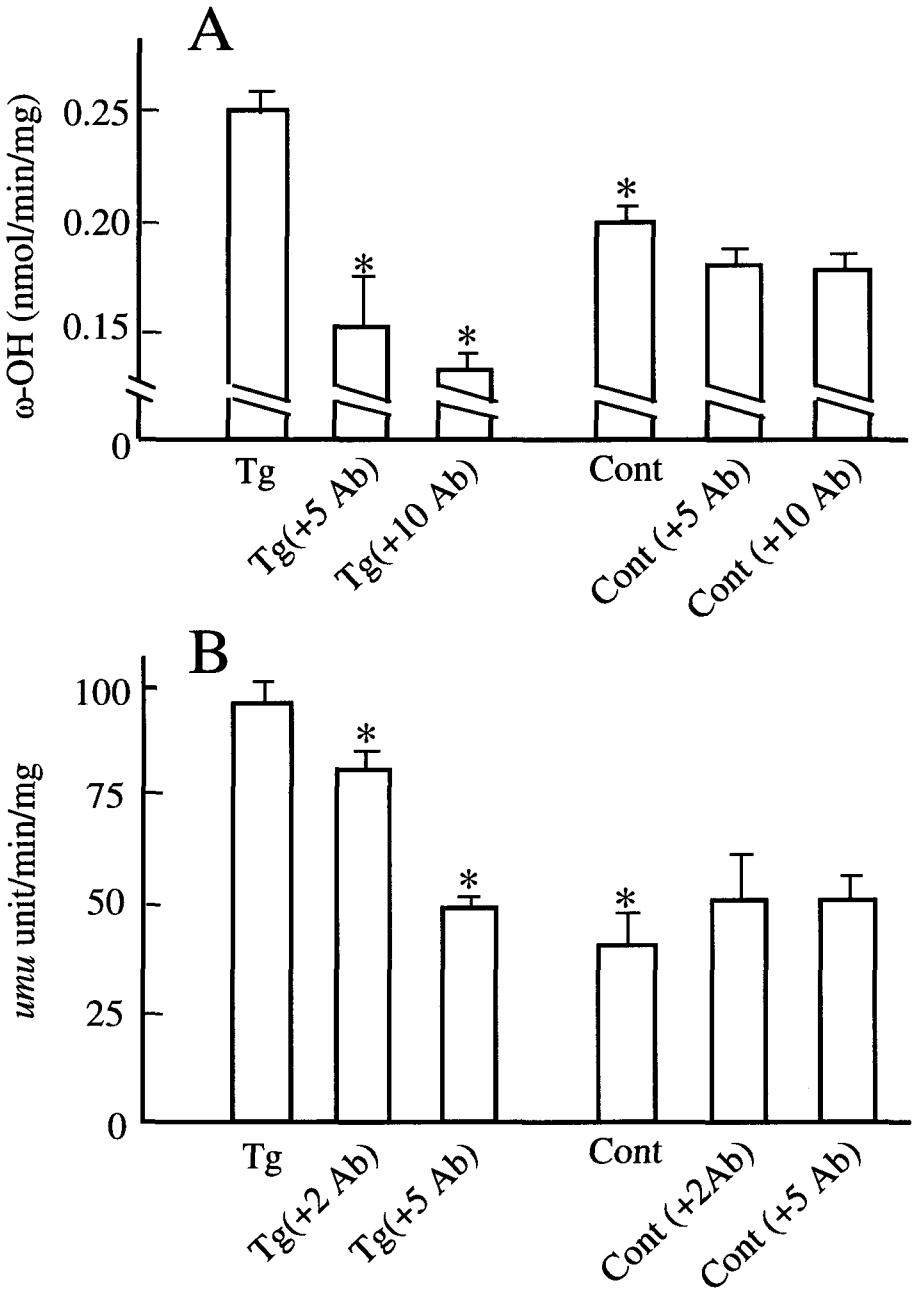

Fig. 2 Catalytic activity of hepatic microsomes of transgenic mice. Hepatic microsomes (100 μg) from transgenic female mice (Tg) and control mice (Cont) were reacted with lauric acid (A). Hepatic microsomes (20 μg) reacted with 2-aminofluorene in presence of *umu* gene expression system (B). +2 Ab, +5 Ab, and +10 Ab indicate addition of 20, 50, and 100 μg of anti-Cyp4b1 antibody IgG into the reaction mixture.
Values are expressed as mean ± SD from four different experiments.
*Significantly different from the value observed in the case of the transgenic mice (Tg), $p<0.01$.

