The genetic bases of inter- and intrastrain differences in CYP2D-dependent drug metabolism in rats

（ラットにおけるCYP2Dの系統差及び個体差の分子機構の解明）

Noriaki Sakai

Contents

Abbreviations

BN: Brown Norway

C/EBP: CCAAT/enhancer binding protein

ChIP: Chromatin immunoprecipitation

Ct: Cycle threshold

DA: Dark Agouti

EM: Extensive metabolizer

EMSA: Electrophoresis mobility shift assay

EM-W: EM from Wistar rat

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GATA: GATA binding protein

G-6-P: Glucose-6-phosphate

G-6-PDH: Glucose-6-phosphate dehydrogenase

hnRNP K: heterogeneous nuclear ribonulceoprotein K

HPLC: High performance liquid chromatography

MALDI-TOF: Matrix-assisted laser desorption/ionization time-of-flight

NMD: Nonsense-mediated mRNA decay

PM: Poor metabolizer

PM-W: PM from Wistar rat

PVDF: Polyvinylidene difluoride

P450: Cytochrome P450

RFLP: Restriction fragment length polymorphism

SD: Sprague-Dawley

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sp1: Specificity protein 1

STAT: Signal transducers and activators of transcription

TFA: Trifluoroacetic acid

Preface

Knowledge of the genome in many organisms is expanding rapidly. In February 2001, the entire nucleotide sequence of human was published (International human genome sequencing consortium, 2001), drawing scientific attentions to the complex networks of genes and their products, and to the complex relations between genes, diseases and drug response. The increase in knowledge has raised the concept of tailor-made medicine. Information of the individual phenotype for metabolizing enzymes can aid in the choice of the most suitable drug, in the adjustment of the dose that is given, and in predicting interactions between different drugs as a result of pharmacokinetic characteristics. The greatest importance on the interindividual differences in drug response, at present, is exerted by the differences in capacity for drug metabolism caused by genetic polymorphism or by inhibition or induction of drug metabolism. Besides drug-drug interactions, pathophysiological factors and environmental factors, the genetics of the drug metabolizing enzymes play a critical role for understanding interindividual differences in drug response and adverse drug reaction. The highest impact among drug metabolizing enzymes is exerted by the cytochrome P450 (P450, CYP), the major phase I enzyme.

P450 is the collective term for a superfamily of heme-containing monooxygenases. Based on the amino acid similarity, P450 enzymes are classified into families (>40% similarity) and subfamilies (>55% similarity) (Nelson *et al.*, 1996). The P450 system is exploited by many organisms in the biosynthesis of endogenous compounds like steroids, vitamins, fatty acids, prostaglandins, leukotrienes, biogenic amines and pheromones. However, the majority of P450 substrates are foreign chemicals, such as drugs and environmental pollutants.

Three families (CYP1, CYP2 and CYP3) are mainly involved in biotransformation of xenobiotics (Kobayashi et al., 2002). Among these, CYP2C9, CYP2C19 and CYP2D6 are highly polymorphic and together account for about 40% of hepatic human phase I metabolism (Ingelman-Sundberg, 2005).

The CYP2D subfamily oxidizes endogenous steroids (Harada and Negishi 1984; Kishimoto et al., 2004) and a large number of drugs (Brosen 1990; Eichelbaum and Gross 1990), including debrisoquine (Distlerath et al., 1985; Kobayashi et al., 1989), sparteine (Osikowska-Evers et al., 1987), bufuralol (Gut et al., 1986), propranolol (Raghuram et al., 1984; Fujita et al., 1993), imipramine (Brosen et al., 1986) and bunitrolol (Narimatsu et al., 1994; Yamamoto et al., 1996,1998) (Table 1).

The CYP2D subfamily was extensively studied from the 1980s due in large part to the role of CYP2D6 in the human debrisoquine/sparteine drug oxidation polymorphism. Accumulating reports revealed that polymorphisms in debrisoquine metabolism are caused by the lack of full expression of CYP2D6 (Gonzalez et al., 1988; Gough et al., 1990). The human enzyme CYP2D6 has received much attention because of its polymorphic expression under genetic control. More than 80 CYP2D6 alleles have been classified by the CYP Allele Nomenclature Committee (http://www.imm.ki.se/CYPalleles/cyp2d6.htm).

Six genes homologues to the human CYP2D6, CYP2D1 through CYP2D5 and CYP2D18, are found in rats. Among these six isoforms, CYP2D5 and CYP2D18 have over 95% homology in amino acid sequence to CYP2D1 and CYP2D4, respectively. CYP2D2 and CYP2D3 mRNAs are mainly expressed in the liver, kidney, and small intestinal mucosa. CYP2D1 and CYP2D5 mRNAs are expressed in various tissues. CYP2D4 and CYP2D18 mRNAs are expressed in liver, kidney, small intestinal mucosa, brain, adrenal gland, ovary, testis and gonecystis. However,

very little study of differences in catalytic properties among these six isoforms is reported.

The polymorphisms in CYP2D-dependent drug metabolisms are also found in rats. The rat CYP2D isoforms have an overlapping substrate specificity with the human CYP2D6. Therefore, rat strains are used as animal models for human poor or extensive metabolizer (PM and EM, respectively). In particular, the female Dark Agouti (DA) rat is used as an animal model for the PM phenotype because of their impaired ability of the CYP2D2 protein to metabolize debrisoquine (Gonzalez et al., 1987), bufuralol (Boobis et al., 1986; Gonzalez et al., 1987) and bunitrolol (Suzuki et al., 1992; Yamamoto et al., 1996, 1998). On the other hand, the Sprague-Dawley (SD) rat is often used as the EM counterpart (Chu et al., 1996; Colado et al., 1995; Stresser et al., 2002). Thus, to clarify the molecular bases of inter- and intrastrain differences catalyzed by the CYP2D subfamily among rat strains is essential to predict the effects of drugs on human. Nevertheless, limited information on genetic variations in rat strains is available compared with human.

Here, I clarified the mechanisms underlying inter- and intrastrain differences in CYP2D-dependent drug metabolism in rat strains. In chapter 1, I elucidated the mechanism of low expression of CYP2D2 mRNA in DA rats, animal model for the PM phenotype of CYP2D6. In chapter 2, I identified CYP2D3 as the major isoform involving in diazepam *p*-hydroxylation, the major metabolic pathway of diazepam at low concentration, in rats. In addition, I explored the relationships between genetic polymorphisms and inter- and intrastrain differences in diazepam *p*-hydroxylation.

Table 1. Drugs known to be substrates or to interact with the CYP2D

Major classes of drugs as substrate

- Antidepressants
  - Tricyclic antidepressants
  - Serotonin reuptake inhibitors
- Neuroleptics
- Beta-blockers
- Antiarrhytmics

Specific drugs as substrate

- Alprenol
- Amiflamine
- Aprindine
- Bufuralol
- Bunitrolol
- Bupranolol
- Chlorpropamide
- Clomipramine
- Clozapine
- Codeine
- Debrisoquine
- Desimipramine
- Desmethylcitalopram
- Dextromethorphan
- Dihydrocodeine
- Encainide
- Ethylmorphine
- Flecainide
- Flunarizine
- Fluperlapine
- Guanoxan
- Haloperidol
- Hydrocodone
- Imipramine
- Indoramin
- Maprotiline
- Methoxyamphetamine
- Methoxyphenamine
- Metiamide
- Metoprolol
- Mexiletine
- Nortriptyline
- Ondansetron
- Otycodone
- Perhexiline
- Perphenaxine
- Phenacetin
- Phenformin
- Propafenone
- Propranolol
- Risiperidone
- Thioridazine
- Timolol
- Tomoxetine
- Tropisetron
- Zuclopenthixol

Inhibitors, drugs

- Chinidin
- Fluoxetin
- Levomepromizine
- Lobelin
- Methadone
- Paroxetine
- Quinidine
- Trifluperidol

Inhibitors, alkaloids

- Ajmalicine
- Berberine
- Coniine
- Ergotamine
- Gramine
- Harmaline
- Laudanosine
- Sempervirine
- Vincamine
- Vinblastine

Chapter 1
Mechanism of low expression of CYP2D2 mRNA in Dark Agouti rats

Introduction

About 30 % of clinically used drugs are metabolized, at least in part, by CYP2D6 despite its low hepatic abundance (Gonzalez, 1996, Shimada et al., 1994). In addition, the existence of both numerous allelic variants and the multiplicity of gene copies complicate the prediction of drug plasma levels. Thus, CYP2D6 is one of the most important polymorphic drug-metabolizing enzymes causing interindividual differences in drug response. In cases where the drug clearance is dependent on CYP2D6, adverse effects due to elevated drug plasma levels occur more frequently in PM than in EM. Such adverse effects are well documented in the metabolism of typical CYP2D6 substrates debrisoquine, spartine and bufuralol.

Adult DA rats have been used as an animal model of the CYP2D6 PM phenotype because their metabolic activity of typical CYP2D6 substrates is significantly lower than that of other rat strains. In particular, DA rats have very low levels of debrisoquine 4-hydroxylation activity. Accordingly, DA rats have been frequently used for the study of CYP2D substrate metabolism as the unique animal model of a PM phenotype for CYP2D6 (Colado et al., 1995, Chu et al., 1996, Masubuchi et al., 1993).

Originally, it was reported that CYP2D1 mRNA is not expressed in the DA rat strain (Matsunaga et al., 1989). Furthermore, only CYP2D1 expression was capable of metabolizing bufurarol, while other CYP2D isoforms was not. Thus, the cause of the deficiency in CYP2D-dependent activity in DA rats was suggested to be the lack of expression of CYP2D1 mRNA. Subsequently, however, several groups

reported that CYP2D2 protein purified from rat hepatic microsomes possessed high catalytic activities toward typical CYP2D6 substrates (Suzuki et al., 1992, Ohishi et al., 1993). In 1998, Yamamoto et al. reported that DA rats exhibited lower levels of CYP2D2 expression of both mRNA and protein than SD rats. Thus, it is at present clear that the deficiency in CYP2D-dependent activity in DA rats is caused by the low level of CYP2D2 mRNA expression.

In contrast to other hepatic xenobiotic-metabolizing P450s, the expression of the CYP2D subfamily seems to be not regulated by any known environmental agent such as polycyclic aromatic hydrocarbons, ethanol or phenobarbital, and is not inducible by known hormones such as steroid and thyroid hormones. Actually, some research groups have reported that several liver-enriched transcription factors and more ubiquitously expressed factors are responsible for the constitutive expression of the CYP2D subfamily in humans and rodents (Cairns et al., 1996, Yokomori et al., 1995, Lee et al., 1994). However, although the transcriptional mechanisms of other CYP2D isoforms in rats are well defined, the transcriptional regulation of CYP2D2 remains to be elucidated. Also, the molecular mechanisms of low expression of CYP2D2 mRNA in DA rats are still unknown. To fully understand the interstrain differences in the CYP2D2 expression, it is essential to clarify the mechanistic bases of gene regulation.

In this chapter, I elucidated a single nucleotide substitution in the promoter region of the CYP2D2 gene, resulting in its attenuated expression of mRNA in DA rats. This is the first report that a mutation alters the expression level of CYP2D2 in rats. In addition, I identified heterogeneous nuclear ribonucleoprotein K (hnRNP K), which serves as a molecular scaffold to bind to the core promoter of the CYP2D2 gene where the mutation was observed in DA rats.

Materials and methods

Materials and animals

All oligonucleotides containing 3'-biotin-labeled oligonucleotides were purchased from Hokkaido System Science (Sapporo, Japan). Specificity protein 1 (Sp1) consensus oligonucleotide was purchased from Promega (Madison, WI). CCAAT/enhancer binding protein (C/EBP), GATA binding protein (GATA), signal transducers and activators of transcription (STAT) 1, STAT3, STAT4, STAT5 and STAT5/6 consensus oligonucleotides were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All chemical reagents were analytical grade.

Adult male and female SD and DA rats (9 weeks old) were obtained from Nihon SLC Co. (Shizuoka, Japan). They were housed under standard laboratory conditions with free access to food and water, and were used for experiments after 1 week of acclimatization. All experiments using animals were performed with the supervision and approval of the Animal Care and Use Committee of Hokkaido University.

Cell lines and culture conditions

H4-II-E rat hepatoma cells and HepG2 human hepatoma cells were obtained from the American Type Culture Collection (Manassas, VA) and the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan), respectively. The cells were routinely grown in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) with 10% fetal bovine serum. Cells were cultured at 37°C in 5% CO_2 in air.

Isolation of genomic DNA from rat liver

Genomic DNA was isolated from rat liver using a DNeasy Tissue Kit

(Qiagen, Hilden, Germany). The concentration and purity of DNA were determined by using a spectrophotometer. The integrity was examined by electrophoresis in a 1% agarose gel with ethidium bromide staining.