same mobility. Mouse Cyp4b1 had more mobility on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) than human CYP4B1. Furthermore, bands of mouse Cyp4b1 were not detected in the hepatic microsomes of both transgenic and control mice with the same mobility; this finding indicate that mouse liver did not express mouse Cyp4b1. Expression levels of human CYP4B1 in hepatic microsomes of mice were much lower than that of Cyp4b1 in the renal microsomes of male mice. This result may be due to the instability of the cytochrome P450.

9.3.2 Catalytic activities of hepatic microsomes from transgenic mice

Hepatic microsomes from transgenic mice, including human CYP4B1, were prepared and their catalytic activities were measured (Fig. 2A). Lauric acid ω-hydroxylation of transgenic mice was higher than that of control mice by 25%. Anti-Cyp4b1 antibody efficiently inhibited lauric acid ω-hydroxylation activity in the transgenic mice but not in the control mice. Lauric acid ω-hydroxylation is catalyzed by CYP4A isoforms as well as by CYP4B1 (18). In control mice, Cyp4a isoforms may have catalyzed ω-hydroxylation, which was not inhibited by Cyp4b1 antibody. 2-AF is a more specific substrate for CYP4B1 than lauric acid. Mutagenic activation of 2-AF by hepatic microsomes of mice was measured by *umu* gene expression system (Fig. 2B). Transgenic mice demonstrated a two-fold increase in the activity over that of the control mice. This activity was efficiently inhibited by anti-Cyp4b1 antibody. These results indicate that human CYP4B1 does exhibit activity similar to the CYP4B1 isoforms found in other experimental animals (9). Human CYP4B1 could not be purified from the hepatic microsomes of transgenic mice because it lost its activity during solubilization.

9.3.3 Heterologous expression of human CYP4B1 and its catalytic activity

Human CYP4B1 alone was expressed in yeast cells. However, it was found to be unstable and to have no activity toward lauric acid and 2-AF. These results are consistent with those reported by Zheng *et al.* (11). CYP4B1 purified from mice or rats is also unstable and readily loses its activity (8). Because of its relative stability, we studied the expression of the human CYP4B1/NADPH-cytochrome P450 reductase fusion protein. The presence of cytochrome b_5 and NADPH-cytochrome P450 reductase stabilizes cytochrome P450s (20). CYP21 is stabilized by fusion with reductase in yeast cells although it is unstable when expressed alone (16). We found that the CYP4B1/reductase fusion protein does exhibit absorption at 450 nm in its CO-reduced form (Fig. 3). The results of the Western blot analysis of expressed CYP4B1 and the CYP4B1 and reductase fusion protein are given in Fig. 4. The molecular weight of the fusion protein was about 120 kDa, as shown by SDS-PAGE. The expression level of the fusion protein in yeast cells was similar to that of CYP4B1 expressed alone. The fusion protein possessed lauric acid ω-hydroxylation activity (2.28 nmol/min/nmol of P450) (Fig. 5A). Furthermore, the fusion protein was able to

activate 2-AF to mutagenic compounds (Fig. 5B); this activation was detected by an *umu* test.