Sequencing of 5'-upstream region of CYP2D2 gene

Sequencing was carried out using genomic DNA samples isolated from SD and DA rats of both sexes. Sequence analysis of a -4127/+3 bp 5'-upstream region of the CYP2D2 gene was performed, and sequences of SD and DA rats were compared. The PCR-amplified fragments were subsequently cloned into a pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA). More than 10 cDNA clones were analyzed to identify a mutation and repeatedly cloned from other samples to exclude PCR errors. The nucleotide sequence was analyzed with a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an automated DNA sequencer (ABI Prism 310 Genetic Analyzer) following the manufacturer's instructions.

CYP2D2 reporter plasmid construction

Various lengths of the 5'-flanking region of the CYP2D2 gene were amplified using the genomic DNA samples with the forward and reverse primers (Table 2). All reaction conditions were as follows: denaturation for 1 min at 94°C, annealing for 1 min at 60°C, and extension for 1 min at 72°C for 35 cycles. The PCR product was cloned into a pCR2.1-TOPO vector (Invitrogen). After confirmation of the correct sequence, this 5'-flanking fragment was inserted into a pGL3-Basic vector (Promega) using *Asp718* and *XhoI* sites. The resulting deletion mutants transcribed the firefly luciferase gene under the control of the 5'-flanking region of the CYP2D2 gene. The structures of these deletion mutants are shown in Fig. 2A

Reporter assay

HepG2 cells were transfected with 100 ng of renilla luciferase expression plasmid pRL-SV40 (Promega) and 1 μg of one of the following firefly luciferase expression plasmids: pGL3-514, pGL3-284, pGL3-166, pGL3-166M, pGL3-132, pGL3-79, or pGL3-Basic (Promega) by using FuGENE 6 transfection reagent (Roche, Lewes, UK). The reporters were cotransfected with 600 ng of pCMV6-hnRNP K expression vector (ORIGENE, Rockville, MD). At 48 h after the transfection, the cells were harvested and the luciferase activity was analyzed using Dual-Glo Luciferase Assay System (Promega) with a Mithras LB 940 luminometer (Berthold Technologies, Bad Wildbad, Germany). Firefly luciferase activity was normalized to renilla luciferase activity. These assays were repeated more than three times. Statistic analysis was performed using Student's *t*-test. Differences were considered significant if $P<0.05$.

Nuclear extraction

For the electrophoresis mobility shift assay (EMSA), nuclear extract from H4-II-E cells was prepared by the mini-extract method of Schreiber et al. (1989) with slight modification. Typically, cells were grown to a density of 1×10^6 to 2×10^6/ml, and approximately 10^7 cells were removed to prepare the mini-extract and frozen immediately without dialysis. Nuclear extract from rat livers was prepared as described by Gorski et al. (1986) with slight modification. In brief, minced rat liver (10 g) was homogenized in 5 volumes of buffer A (10 mM Hepes, pH 7.9, 15 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2.2 M sucrose, 5% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol and 14 μg/ml aprotinin) using a Teflon-glass homogenizer. The filtered homogenate was layered

over 10 ml of buffer B (buffer A with 10% glycerol) and then centrifuged at 76,000 g for 50 min at 4°C in P50-AT2 rotor. The resulting pellets were suspended with a volume of 5 ml of buffer C (10 mM Hepes, pH 7.9, 100 mM KCl, 1 mM EDTA, 10% glycerol, 3 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol and 14 μg/ml aprotinin). After adjusting the final concentration of KCl to 0.55 M, the suspension was gently agitated for 15 min. This suspension was centrifuged at 113,000 g for 60 min at 4°C. The supernatant volume was measured, and powdered ammonium sulfate (0.3 mg/ml) was added. The solution was gently stirred for 30 min. The precipitated proteins were sedimented by a centrifugation at 100,000 g for 30 min. The protein pellet was then dissolved in the dialysis buffer (25 mM Hepes, pH 7.9, 40 mM KCl, 0.1 mM EDTA, 10% glycerol and 1 mM dithiothreitol) and dialyzed against the same buffer overnight with one change of buffer. Protein precipitates formed during dialysis were removed by centrifugation in a microcentrifuge at the end of dialysis. The resulting supernatant was the final nuclear preparation. The protein concentrations of all extracts were determined by the Bradford assay with bovine serum albumin as the standard (Bio-Rad, Hercules, CA).

EMSA

A biotin-labeled double-strand DNA probe containing the promoter region of the CYP2D2 gene was synthesized by heating sense and antisense oligonucleotides in sterilized water for 5 min at 95°C, and then slowly cooling to room temperature over a 4 h period. Oligonucleotides represented the following sequences: probe WT-50, positions -129 to -80; probe WT-20, positions -113 to -94; or probe M-20, the same positions as WT-20 but containing C-104T transition (numbered from the primary transcription site). The homogeneity of annealed oligomers was examined

by gel electrophoresis.

With a LightShift Chemiluminescence EMSA kit (Pierce, Rockford, IL), the reaction mixture for the EMSA contained 10 mM Tris-HCl, pH 7.9, 50 mM KCl, 1 mM dithiothreitol, 50 ng of poly(dI·dC) (GE Health Care, Piscataway, NJ) and 5 μg of nuclear extract. After preincubation for 10 min, biotin-labeled probe DNA (20 fmol) was added to the mixture, and the binding reaction was incubated for 20 min at room temperature. The product was then resolved by electrophoresis through a 5% native polyacrylamide gel in 25 mM Tris borate, 1 mM EDTA buffer, pH 8.0. Electrophoresis was performed at 4°C at 80 V. The separated proteins were transferred to a Hybond-N^+ membrane (GE Health Care). After ultraviolet cross-linking, the membrane was blocked and incubated with LightShift-stabilized streptoavidin-horseradish peroxydase conjugate (Pierce) for 60 min. Positive reactions were detected by incubating the membrane in LightShift Luminol/Enhancer solution (Pierce). All procedures were performed according to the manufacturer's instructions. For competition assays, 100-400 fold molar excesses of unlabeled, competitor DNA were pre-incubated with nuclear extracts for 30 min at room temperature. The supershift gel mobility assay was performed with minor changes. The nuclear extract was preincubated with the specific antibody to hnRNP K (Santa Cruz Biotechnology) for 30 min at room temperature before incubation with the labeled probe, as described above, followed by loading onto the gel.

DNA affinity precipitation assay

DNA affinity precipitation assay was performed as described by Suzuki et al. (1993) with slight modification. In brief, the biotin-labeled double-stranded DNA probe (150 pmol, 1 μg) was mixed with 100 μg HeLa nuclear extract (Promega) containing the buffer used in EMSA and poly(dI·dC) (15 μg) in the presence or

absence of the competitor, and the mixture was incubated for 20 min at room temperature. Then, streptoavidin-Dynabeads (Dynal, Oslo, Norway) were added with mixing by rotation for 30 min. The Dynabeads were collected with a magnet and washed twice with the same buffer. The trapped proteins were resolved by electrophoresis through a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel, and stained using Coomassie brilliant blue R-250. The stained gels were subsequently utilized for the in-gel digestion.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry

Sample purification for MALDI-TOF mass spectrometry was performed as described by Kumarathasan et al. (2005) with slight modification. Protein bands were excised and destained with 25 mM NH_4HCO_3 in 30% acetonitrile at room temperature for 10 min. After removing the solvent, the gels were dried using an evaporator. Dried gels were incubated in a reduction buffer (25 mM NH_4HCO_3, and 10 mM dithiothreitol) at 56°C for 1 h, and then alkylated with 55 mM iodoacetamide at room temperature for 45 min with shading. After alkylation, the gels were dried and digested with trypsin solution (2 μg trypsin in 200 μl of 50 mM NH_4HCO_3), initially for 30 min on ice, then left at 37°C for 16 h. After incubation, gels were extracted with 50% acetonitrile and 5% trifluoroacetic acid (TFA) in a shaker. The supernatant was collected, dried and stored at -80°C until mass spectrometry analysis.

The peptides were dissolved with 0.1% TFA, desalted and cleaned with C18 ZipTip pipette tips (Millipore, Billerica, MA). Co-elution was performed directly onto the MALDI target with 2 μl α-cyano-4-hydroxycinnamic acid matrix (5 mg/ml in 50% acetonitrile, 0.1% TFA) (Bruker Daltonics, Billerica, MA). Mass spectra were

obtained on a Bruker Autoflex MALDI-TOF mass spectrometer in reflector mode. Protein database searching was performed using the Mascot program (Matrix Science, London, UK) with 100 ppm mass tolerance. Scores greater than 66 were considered significant, meaning that for scores higher than 66 the probability of that the match is a random event is lower than 0.05.

Chromatin immunoprecipitation (ChIP) assay

H4-II-E cells were grown to 70% confluency on three 15 cm plates. Transcriptional factors were cross-linked to DNA by adding 1% formaldehyde directly to the culture medium for 15 min at 37°C. An EZ ChIP Chromatin Immunoprecipitation kit (Upstate, Chicago, IL) was used with some modifications. The cells were lysed with SDS lysis buffer containing protease inhibitors. Cell lysates were enzymatically treated to shear DNA in a range of 200-1000 bp with an Enzymatic Shearing Kit (Active Motif, Carlsbad, CA). Cross-links of the chromatin were, in part, recovered for visualization of shearing efficiency on an agarose gel. Twenty-five μl of chromatin in the supernatant of sheared cell lysates was saved as input DNA, whereas the rest of the chromatin in the supernatant was utilized for immunoprecipitation. Fifty μl of chromatin was added to 100 μl of anti-hnRNP K antibody/magnetic bead mixture, which was prepared as described by Lee et al. (2006), and incubated overnight on a rotator at 4°C. Anti-mouse IgG and anti-RNA polymerase II antibodies were also included to prepare DNA as a negative control and a ChIP quality control, respectively. After immunoprecipitation, hnRNP K-bound chromatin was then dissociated with proteins through the procedures recommended by the manufacturer. The purified DNA from hnRNP K-bound chromatin and controls was finally used as a template for PCR amplification. The primers used for the amplification of CYP2D2 were as follows: Forward,

5'-AAAGGGCAAGAACCTCTGATG-3' and Reverse, 5'-GAGCCAAGTAGCTGTGT TAAT-3'. The primers used for the amplification of glyceraldehde-3-phosphate dehydrogenase (GAPDH), which was cross-linked to acetyl histone H3, were also designed for quality control of DNA: GAPDH-F, 5'-CATTGACCTCAACTACATGG-3'; and GAPDH-R, 5'-TGACCAGCTTCCCATTCTCA-3'. One μl of template was added to 20 μl of PCR mixture containing 0.5 units of Ex Taq (TaKaRa Bio, Shiga, Japan), 0.2 mM dNTPs, 1x PCR buffer with $MgCl_2$, and 500 nM of each primer. PCR conditions were as follows: 94°C for 4 min for 1 cycle, then 94°C for 30 seconds, 61°C for 30 seconds, 72°C for 30 seconds for 30 cycles, and a final extension at 72°C for 7 min.

Table 2. Oligonucleotide primers used for construction of reporter vectors

Primer	Sequence 5' to 3'	Positions	Construction
1	TTCACAGGCTGGAGTTACCTT	-514 to -494	pGL3-514
2	GCACACTGAGGAAGGGTTCA	-284 to -265	pGL3-284
3	GGAGAGAAGAAAGGGCAAGAA	-166 to -146	pGL3-166, 166M
4	TTTTCCCTGGACTTTTCTGA	-132 to -113	pGL3-132
5	TTAACACAGCTACTTGGC	-79 to -62	pGL3-79
6	TACAACAGACTGGGAACCTGG	+3 to -18	All

Results

Sequencing and comparison of 5'-flanking region of CYP2D2 gene of SD and DA rats

The expression level of CYP2D2 mRNA has previously been demonstrated to be significantly lower in DA rats than in SD rats. Sex differences have also been reported (male SD>female SD>male DA>female DA) (Yamamoto et al., 1998). To compare the transcriptional regulatory region between SD and DA rats, I cloned and sequenced a segment from the primary transcription site to 4 kb upstream of the CYP2D2 gene (Fig. 1). Then, I compared the analyzed sequence with SD rat-derived data from the NCBI database. In comparison with the database, there was no mutation within the 4 kb sequence in SD rats. On the other hand, in DA rats, a cytosine-to-thymine substitution localized at nt -104 (numbered from the primary transcription site) was found in the analyzed sequence. This mutation (C-104T) was detected in both male and female DA rats. The putative TATA box is

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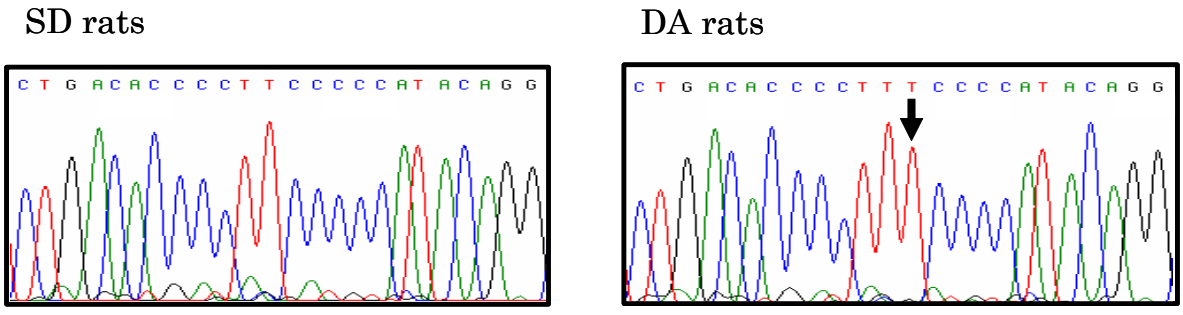

Fig. 1. Comparison of 5'-flanking regions of CYP2D2 gene of SD and DA rats

The 5'-flanking region corresponding to nucleotides -93 to -116 is shown. The substitution (C to T) was observed in male and female DA rats. This was the only mutation in the 4 kb sequence of the 5'-upstream region of CYP2D2. The mutation is indicated by an arrow.

present 26 nucleotides upstream from the transcription initiation site based on the TFSEARCH ver1.3 computer program (http://mbs.cbrc.jp/research/db/TFSEARCHJ.html). This mutation was located at 78 bp upstream from the TATA box, implying its impact on transcriptional activity.