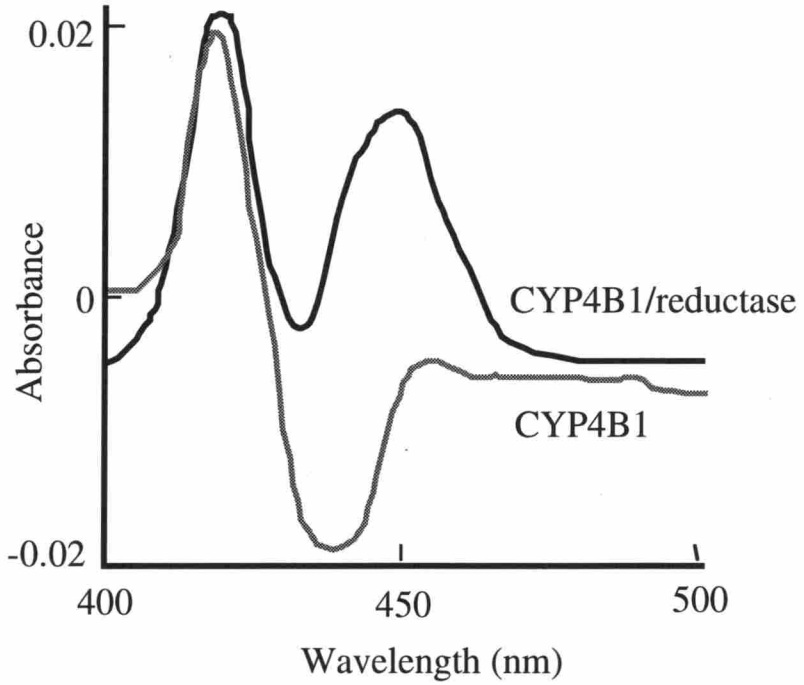

Fig. 3 Expression of CYP4B1 and the fusion protein of CYP4B1 and NADPH-cytochrome P450 reductase (CYP4B1/reductase). The CO-reduced difference spectra using yeast microsomes (4 mg) was measured in 20 mM Tris-acetate buffer (pH 7.2) containing 20% glycerol.

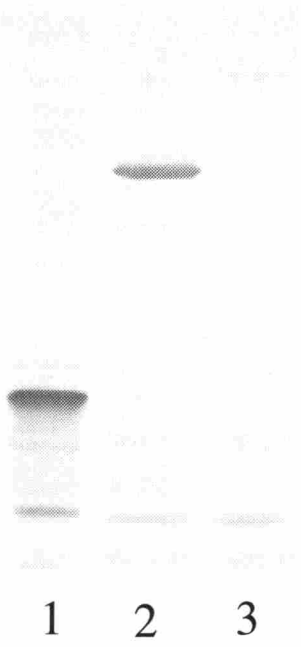

Fig. 4 Immunoblot analysis of yeast microsomes expressing human CYP4B1 and the fusion protein of CYP4B1 and NADPH-cytochrome P450 reductase with anti-Cyp4b1 antibody. Lanes 1-3 depict CYP4B1 alone, CYP4B1/NADPH-cytochrome P450 reductase fusion protein, and pGYR1 plasmid including the reverse direction of CYP4B1 cDNA, respectively. Yeast microsomes (2 μg) were analyzed by SDS-polyacrylamide gel electrophoresis using 7.5% acrylamide gels.