Transcriptional activity of CYP2D2 gene

To identify the positive regulatory elements specifically responsible for the transcriptional regulation of the CYP2D2 gene, a series of deletion derivatives of the luciferase construct were constructed from 5'-flanking region of the CYP2D2 gene (Fig. 2A). These deletion mutants were transfected into HepG2 cells and luciferase activity was measured. The level of luciferase activity was normalized by pRL-SV40 plasmid. Fusion of the CYP2D2 nucleotide sequence derived from -514 to +3 upstream of the luciferase gene resulted in a 20-fold increase of luciferase activity compared to the empty pGL3 basic vector (Fig. 2B). This result indicates that a positive regulatory element is present in the region from -514 to +3 bases of the CYP2D2 gene. To further clarify the core region involved in the gene regulation, the effects of deletion constructs were analyzed in detail. Deletion to -79 bases decreased the luciferase activity by 70% compared with pGL3-132 control. These results indicate that the positive regulatory element locates in the region from -132 to -79 of the CYP2D2 gene.

To investigate the effect of the mutation on transcriptional activity, the deletion mutant containing a C-104T substitution (pGL3-166M) was transfected into HepG2 cells. The pGL3-166M construct showed about 30% luciferase activity compared to the pGL3-166 construct containing the wild-type (Fig. 2C). This result indicates that this single nucleotide substitution plays a crucial role on the low expression of CYP2D2 mRNA in DA rats.

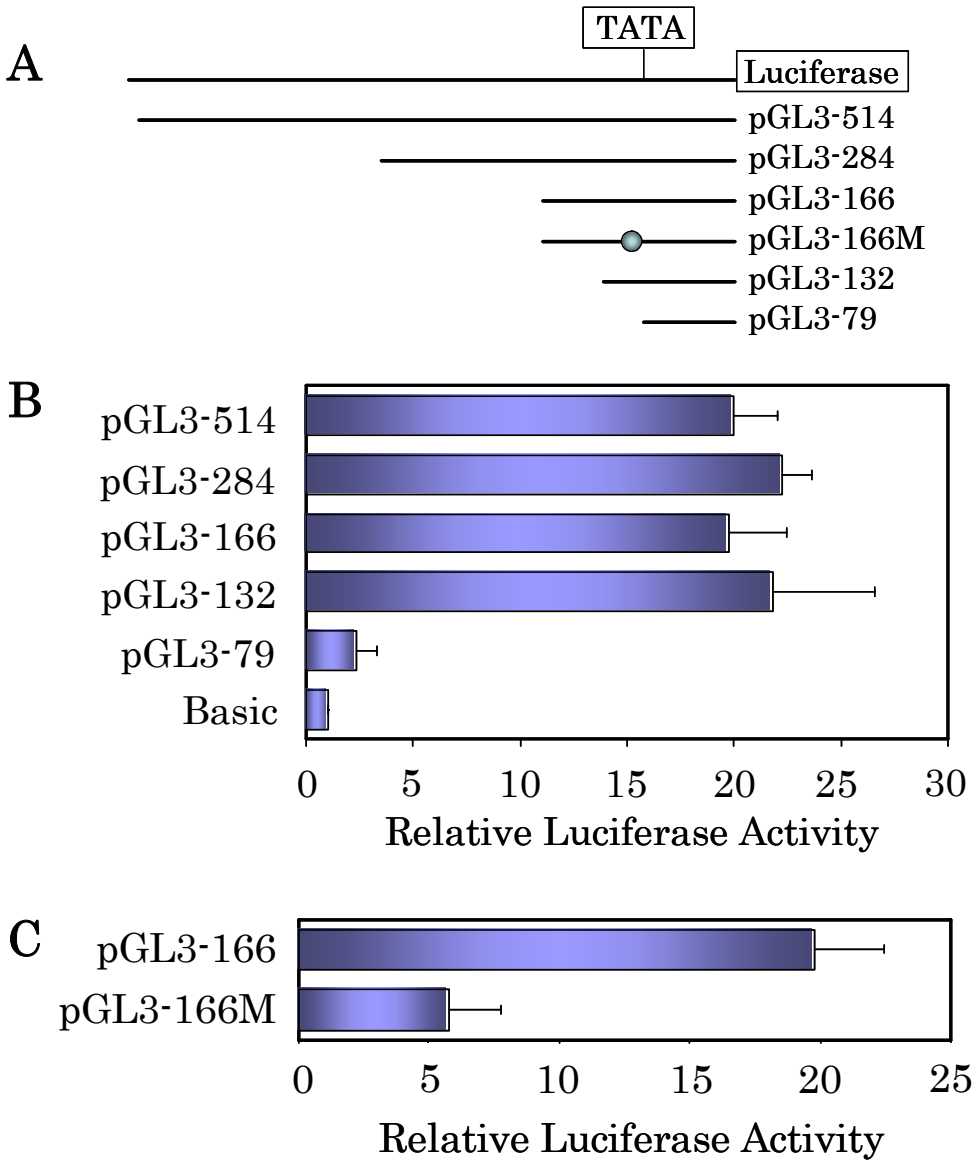

Fig. 2. Transcription activities of CYP2D2 deletion mutants in HepG2 cells

A, Luciferase reporter plasmids were constructed using pGL3-Basic vector (Promega) and 5'-flanking fragments of the CYP2D2 gene. The numbers given to the deletion mutants indicate the 5'-ends of the 5'-flanking sequence of CYP2D2. The position of the TATA box and the relative lengths and positions of the fragments (solid lines) are indicated. The substitution found in DA rats is indicated by a circle. B, Luciferase activities generated using the reporter plasmid were compared with that using the negative control plasmid (no insert) pGL3-Basic vector (Basic). These data represent means ± SEM of three independent experiments.

Effects of cis-element or trans-acting factors on binding affinity

EMSA was performed to detect nuclear protein complexes with the positive regulatory element of the CYP2D2 gene. The nucleotide sequence of the positive regulatory element of the CYP2D2 gene is shown in Fig. 3A, and the probes and competitors used for EMSA are also shown. The binding of the nuclear factor extracted from H4-II-E rat hepatoma cells to the WT-50 probe showed a single specific band, complex A (Fig. 3B, lane 2). To further investigate the core binding region of a nuclear protein to probe, EMSA was performed in the presence of a 100- to 400-fold molar excess of unlabeled oligonucleotides as shown in Fig. 3A. The band of complex A disappeared in the presence of competitor 2 (lanes 5-7). These results indicate that the nucleotide sequence from -94 to -113 is a core binding region of a transcription factor.

To clarify whether the probe with a mutation affects the binding affinity of the nuclear protein, I performed EMSA by mixing the nuclear protein extracted from SD rat liver and the WT-20 or M-20 probe (Fig. 4, lanes 1-4). The binding affinity of the nuclear protein to the mutant-type probe was drastically decreased compared to wild-type probe (lanes 2, 4). Subsequently, I compared the binding affinity of nuclear protein extracted from SD or DA rat liver to the WT-20 probe (lanes 5-7). There was no strain difference in the nuclear proteins in the binding affinity to wild-type probes (lanes 6, 7).

Identification of transcription factor involving CYP2D2 gene regulation

The core binding region of the transcription factor was analyzed using the TFSEARCH ver1.3 computer program. The transcription factors predicted by the TFSEARCH program are shown in Fig. 5A. Initially, to identify the nuclear factor constructing complex A, EMSA was carried out in the presence of 200-fold molar

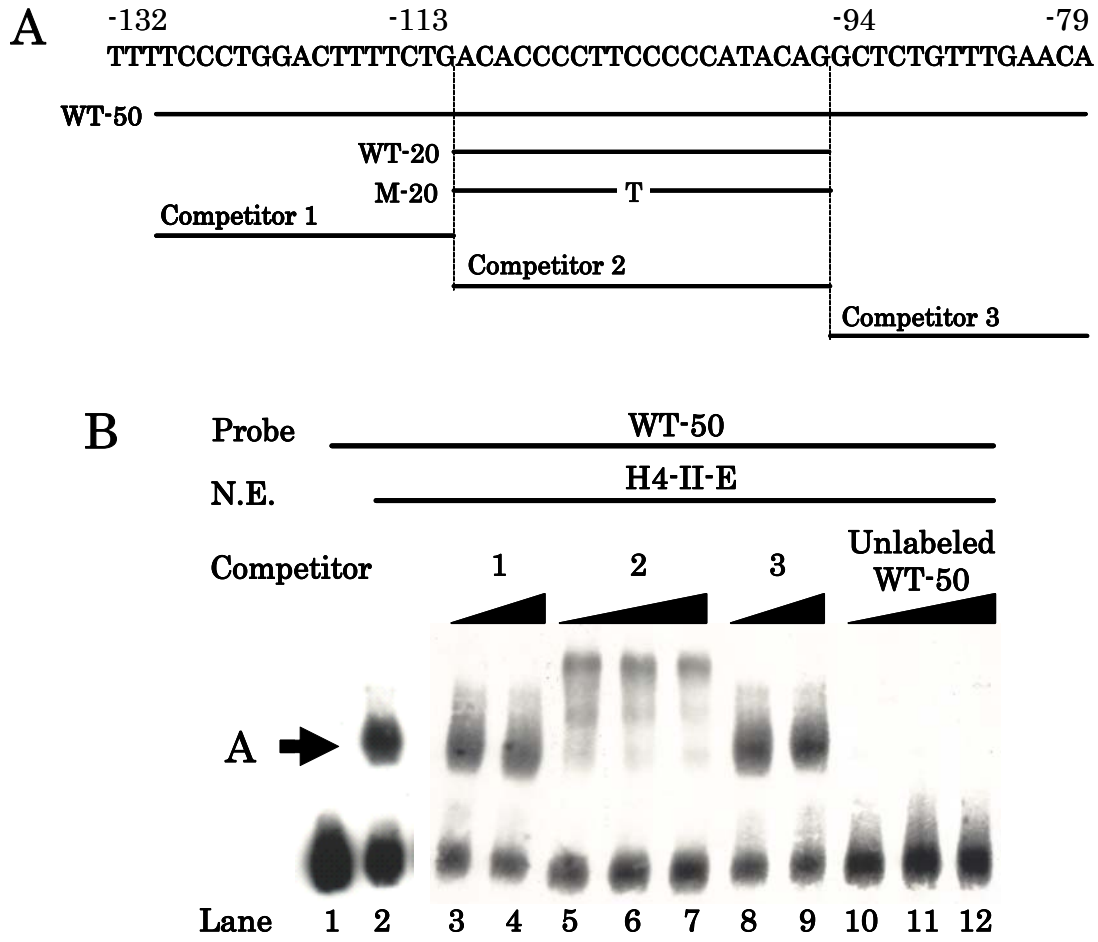

Fig. 3. EMSA of nuclear protein factors for transcriptional element of CYP2D2

A, The transcriptional element corresponding to the probes and competitors used for EMSA are shown. The solid lines represent each probe and competitor sequence. B, EMSA was performed as described in Materials and methods. Competition analysis was carried out by the addition of a 100- to 400-fold molar excess of the unlabeled competitors (lanes 3-12). In lanes 3 and 4, 200- and 400-fold molar excesses of competitor 1 were used. In lanes 5 to 7, 100-, 200- and 400-fold molar excesses of competitor 2 were used. In lanes 8 and 9, 200- and 400-fold molar excesses of competitor 3 were used. In lanes 10 to 12, 100-, 200- and 400-fold molar excesses of unlabeled WT-50 were used, respectively. N.E., nuclear extract.