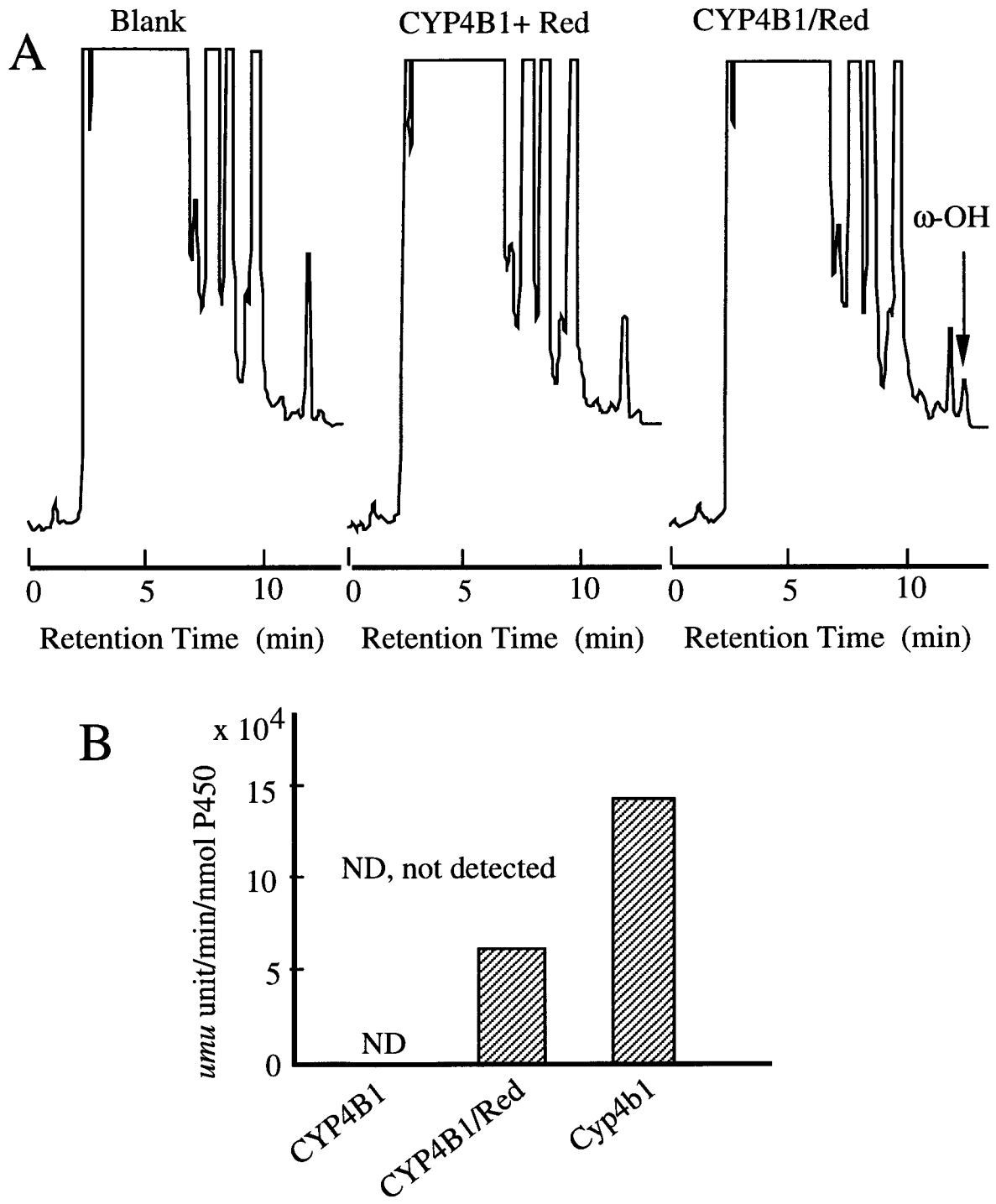

Fig. 5 Catalytic activities of human CYP4B1 expressed in yeast cells. Lauric acid (100 μM) was reacted with yeast microsomes (500 μg) expressing CYP4B1 alone or the fusion enzyme. Metabolites were analyzed by HPLC (A). An *umu* test using 2-aminofluorene was performed in the presence of the yeast microsomes (50 μg).