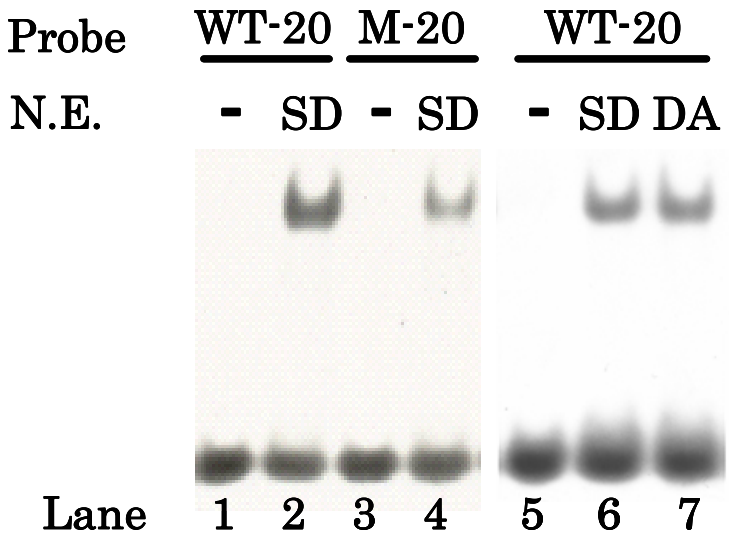

Fig. 4. Effect of cis-element or trans-acting factors on binding affinity

EMSA was performed as described in Materials and methods. The WT-20 probe was incubated with nuclear extracts prepared from SD rat liver for 20 minutes at room temperature (lane 2). The M-20 probe was incubated with nuclear extracts prepared from SD rat liver under the same conditions (lane 4). Under the same binding reaction conditions, the WT-20 probe was incubated with the nuclear protein extracted from SD and DA rat liver, respectively (lanes 6, 7).

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excess of unlabeled consensus oligonucleotides for the predicted transcriptional factors. However, all competitors examined in this study failed to abolish the band of complex A (Fig. 5B).

Then, I attempted to isolate the nuclear factor involved in the formation of complex A using a DNA affinity precipitation assay. The biotinylated WT-20 probe was incubated with a HeLa nuclear protein in the presence or absence of a 400-fold molar excess of the unlabeled WT-20, and the complexes formed were isolated with streptoavidin-conjugated magnet beads. The molecular weight of the isolated

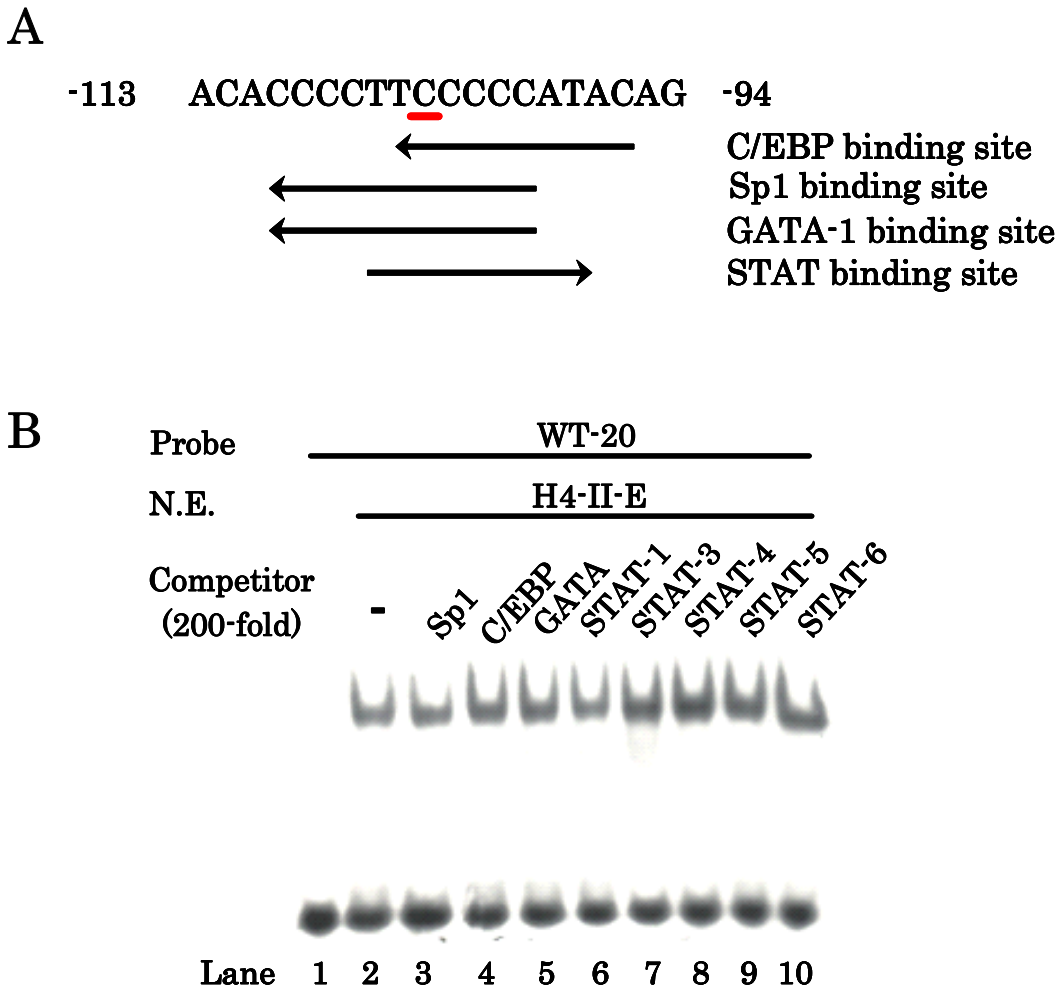

Fig. 5. Competition EMSA for transcription factors predicted by TFSEARCH

A, The nucleotide sequence between -113 to -94 is shown. Analysis for consensus transcription factor binding motifs was performed using the TFSEARCH program with a threshold of 70.0 points. Predicted binding sites for C/EBP, Sp1, GATA-1 and STAT are indicated by arrows. The mutated site found in DA rats is underlined. B, EMSA was performed as described in Materials and methods. A 200-fold molar excess of each competitor was added to the reaction mixture.

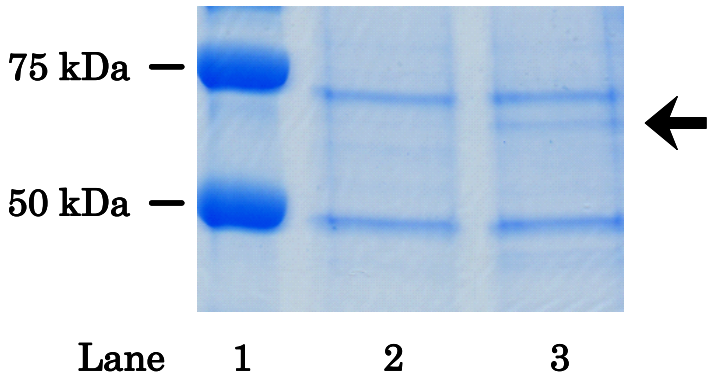

Fig. 6. Isolation of nuclear factor involving DNA-protein complex

A nuclear extract from HeLa cells was incubated with the biotinylated WT-20 probe in the presence or absence of the unlabeled WT-20 (lanes 2, 3). The DNA-protein complex was isolated, and analyzed by SDS-PAGE. The molecular weight of a protein was estimated by comparison with Bio-Rad precision protein standards (lane 1). The specific band involving the DNA-protein complex is indicated by an arrow.

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proteins was examined by SDS-PAGE (Fig. 6). Bands of about 70 kDa, 65 kDa and 50 kDa were detected in the absence of the unlabeled WT-20 (lane 3). On the other hand, the 65 kDa band completely disappeared after the addition of the unlabeled WT-20 (lane 2). Thus, I expected a 65 kDa protein to be the main transcription factor involved in CYP2D2 gene regulation.

Identification of the target 65 kDa protein was achieved by using mass spectrometry analysis. The protein band used for MALDI-TOF mass spectrometry analysis was excised from the polyacrylamide gel and subjected to trypsin digestion. The protein was successfully identified to be hnRNP K by peptide mass fingerprinting. A typical mass spectrum corresponding to tryptic digests of hnRNP K is shown in Fig. 7. Using MASCOT, the probability-based MOWSE score for

hnRNP K was 104 ($p<0.05$) with 12 peptide matches.

To confirm that hnRNP K protein binds to the core binding region in CYP2D2 gene regulation, a supershift assay using a specific antibody against hnRNP K was performed. A supershifted band appeared after addition of the antibody, and the shifted band increased in a dose-dependent manner (Fig. 8A, lanes 2-5). A ChIP assay was then performed to directly verify the binding of hnRNP K protein to the 5' flanking region of the CYP2D2 gene *in vivo*. Purified DNA was analyzed by PCR using specific primers for the CYP2D2 promoter. The PCR product was observed in the anti-RNA polymerase II sample, hnRNP K sample and input sample, but not in an anti-mouse IgG sample (Fig. 8B). These results indicate that hnRNP K protein can bind DNA both *in vitro* and *in vivo*, and they provide strong evidence that hnRNP K is associated with CYP2D2 gene regulation. To confirm the effect of hnRNP K on CYP2D2 transcription, the hnRNP K expression plasmid was cotransfected with pGL3-514 in HepG2 cells. Overexpression of hnRNP K stimulated the transcription activity (Fig. 8C). Thus, the fact that hnRNP K acts on the positive regulation of the CYP2D2 gene is consistent with results of the reporter assay using deletion mutants.

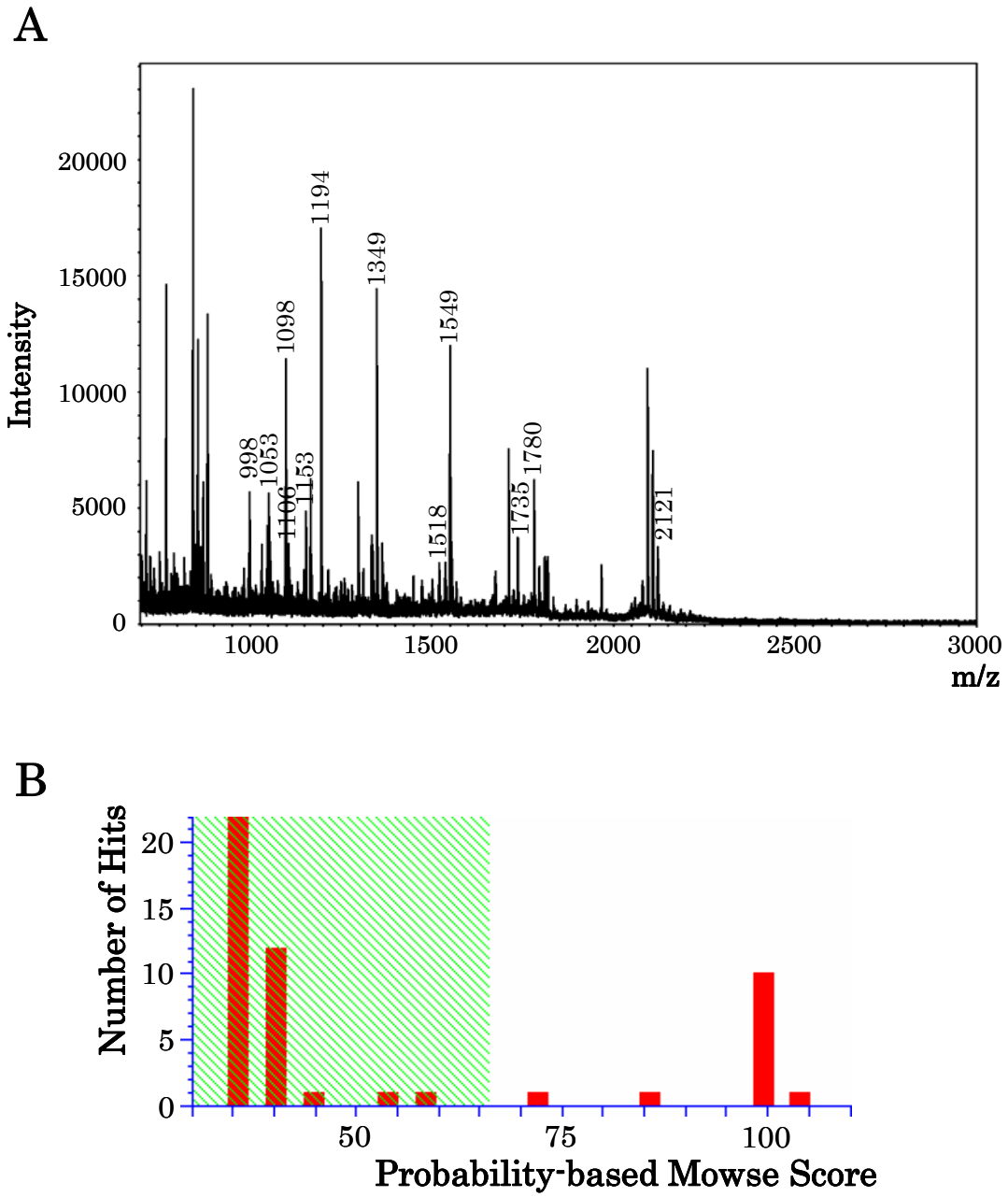


Fig. 7. MALDI mass spectrum and probability plot corresponding to hnRNP K

A, The m/z-value was used to identify the protein by searching protein databases. B, Identification of the protein was obtained with a high probability based score. A score higher than 66 was considered to be significant.

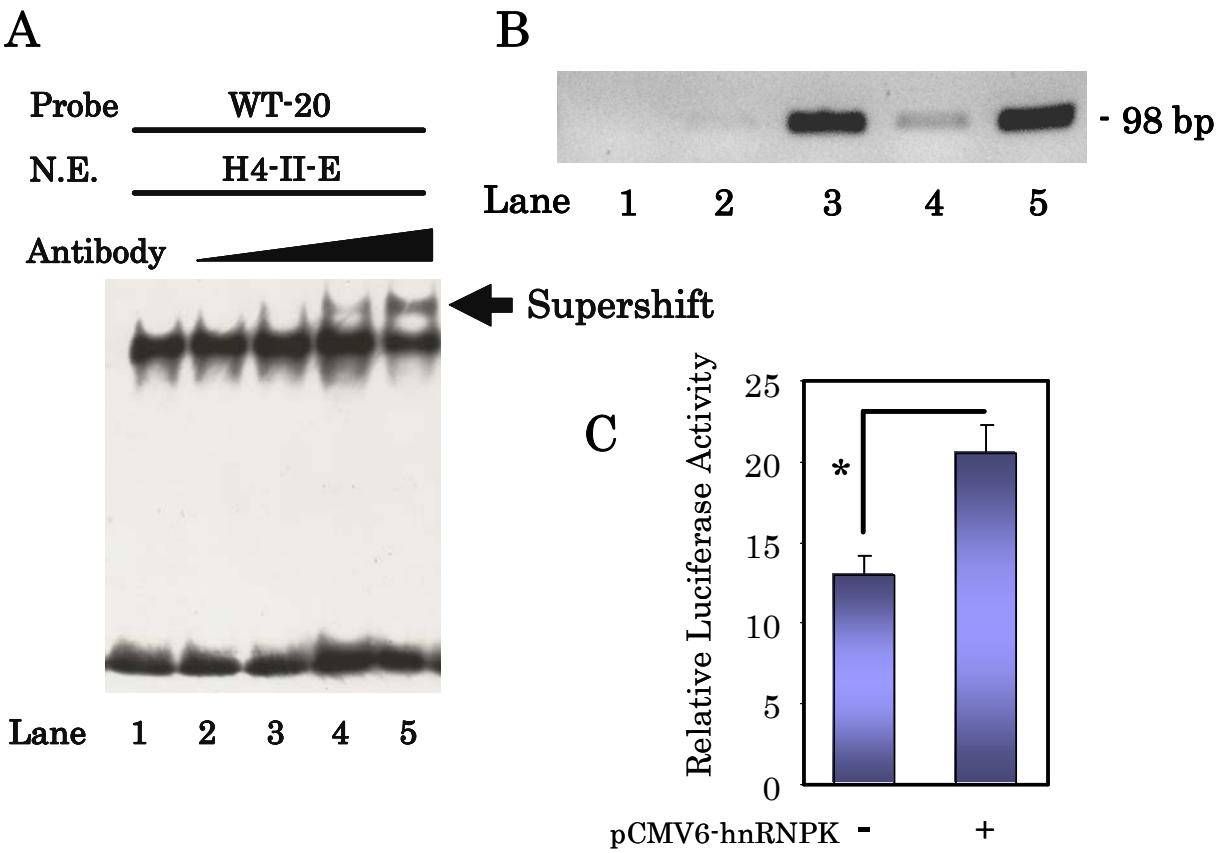


Fig. 8. hnRNP K protein binds to core binding region of CYP2D2 gene regulation

A, A supershift assay was performed by the addition of 0.2, 0.5, 1 and 2 μg of the specific anti-hnRNP K antibody (lanes 2-5). B, ChIP assay was carried out using chromatin from H4-II-E cells and anti-mouse IgG (lane 2), anti-RNA polymerase II (lane 3) and anti-hnRNP K (lane 4) as the immunoprecipitating antibody. Purified DNA was analyzed by PCR using specific primers for the CYP2D2 promoter. Distilled deionized water and input chromatin was also amplified as a PCR control (lanes 1, 5). C, A reporter plasmid, pGL3-514, was cotransfected with the expression plasmid for pCMV6-hnRNPK into HepG2 cells. These data represent means ± SEM of three independent experiments. *$p<0.05$.

Discussion

To date, several groups have reported the transcriptional regulation of CYP2D enzymes in several species. In human CYP2D6, several transcription factors such as hepatocyte nuclear factor 4 are responsible for controlling the transcriptional activity (Cairns et al., 1996). In mouse Cyp2d9, male-specific demethylation occurs at the CpG site upstream of the Cyp2d9 gene (Yokomori et al., 1995a, 1995b). In the rat CYP2D4 specific regulatory element (between nucleotides -116 and -90), the competitive interference of transcriptional factors may specifically regulates the high expression of CYP2D4 in the brain (Mizuno et al., 2003). In rat CYP2D5, two transcription factors, C/EBP and Sp1, work in conjunction to activate the CYP2D5 gene (Lee et al., 1994). However, the transcriptional regulation of CYP2D2, which metabolizes the majority of typical CYP2D6 substrates, remained to be elucidated. Furthermore, the genetic basis of the extremely low expression of CYP2D2 mRNA in DA rats was not fully understood.

In this chapter, I identified the transcriptional regulatory element of the CYP2D2 gene responsible for the expression of this enzyme in the 5' flanking region from -94 to -113. The transcription factor hnRNP K, which is known as a poly(C)-binding protein, formed a complex with the transcriptional regulatory element. A polypyrimidine sequence (5'-CCCCTTCCCCC) within the transcriptional regulatory element was a preferred target for the hnRNP K protein. Thus, I identified hnRNP K protein as a novel regulator of CYP2D2 gene transcription.

The hnRNP K protein is implicated in chromatin modeling, transcription, splicing and translation processes. It has been reported that the hnRNP K protein is a versatile molecule that interacts with RNA, DNA, tyrosine and serine/threonine kinases, the transcriptional factor TATA-binding protein and a number of

zinc-finger transcriptional factors (Ostrowski et al., 2001; Tomonaga et al., 1995; Michelotti et al., 1996; Denisenko et al., 1996). The hnRNP K protein recruits a variety of molecular partners and may act as a docking platform or scaffold in these processes. Thus, other transcription factors such as TATA-binding proteins may synergistically activate the CYP2D2 transcription activity with hnRNP K protein, although the overexpression of hnRNP K stimulated the CYP2D2 transcriptional activity through binding to the transcriptional regulatory element. To date, little is known about the potential role of hnRNPs in P450 gene regulation. It has only been reported that the hnRNP A1 interaction with the CYP2A5 and CYP2A6 mRNA is a key post-transcriptional regulation of each gene (Raffalli-Mathieu et al., 2002; Christian et al., 2004). It binds to the CYP2A5 and CYP2A6 mRNA 3′ untranslated region, and it is involved in the stabilization of the transcript, most likely by controlling the length of its poly(A) tail.

Additionally, I clarified that a single mutation within the transcription regulatory element abolished the binding of hnRNP K protein in DA rats. Considering that a mutation was found in the polypyrimidine sequence (5'-CCCCTTTCCCC, substitution is underlined), the low binding affinity of hnRNP K to the mutated sequence was a plausible result. Thus, decreased recruitments of hnRNP K protein to the mutated sequence are in good agreement with the markedly attenuated transcription activity of CYP2D2 gene in DA rats.

In conclusion, I ascertained that hnRNP K protein plays an important role in CYP2D2 gene regulation. Furthermore, I clarified that a single mutation within the transcription regulatory element resulted in the low expression of CYP2D2 mRNA in DA rats, due to the weak affinity to hnRNP K protein.

Chapter 2
The genetic polymorphism of diazepam metabolism among laboratory rat strains

Introduction

Diazepam is one of the benzodiazepines that are widely used in the treatment of anxiety disorders, depression and insomnia. Inaba et al. (1988) reported inter-individual variability of diazepam metabolism in humans. Similar variations in diazepam metabolism are also observed in mice and rats (Griebel et al., 2000). Intrastrain differences in the pharmacokinetics and pharmacodynamics of diazepam were documented among outbred Wistar rats (van der Laan et al., 1993; Bert et al., 2001). On the other hand, Mechan et al. (2002) reported strain differences between SD and DA rats in the pharmacodynamics of diazepam. The considerable differences in the plasma levels of diazepam and its metabolites indicated inter- and intra-strain variations in diazepam metabolism.

Neville et al. (1993) reported that diazepam was metabolized to three primary metabolites, 3-hydroxy-diazepam, N-desmethyl-diazepam and *p*-hydroxy-diazepam, in liver microsomes of adult male Wistar rats. These metabolic pathways were reported to be catalyzed by CYP3A2, CYP2C11 and CYP2D1, respectively (Fig. 9). Saito et al. (2004a) found the existence of EM and PM of diazepam metabolism in liver microsomes from Wistar rats at low concentration of substrate. EM from Wistar rats (EM-W) had markedly higher activity toward diazepam *p*-hydroxylation than PM from Wistar rats (PM-W). In addition, Saito et al. (2004b) reported strain differences in diazepam *p*-hydroxylation in SD, BN and DA rats. SD and Brown Norway (BN) rats had 300-fold higher diazepam *p*-hydroxylation activity than DA rats at low concentration of substrate (Table 3). As

a result of diazepam *p*-hydroxylation activity, the major metabolic pathways of diazepam differed among rat strains.

The reaction of *p*-hydroxylation was suggested to be catalyzed by CYP2D1. However, it was demonstrated that there was no significant difference in the expression levels of CYP2D1 in liver microsomes between EM and PM. Moreover, the patterns of debrisoquine 4-hydroxylation activity, which is CYP2D2-dependent activity, did not coincide with those of diazepam *p*-hydroxylation activity in the liver of four rat strains. Then, it was concluded that diazepam *p*-hydroxylation was neither catalyzed by CYP2D1 nor CYP2D2, which contributed to the metabolism of debrisoquine, bunitrolol and bufuralol (Boobis et al., 1986; Gonzalez et al., 1987;

Table 3. Total P450 contents and diazepam metabolic activities in liver microsomes from four rat strains

	Total P450 contents	Activities (pmol/min/mg protein)		
	(nmol/mg protein)	*p*-hydroxylation	3-hydroxylation	*N*-desmethylation
SD	0.80 ± 0.08	171.9 ± 3.0 *	146.0 ± 26.1	27.5 ± 0.9
BN	0.73 ± 0.05	156.4 ± 8.3 *	115.6 ± 8.3	31.3 ± 1.0
DA	0.83 ± 0.03	0.4 ± 0.0	457.2 ± 35.7 *	67.3 ± 1.8 *
PM-W	0.73 ± 0.08	0.5 ± 0.0	141.3 ± 19.3	24.8 ± 1.6
EM-W	0.62 ± 0.05	94.6 ± 16.0 *	146.3 ± 38.7	31.1 ± 2.7

The concentration of diazepam was 3.13μM. Values are expressed as the mean ± SEM. *Significant difference from PM-W rats ($p < 0.01$). This data was cited from Saito et al. (2004a, 2004b).

Suzuki et al., 1992; Yamamoto et al., 1996, 1998). The enzyme which catalyzes diazepam *p*-hydroxylation is still not clear.

It is reported that the antibody against CYP2D1 or CYP2D2 isoforms inhibited the activity of diazepam *p*-hydroxylation in rat liver microsomes (Neville et al., 1993; Saito et al., 2004b). Since the rat CYP2D subfamily conserves a high similarity of amino acid sequence (>70%) among its members, the antibody against the full length of CYP2D isoform might inhibit drug-metabolizing activities catalyzed by any form of CYP2D without discrimination. In addition, antibodies generated against the purified P450 enzymes are often not specific. Despite the structural similarities among CYP2D isoforms, significant differences in the ability to metabolize drugs have been observed among these CYP2D isoforms (Wan et al., 1997; Chow et al., 1999; Hiroi et al., 2002).