9.4 DISCUSSION

A transgenic mouse line that expresses human CYP4B1 in the liver was generated using a construct containing the human CYP4B1 cDNA connected to the apo E gene promoter. The liver-specific promoter was chosen to express CYP4B1 because constitutive mouse Cyp4b1 was not expressed in mouse liver and liver expressed sufficient amounts of NADPH-cytochrome P450 reductase and cytochrome b_5, which are necessary for CYP4B1 to reveal the activity. Heterologous expression of human CYP4B1 has not succeeded. Expressed proteins were a form of P420 that did not have any activity (11, 21). Zheng *et al.* (11) reported that amino acid exchange at the 427 position from Pro to Ser, which is conserved in many cytochrome P450s, renders human CYP4B1 unstable. In the present study, we succeeded in expressing active human CYP4B1 by constructing the fusion protein in yeast cells or by introducing CYP4B1 gene into transgenic mice. Recently, Yamamoto *et al.* (22) expressed CYP2D2 in insect cells with a baculovirus system, but they obtained a form of P420 only. We were able to express active CYP2D2 in yeast cells by co-expression of NADPH-cytochrome P450 reductase (23). Insect cells have very low levels of NADPH-cytochrome P450 reductase, but our yeast system expressed sufficient amounts of reductase, suggesting that the reductase stabilized CYP2D2 in the yeast cells. In the current study, it was possible that CYP4B1 was stabilized by the reductase and cytochrome b_5. Furthermore, CYP4B1 cDNA isolated in this study showed insertion of the Ser residue at the 207 position. This finding is differs from those reported by Nhamburo *et al.* (10) and Zheng *et al.* (11). Nonetheless, the nucleotide sequence of 50 human genes was investigated and it appeared that all of them had the Ser insertion, thus suggesting that this type of CYP4B1 is a major variant in humans (data not shown). The Ser insertion may also stabilize CYP4B1. As native human CYP4B1 has not yet been purified, it cannot simply assumed that human CYP4B1 acts as a catalyst. However, there is some evidence from our previous study suggesting that human CYP4B1 does indeed posses catalytic activity (12). We have detected CYP4B1 by immunoblot analysis of human kidney and bladder tissues. These microsomes revealed lauric acid ω-hydroxylation activity and mutagenic activation of 2-AF, which are typical for CYP4B1. These activities were inhibited by CYP4B1 antibody (12).

Recently, several interesting studies have discussed the biological role of CYP4B1 (13, 24, 25). The rabbit corneal epithelium metabolizes arachidonic acid to 12-hydroxy-5,8,11, 14-eicosatetraenoic acid (12(R)-HETE) and 12-hydroxy-5,8, 14-eicosatrienoic acid (12(R)- HETrE), both of which possess potent inflammatory properties. Rabbit CYP4B1 contributes to the production of these endogenous metabolites and it is induced under ischemic conditions (24). Mastyugin *et al.* (24) assume the role of inflammatory mediators in hypoxia- and chemical-induced injury in

the cornea; CYP4B1 may play an important role in such a biological pathway. The CYP4B1 transgenic mice can also be used as an in vivo system to study human CYP4B1-mediated endogenous metabolism. For instance, analyzing in vivo reactions of arachidonic acid metabolites produced by human CYP4B1 may provide information about such endogenous metabolism.

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CHAPTER 10
10.1 ABBREVIATIONS

2-AA	2-aminoanthracene
2-AAF	2-acetylaminofluorene
2-AF	2-aminofluorene
apo E	apolipoprotein E
CYP or P450	cytochrome P450
DCB	3,3'-dichlorobenzidine
DEAE	diethylaminoethyl
DLPC	dilauroylphosphatidylcholine
DM	diabetes mellitus
DMAB	3,2'-dimethyl-4-aminobiphenyl
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EET	epoxyeicosatrienoic acid
Fam	6-carboxyfluorescein
HETE	hydroxyeicosatetraenoic acid
HPLC	high-performance liquid chromatography
MeIQ	2-Amino-3,5-dimethylimidazo[4,5-f]quinoline
3-MeO-AAB	3-methoxy-4-aminoazobenzene
2-NA	2-naphthylamine
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethylsulfonylfluoride
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS	sodium dodecyl sulfate
STZ	streptozotocin
Tamra	6-carboxy-tetramethylrhodamine

10.2 ACKNOWLEDGEMENTS

I express my courteous thanks to Prof. Yoshihiko Funae of Osaka City University Medical School for valuable suggestions and encouragement through this study.
I wish to thank Prof. Toshiharu Hase, Prof. Satoru Kawamura, and Prof. Seiki Kuramitsu of Osaka University Graduate School and Prof. Norio Shimamoto of Takeda Chemical Industries for their helpful suggestions and criticisms.
I am indebted to my collaborators at Keio University, Tohoku University, Hokkaido University, Osaka Prefectural Institute of Public Health, and University of Osaka Prefecture, National Institutes of Health of USA and Osaka City University and to my colleagues at Department of Chemical Biology of Osaka City University Medical School.

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