In this chapter, I identified the enzyme involved in diazepam *p*-hydroxylation to be CYP2D3 using yeast recombinant CYP2D isoforms. This is the first report indicating that CYP2D3 has catalytic activity towards xenobiotic metabolism. In addition, the polymorphism of diazepam *p*-hydroxylation was revealed to be due to inter- and intrastrain differences in expression levels of CYP2D3 protein in four strains of rats. I clarified that single nucleotide mutation in coding region of CYP2D3 gene caused the nonfunctional truncated protein in PM rats. Here, I describe the relationship between a defect in the CYP2D3 gene (genotype) and inter- and intrastrain differences in diazepam *p*-hydroxylation (phenotype) among four rat strains.

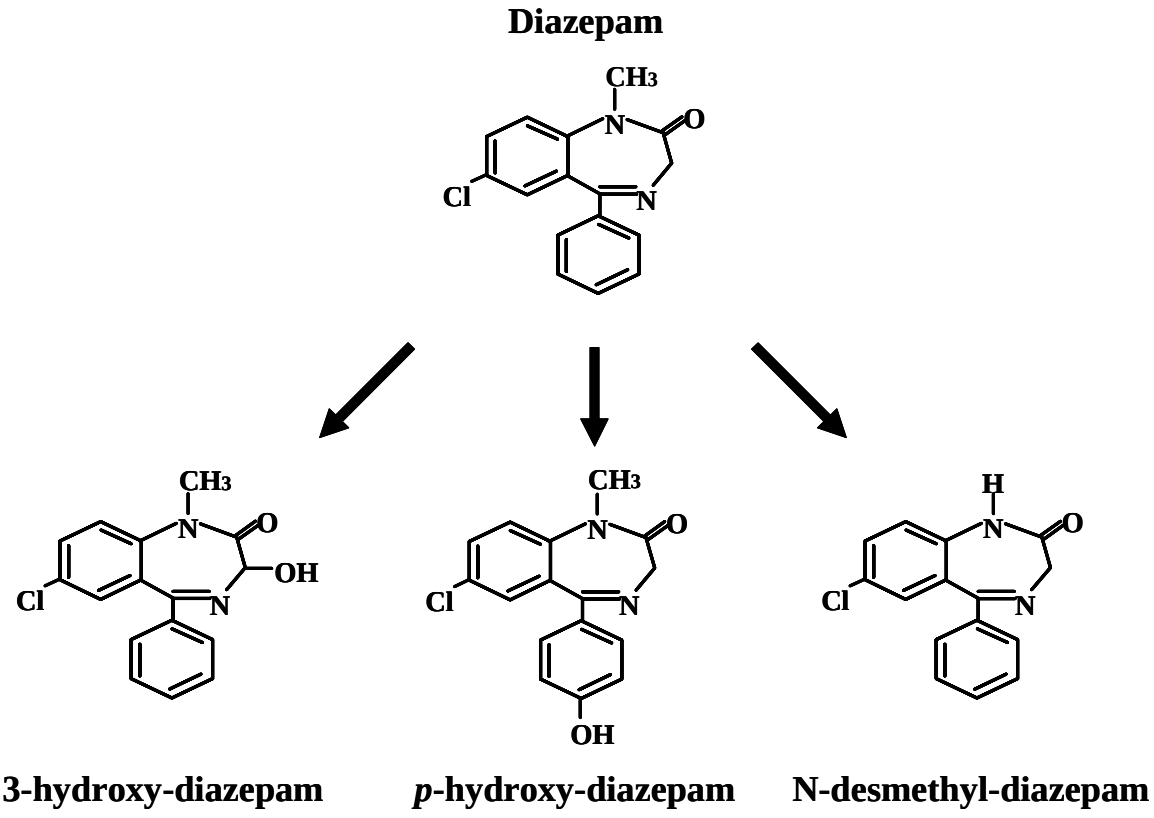


Fig. 9. Metabolic pathways of diazepam in male rat liver microsomes.

Diazepam 3-hydroxylation, *p*-hydroxylation and N-desmethylation were reported to be mainly catalyzed by CYP3A2, CYP2D1 and CYP2C11, respectively (Neville et al., 1993).

Materials and methods

Materials and animals

Diazepam (7-Chloro-1,3-Dihydro-1-Methyl-5-Phenyl-2H-1,4-Benzodiazepin-2-One) and the internal standard, nitrazepam (1,3-Dihydro-7-nitro-5-phenyl-2H-1,4-benzodiazepin-2-one), were purchased from Wako Pure Chemicals Co. (Osaka, Japan). The three primary diazepam metabolites, *p*-hydroxy-diazepam, 3-hydroxy-diazepam, and N-desmethyl-diazepam, were gifts from Japan Hoffman La Rosch Pharmaceutical Co. (Tokyo, Japan). Glucose-6-phosphate (G-6-P), NADPH and glucose-6-phosphate dehydrogenase (G-6-PDH) were purchased from Oriental Yeast Co. (Tokyo, Japan). The other reagents were of analytical grade.

Anti-CYP2D2 antibody was prepared as described previously (Suzuki et al., 1992; Nakamura et al. 1995; Yamamoto et al., 1996). Anti-CYP2D4 peptide antibody (CR 3240) was purchased from AFFINITI Research Products Ltd. (Exeter, UK). Adult male SD, DA and Wistar rats (9 weeks old) were obtained from Japan SLC Co. (Shizuoka, Japan). Adult male BN rats (9 weeks old) were obtained from Kyudo Co., Ltd. (Fukuoka, Japan). They were housed under standard laboratory conditions with free access to food and water, and were used for experiments after 1 week of acclimatization. All experiments using animals were performed with the supervision and approval of the Institutional Animal Care and Use Committee of Hokkaido University. CYP2D1, CYP2D2, CYP2D3 and CYP2D4 proteins, which were expressed in yeast (Wan et al., 1997), were gifts from Dr Y. Funae.

Preparation of liver microsomes

Liver microsomes were prepared according to the method of Omura and Sato (1964). The samples were homogenized with three volumes of ice-cold 1.15% KCl. The homogenates were centrifuged at 9,000 g at 4℃ for 20 min, and then

ultracentrifuged two times at 105,000 g at 4℃ for 70 min. The pellets were resuspended in 0.1 M potassium phosphate buffer (pH 7.4) and frozen in liquid nitrogen, and stored in the freezer at -80℃ until use.

Immunoprecipitation and immunoblotting of the microsomal protein

Immunoprecipitation was carried out using an Immunoprecipitation Kit (Roche, Mannheim, Germany) as recommended by the manufacture. The proteins were concentrated and immunoaffinity-purified with anti-CYP2D2 antibody. SDS-PAGE was carried out according to the methods of Laemmli (1970), using a 12% polyacrylamide gel. Proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane and immunostained with diaminobenzidine as substrate. The relative intensities of the immunoblotting were analyzed using the NIH Image v. 1.63 (Lennard 1990).

Analysis of amino acid sequence

Liver microsomes were subjected to SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. The blotted PVDF membrane was stained with 0.1% Coomassie Brilliant Blue R-250 containing 1% acetic acid and 40% methanol. After washing with 50% methanol, the band which was specifically expressed in liver microsomes of EM in diazepam *p*-hydroxylation was cut off from the membrane. The N-terminal sequence of the protein on PVDF membrane was determined using a Procise 492 Protein Sequencer (PerkinElmer Life Sciences).

Measurement of enzyme activities

Diazepam metabolites were detected using high performance liquid

chromatography (HPLC) according to the method described previously (Saito et al., 2004b) with some modifications. The microsomal fraction containing CYP2D1, CYP2D2, CYP2D3 and CYP2D4 prepared from yeast cells was used as recombinant enzyme. The reaction mixture (total volume, 0.2 ml) contained 50 μM diazepam, 3 mM $MgCl_2$, 5 mM G-6-P, and 1 mg/ml for yeast microsomes. The reaction was started by adding 1 mM NADPH and 1 enzyme unit of G-6-PDH after preincubation at 37℃ for 5 min. Incubation was carried out at 37℃ for 20 min. The reaction was terminated by the addition of 1.5 ml of ethyl acetate.

Isolation of total RNA and genomic DNA

Total RNA was isolated from rat liver using Isogen (Nippon Gene, Toyama, Japan). Genomic DNA was isolated from rat liver using a DNeasy Tissue Kit (Qiagen, Hilden, Germany). The concentration and purity of both DNA and RNA were determined by using a spectrophotometer. The integrity was examined by electrophoresis in a 1% agarose gel with ethidium bromide staining.

Quantitative real-time PCR analysis

The cDNA samples were obtained using 1 μg of total RNA as the template for reverse transcription with ReverTraAce and Oligo (dT) 20 primer (Toyobo, Osaka, Japan). TaqMan MGB probes and primers for CYP2D3 and beta-glucuronidase, as an endogenous control, were purchased from ABI (Applied Biosystems, Foster City, CA). According to the manufacturer's instructions, a primer for CYP2D3 was designed around the boundary between exons 4 and 5. TaqMan PCR reaction was carried out using Realtime PCR Master Mix (Toyobo). The first step was performed for 10 min at 95 ℃, followed by 40 cycles at 95℃ for 15 s and 60℃ for 1 min, as recommended by the manufacturer using an ABI Prism 7700 Sequence Detection

system (Applied Biosystems). The real-time PCR products of CYP2D3 and beta-glucuronidase were cloned and sequenced to verify the analytical specificity.

Relative quantification was carried out using the comparative cycle threshold (Ct) method as recommended by the manufacturer. To validate the comparative Ct calculations, the amplification efficiencies of CYP2D3 and beta-glucuronidase were confirmed. The amount of target was obtained by normalizing to an endogenous reference and relative to SD rats. Each reaction was done in duplicate on 96-well optical PCR plates (Applied Biosystems).

Sequencing of CYP2D3 cDNA

Sequencing was carried out using the cDNA samples used in quantitative real-time PCR. The full length of CYP2D3 cDNA was amplified using synthesized oligonucleotide primers of 5'-GGCCAGTGGTCTTTGGTAGC-3' (sense, nucleotide positions 15-34, NM_173093) and 5'- GGCAGCCACAGAACTGTTTTA -3' (antisense, nucleotide positions 1607-1627). The reaction condition was denaturation for 1 min at 94℃, annealing for 1 min at 57℃, and extension for 2 min at 72℃ for 35 cycles. This PCR-amplified fragment was subsequently cloned into a pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA). More than 10 cDNA clones from each of the four strains were analyzed to identify a mutation and repeatedly cloned from other samples to exclude PCR errors. The nucleotide sequence was analyzed with a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) and an automated DNA sequencer (ABI Prism 310 Genetic Analyzer) following the manufacturer's instructions.

CYP2D3 genotyping using PCR-RFLP

The single PCR and restriction fragment length polymorphism (RFLP)

methods were used to identify the wild-type and mutant CYP2D3 alleles. CYP2D3-specific PCR was accomplished with the primer pairs 5'-TCACATATCCCTGTCATA-3' and 5'-TCAGTCAGTCTGGGGCT-3', yielding a single 384 base pair product. The reaction mixture contained approximately 100 ng genomic DNA, 0.5 μM of each primer, 0.2 μM of each dNTP, 2 mM $MgCl_2$, and 1 U of ExTaq polymerase (TaKaRa Bio, Shiga, Japan) in a total volume of 50 μl. Touchdown PCR was performed as follows: the first cycle of denaturation at 94 ℃ for 60 s, annealing at 65 ℃ for 30 s, elongation at 72 ℃ for 30 s; the 2nd-30th cycles of denaturation at 94 ℃ for 30 s, annealing at 63-48.5 ℃ for 30 s, elongation at 72 ℃ for 30 s; the 31st cycle of denaturation at 94 ℃ for 30 s, annealing at 45 ℃ for 30 s, elongation at 72 ℃ for 30 s; and the final elongation at 72 ℃ for 5 min. In touchdown PCR, the annealing temperature began at 63 ℃ and was reduced by 0.5 ℃ every cycle. Six μl of each of the PCR products was then digested by the restriction enzyme *XmnI* (NEB, Ipswitch, MA, USA) without further purification. The digested PCR products were electrophoresed in a 2% agarose gel with ethidium bromide staining. The fragment patterns determined the presence of the CYP2D3 wild and mutant alleles, as indicated in Fig. 15.

Other methods

The protein concentrations were determined by the method of Lowry et al. (1951). The amounts of P450 were estimated by the method of Omura and Sato (1964). Statistical analysis was performed using the method of Tukey-Kramer.

Results

Isolation of the specific protein which expressed in EM rats

It has been reported that anti-CYP2D2 antibody inhibited diazepam *p*-hydroxylase activity in rat liver microsomes (Saito et al., 2004b). Moreover, both CYP2D1 and CYP2D2 did not catalyze diazepam *p*-hydroxylation. Thus, I attempted to isolate the protein that might contribute to *p*-hydroxylation of diazepam. After immunoprecipitation of liver microsomes of four rat strains using anti-CYP2D2 antibody, I separated proteins in low-bis SDS-PAGE and compared expression levels of the CYP2D subfamily using anti-CYP2D2 antibody (Fig. 10). I detected a band of protein that was expressed in all EM rats (SD, BN and EM-W), but not in PM rats (DA and PM-W).

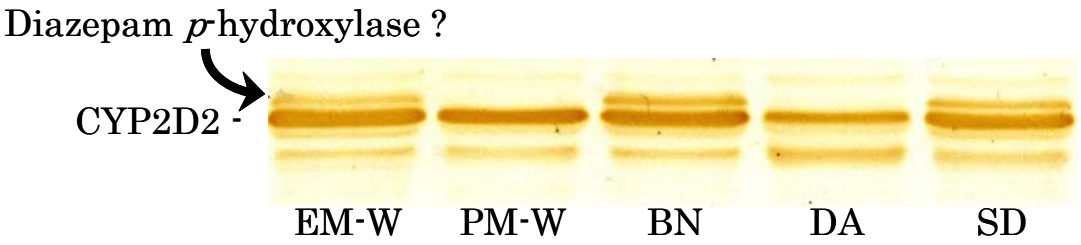


Fig. 10. Western blotting analyses of liver microsomes from four rat strains using anti-CYP2D2 antibody.

Microsomal proteins (10 μg) were separated by SDS-PAGE using 12 % low-bis polyacrylamide gel. After the transfer of protein onto a PVDF membrane, CYP2D isoforms were reacted with peptide antibody against CYP2D2 and immunostained with diaminobenzidine as a substrate.

Comparison of N-terminal amino acid sequences of the specific protein with those of the CYP2D subfamily

To analyze the amino acid sequence of the specific band to EM rats, PVDF membrane corresponding to the specific band was cut off. N-terminal 20-amino acid sequences of the specific band were analyzed with peptide sequencer. I searched the sequence similarity between N-terminal amino acid sequences of specific band and those of protein database using FASTA (GenomeNet, Kyoto Univ.). As a result of the comparison, N-terminal amino acid sequences of the specific protein exactly corresponded to those of CYP2D3 (Table 4). It raises the possibility that CYP2D3 is involved in diazepam *p*-hydroxylation.

Table 4. Comparison of the N-terminal amino acid sequence of the specific protein which expressed in EM with the sequences of the CYP2D subfamily members.

Protein	N-terminal amino acid sequence
DZ *p*-hydroxylase ?	M E L L A G T G L W P M A I F T V I F I
CYP2D1	M E L L N G T G L W S M A I F T V I F I
CYP2D2	M G L L I G D D L W A V V I F T A I F L
CYP2D3	M E L L A G T G L W P M A I F T V I F I
CYP2D4	M R M P T G S E L W P I A I F T I I F L
CYP2D5	M E L L N G T G L W P M A I F T V I F I
CYP2D18	M R M P T G S E L W P I A I F T I I F L

Western blotting analyses of liver microsomes and CYP2D isoforms expressed in yeast with anti-CYP2D2 antibody

To make sure anti-CYP2D2 antibody can recognize CYP2D3, I carried out Western blotting analyses of liver microsomes from SD rats and pure CYP2D isoforms (CYP2D2 and CYP2D3) expressed in yeast using anti-CYP2D2 antibody (Fig. 11). Anti-CYP2D2 antibody recognized CYP2D3, and CYP2D3 showed the same molecular size as the specific band expressed in SD rats. This result strongly supports my hypothesis that diazepam *p*-hydroxylase is CYP2D3.

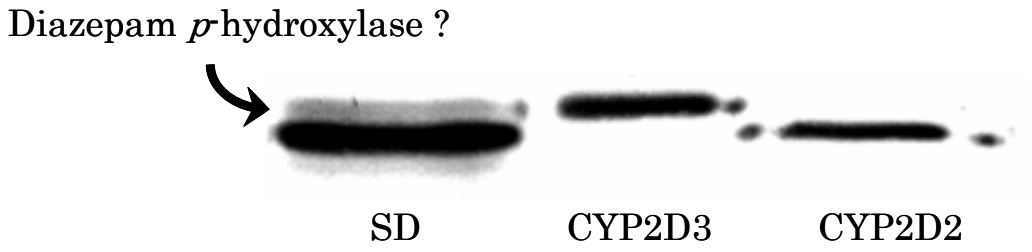


Fig. 11. Western blotting analyses of liver microsomes from SD rat and pure CYP2D isozymes expressed in yeast using anti-CYP2D2 antibody.

Microsomal protein (10 μg) or CYP2D2 and CYP2D3 (each 10 μg) expressed in yeast was applied to each lane. Other experimental details are described in the legend of Fig. 10.

Diazepam-metabolizing activities of CYP2D subfamily

To confirm the above hypothesis, assay of diazepam metabolism in the reconstituted system was performed by using rat CYP2D3 as well as other CYP2D subfamilies expressed in yeast as recombinant enzymes (Table 5). CYP2D3 had a high diazepam *p*-hydroxylation activity, but none of the other CYP2D isoforms have the activity. Also, CYP2D3 possessed moderate activity toward diazepam N-desmethylation. Interestingly, CYP2D4 exhibited a high diazepam N-desmethylation activity. The activity of CYP2D1 was low toward diazepam N-desmethylation. CYP2D2 had no activity toward three metabolic pathways. None of the CYP2D isoforms had diazepam 3-hydroxylation activity. These results support the previous studies and my expectation that CYP2D3, not CYP2D1 and CYP2D2, catalyzes diazepam *p*-hydroxylation.

Table 5. Diazepam metabolic activities of CYP2D isoforms

The concentration of substrate (diazepam) was 50 μM. Other experimental details are described under Materials and methods. N.D., not detectable.

	Activities (*nmol/min/nmol of P450*)		
	p-Hydroxylation	3-Hydroxylation	N-Desmethylation
CYP2D1	N.D.	N.D.	0.02
CYP2D2	N.D.	N.D.	N.D.
CYP2D3	1.19	N.D.	0.29
CYP2D4	N.D.	N.D.	0.89

Expression level of CYP2D4 in liver microsomes among four strains

Since I found that CYP2D4 possessed a high diazepam N-desmethylation activity, which is catalyzed by CYP2C11 or, partially, CYP3A2, I examined the expression level of CYP2D4 in liver microsomes among the four strains. Western blotting analysis showed that DA rat liver microsomes had a high expression level of CYP2D4 protein (Fig. 12). The expression levels of other strains except for DA rats had no differences. This result was in good agreement with the previous study (Schulz-Utermoehl et al., 1999). Although it was reported that DA rats had a high diazepam N-desmethylation activity and attributed this to high levels of CYP3A2, the high expression of CYP2D4, which had a high diazepam N-desmethylation activity, in DA rats also supported the observation.

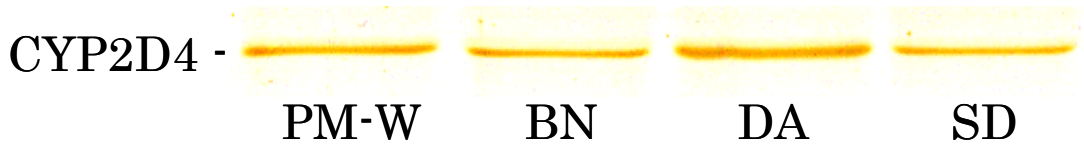


Fig. 12. Western blotting analyses of liver microsomes from four rat strains using anti-CYP2D4 peptide antibody.

Microsomal protein (10 μg) were separated by SDS-PAGE using 12 % polyacrylamide gel. After the transfer of protein onto a nitrocellulose membrane, CYP2D4 was reacted with peptide antibody against CYP2D4 and immunostained with diaminobenzidine as a substrate.

Expression level of CYP2D3 mRNA in livers of four rat strains

I expected a positive correlation of CYP2D3 mRNA levels with the protein levels among the four strains. Thus, the expression levels of CYP2D3 mRNA in liver of the four strains were examined by quantitative real-time PCR. I could not find enough inter- and intrastrain differences to explain the differences in expression levels of CYP2D3 protein among the four rat strains (Fig. 13). The analytical specificity was assured by the sequencing of the PCR products. No amplicon other than CYP2D3 or beta-glucuronidase was detected.

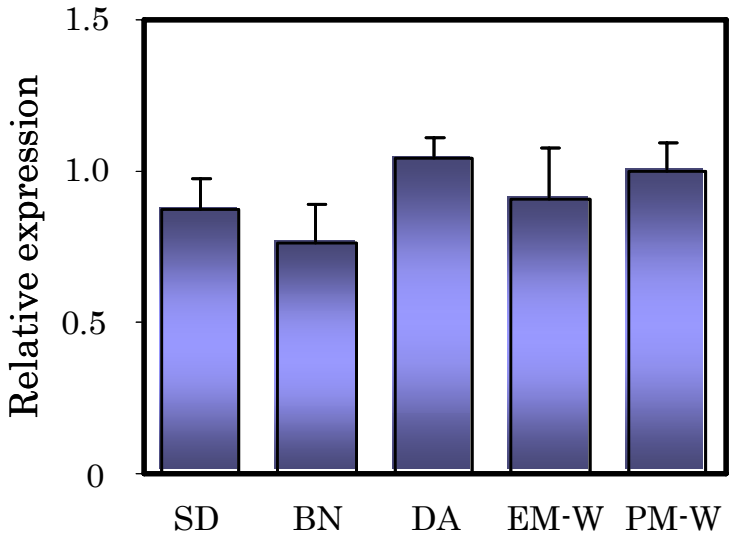


Fig. 13. Expression of CYP2D3 mRNA in livers of four rat strains.

Quantitative real-time PCR for CYP2D3 mRNA was performed on livers from four strains of rats. Relative quantitative analysis was carried out using the comparative Ct method. For the comparative Ct method to be valid, the amplification efficiencies of the target (CYP2D3) and endogenous reference (beta-glucuronidase) were confirmed. The data shown was obtained by normalizing to an endogenous reference. The values are means ± S.D. of 3 samples (SD and BN rats were 5).

Sequence analysis of CYP2D3 cDNA

To explore the possibility that CYP2D3 mRNA does not encode the functional protein in DA and PM-W rats, I subsequently cloned and sequenced the full length of CYP2D3 cDNA. There was a single nucleotide mutation in exon 8 of the CYP2D3 gene of DA and PM-W rats (Fig. 14B). The mutation was a frameshift mutation with a single thymine base insertion at nucleotide 1269. This insertion resulted in a premature termination at codon 434 of CYP2D3 protein upstream of the heme-binding region (Fig. 14C). Due to the deletion of the heme-binding region, the derived mutant CYP2D3 protein must be non-functional, implying impairment of diazepam *p*-hydroxylation. In contrast, no mutation was detected in the sequences of SD and BN rats (Fig. 14A). Interestingly, EM-W rats had both wild and mutant CYP2D3 cDNA sequences, suggesting heterozygosity of the CYP2D3 gene in EM-W rats.

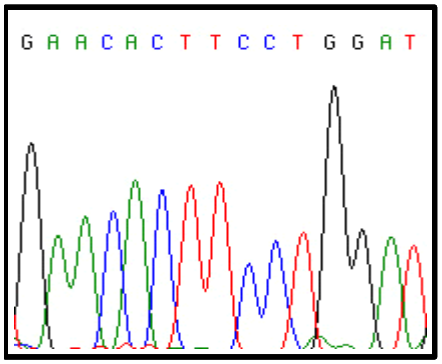
Establishment of genotyping system for CYP2D3 by PCR-RFLP

A genotyping assay using PCR-RFLP was developed to distinguish homozygote from heterozygote. According to the nucleotide sequence analysis, the mutant allele had a thymine base inserted. This insertion was expected to produce an *XmnI* recognition site (5'-GAANNNNTTC-3'). The genotype of the touchdown PCR product was determined according to RFLP. The mutant allele had one *XmnI* digestion site within the amplified region (Fig. 15A). In contrast, the wild allele had no digestion site. Therefore, the genotype of CYP2D3 would be determined by RFLP using *XmnI*. An electropherogram, as shown in Fig. 15B, shows the enzyme digestion pattern of the touchdown PCR product.

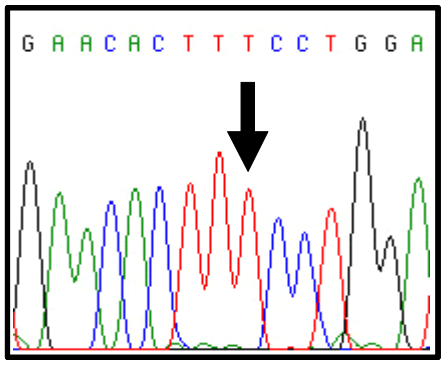
The genotype frequencies of the CYP2D3 gene were studied using genomic DNA samples from the four rat strains (Table 6). In contrast to both SD and BN rats,

all DA rats were homozygous carriers of the CYP2D3 mutant allele. In Wistar rats, genotype frequencies of the mutant homozygote and heterozygote were 80.5% (33/41) and 19.5% (8/41), respectively, while the wild homozygote was not detected in the 41 samples examined. RFLP analysis of the PCR product gave results fully consistent with those of inter- and intrastrain differences in diazepam *p*-hydroxylation. Thus, the CYP2D3 genotyping method developed in this study appeared to be free from any typing errors.

A



B



C

Mutant	cDNA	CTGAAGGATGAGACTGTCTGGGAGAAGCCCCTCCGATTCCAC													1257
Wild	cDNA	CTGAAGGATGAGACTGTCTGGGAGAAGCCCCTCCGATTCCAC													1257
Wild	AA		L	K	D	E	T	V	W	E	K	P	L	R	F	H	419
Mutant	AA		L	K	D	E	T	V	W	E	K	P	L	R	F	H	419

Mutant	cDNA	CCTGAACACTTTCCTGGATGCCCAGGGCAACTTTGTGAAGCA													1299
Wild	cDNA	CCTGAACACTTCCTGGATGCCCAGGGCAACTTTGTGAAGCAT													1299
Wild	AA		P	E	H	F	L	D	A	Q	G	N	F	V	K	H	433
Mutant	AA		P	E	H	F	P	G	C	P	G	Q	L	C	E	A	433

Mutant	cDNA	TGAGGCCTTCATGCCATTCTCAGCAGGCCGCAGAGCATGCCT													1341
Wild	cDNA	GAGGCCTTCATGCCATTCTCAGCAGGCCGCAGAGCATGCCTG													1341
Wild	AA		E	A	F	M	P	F	S	A	G	R	R	A	C	L	447
Mutant	AA	***															

Fig. 14. Comparison of nucleotide and amino acid (AA) sequences of wild- and mutant-type CYP2D3.

A, the wild-type CYP2D3 cDNA sequence corresponding to codons 421-25 is shown. Wild-type CYP2D3 cDNA was isolated from SD, BN and EM-W rat liver. B, a single thymine insertion at nucleotide 1269 is marked by the arrow. Mutant-type CYP2D3 cDNA was isolated from DA, PM-W and EM-W rat livers. C, the single thymine insertion is underlined. This mutation creates a premature termination at codon 434 (TGA) upstream of the heme binding region. The heme binding cysteine, which is perfectly conserved in P450, is enclosed in a box. The terminal signal is indicated by asterisks.

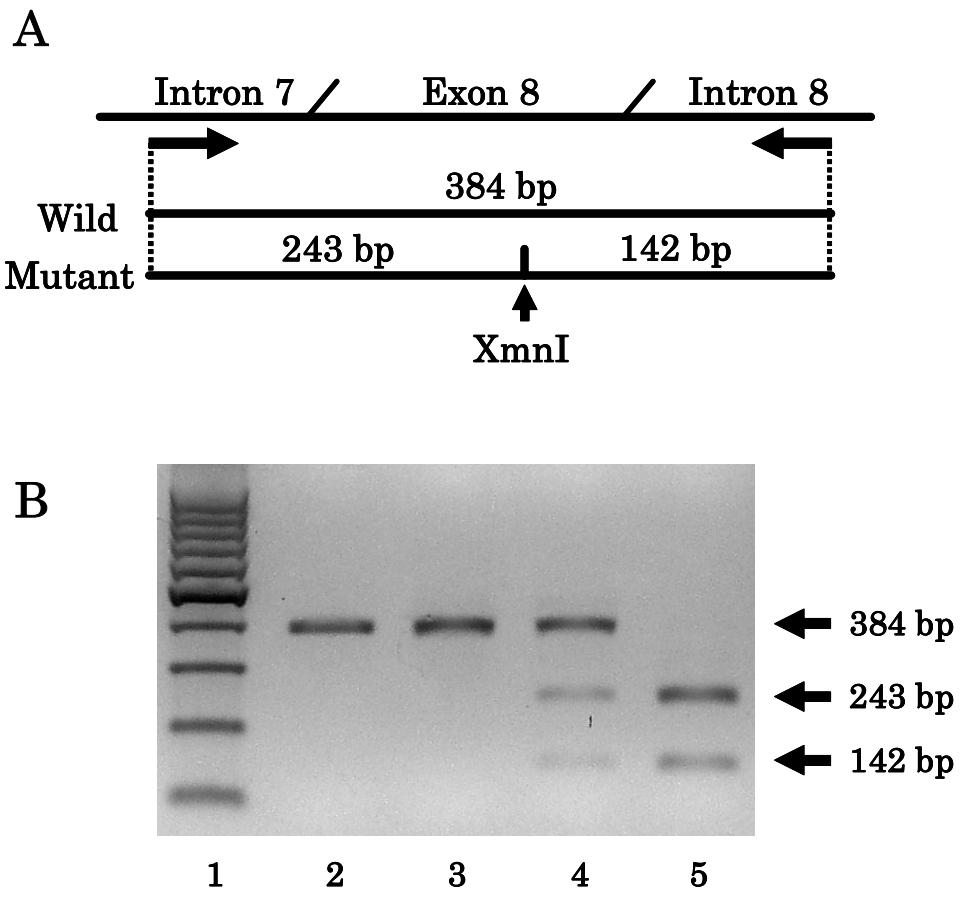


Fig. 15. Genotyping strategy using the PCR-RFLP method for the CYP2D3 gene.

A, horizontal arrows show specific primers used for the target amplification. The vertical arrow and vertical bar show the recognition site for the restriction enzyme *XmnI*. Figures indicate the undigested and digested fragment lengths of the PCR product. B, lane 1 shows a 100 bp DNA ladder-size marker (Toyobo); lane 2 shows the undigested PCR product (384 bp); and lanes 3 to 5 show the RFLP of the PCR product amplified from BN, EM-W and DA rat genomic DNA, respectively.

Table 6. Relationship between the metabolic activity and the genotype frequency among four rat strains using PCR-RFLP analysis.

	Total	Genotype (frequency)			Activity
		Wild homozygote	Heterozygote	Mutant homozygote	Diazepam *p*-hydroxylation
					pmol/min/mg protein
SD	9	9 (100%)	0	0	171.9±3.0*
BN	9	9 (100%)	0	0	156.4±8.3*
DA	8	0	0	8 (100%)	0.4±0.0
PM-W	41	0	0	33 (80.5%)	0.5±0.0
EM-W		0	8 (19.5%)	0	94.6±16.0*

*Significant difference from PM-W rats ($p < 0.01$).

Discussion

In this chapter, I aimed to identify the rat P450 isoform involved in diazepam *p*-hydroxylation, and to disclose the mechanism underlying inter- and intrastrain differences in the diazepam metabolism.

Until recently, diazepam *p*-hydroxylation was thought to be catalyzed by CYP2D1. However, it was reported that diazepam *p*-hydroxylation was not catalyzed by CYP2D1 or CYP2D2. I initially managed to separate the specific protein expressed in liver microsomes of SD, BN and EM-W rats, while not expressed in DA and PM-W rats (Fig. 10). Since the specific protein was observed only in EM rats, there is a strong possibility that the protein is diazepam *p*-hydroxylase. Thus, the amino acid sequence analysis was carried out to clarify the relationships between the specific protein and diazepam *p*-hydroxylase. The N-terminal amino acid sequence of the specific protein exactly corresponded to that of CYP2D3 (Table 4).

Among the rat CYP2D subfamily, the mRNA level of CYP2D3 increased with development (Matsunaga et al., 1990), while similar developmental change was not observed in the mRNA levels of CYP2D1 and CYP2D2 (Chow et al., 1999). Neville et al. (1993) described in their paper that diazepam *p*-hydroxylation was more active in young adult male rats (>5 weeks) than in neonates that are still in the early stages of development. Therefore, the mRNA expression pattern of CYP2D3 is highly consistent with developmental changes of diazepam *p*-hydroxylation. Results from these studies strongly indicate that CYP2D3 is diazepam *p*-hydroxylase.

In order to confirm that CYP2D3 has a diazepam *p*-hydroxylation activity, diazepam metabolism was performed by using the rat CYP2D isoforms expressed in yeast as recombinant enzyme (Table 5). As expected, only CYP2D3 but no other isoforms possessed a high diazepam *p*-hydroxylation activity. In addition, I carried

out the immunoblotting of liver microsome and CYP2D3 expressed in yeast with anti-CYP2D2 antibody. Anti-CYP2D2 antibody recognized CYP2D3, and diazepam *p*-hydroxylase was the same as CYP2D3 in molecular weight (Fig. 11). This is the first report that diazepam *p*-hydroxylation is specifically catalyzed by CYP2D3. CYP2D1 had a low diazepam N-desmethylation activity, while CYP2D2 had no activity toward diazepam metabolism. Although CYP2D1 and CYP2D2 play an important role in the metabolism of numerous drugs such as debrisoquine (Kobayashi et al., 1989), bunitrolol (Suzuki et al., 1992; Yamamoto et al., 1996, 1998) and propranolol (Fujita et al., 1993), it seems that they do not participate in diazepam metabolism so much.

In previous research, DA rats had the high activities toward diazepam 3-hydroxylation and diazepam N-desmethylation. Saito et al. (2004b) showed that the expression level of CYP3A2, which are responsible for diazepam 3-hydroxylation and partially diazepam N-desmethylation, was higher in DA rats than other strains. They also showed that the expression levels of CYP2C11, which is mainly responsible for diazepam N-desmethylation, were not different among four strains. Therefore, it was concluded that the high activity toward diazepam N-desmethylation in DA rats was caused by the high expression of CYP3A2. In the current study, I found that CYP2D4 exhibited a high diazepam N-desmethylation activity, and that DA rats had the higher expression level of CYP2D4 than other strains (Fig. 12). Therefore, not only CYP3A2 but also CYP2D4 may be involved in strain differences in diazepam N-desmethylation among four strains.

Up to date, there is little information about the catalytic specificity of CYP2D isoforms, especially CYP2D3 (Chow et al., 1999; Hiroi et al., 2002). In this study, I demonstrated that CYP2D3 was involving in diazepam *p*-hydroxylation in rats. Moreover, I also showed that the polymorphic expressions of CYP2D3 protein

are responsible for the significant inter- and intrastrain differences in diazepam *p*-hydroxylation. However, the mechanism underlying the polymorphic expression of CYP2D3 protein among the four rat strains remains to be described.

I initially analyzed the expression level of CYP2D3 mRNA by quantitative real-time PCR. However, the expressions of CYP2D3 mRNA in DA and PM-W rats were comparable to that of SD rats (Fig. 13). In order to investigate whether CYP2D3 mRNA encodes the functional protein, I subsequently cloned and sequenced the full length of CYP2D3 cDNA. I found a mutation within a highly similar sequence region among the rat CYP2D subfamily, spanning exon 8, intron 8, and exon 9. In DA and PM-W rats, there was a common frameshift mutation with a single thymine insertion at nucleotide 1269 in exon 8 (Fig. 14). This mutation consequently created a premature termination at codon 434 of the CYP2D3 gene. Importantly, the region deleted by the premature termination codon contains the heme-binding region and the highly conserved cysteine-containing portion of the P450 primary sequence in all species (Gonzalez, 1988). Deletion of the heme-binding region, which is essential to maintain proper heme binding and active P450 enzymes, leads to the creation of a truncated protein that must function improperly or not at all. Therefore, the mutation in the sequence of CYP2D3 of DA and PM-W rats must be plausibly the cause of the significantly low diazepam *p*-hydroxylation. In contrast, SD and BN rats had no mutation in CYP2D3 cDNA, leading to the production of a functional protein. Nucleotide sequence analyses indicated that the genotypes of the extensive metabolizers (SD and BN rats) and that of poor metabolizers (DA and PM-W rats) are homozygous for wild and mutant types in the CYP2D3 gene, respectively. On the other hand, genomic DNA derived from EM-W rat liver showed the heterozygosity of the CYP2D3 gene. This result is plausibly consistent with the fact that EM-W rats possess intermediate

p-hydroxylation activity toward diazepam among the four rat strains.

Usually, mRNAs encoding mutant proteins that are inactive are considered to be unstable. This raises the question why the mutant-type CYP2D3 mRNA stably expressed in PM rats. In eukaryotic cells, nonsense-mediated mRNA decay (NMD) acts as an mRNA surveillance system to get rid of aberrant mRNAs containing premature termination codons. By so doing, NMD eliminates the production of the encoded truncated protein. According to the established rule, premature termination codons that are followed by an exon-exon junction that is located more than 50-55 nucleotides downstream generally elicit NMD (Maquat, 2004). However, the premature termination codon observed is followed by an exon-exon junction that is located in 25 nucleotides downstream, suggesting that this does not elicit NMD. This is the possible reason why CYP2D3 mRNA encoding the truncated protein is stable.

I then developed a convenient and accurate genotyping assay based on PCR-RFLP in order to distinguish homozygote from heterozygote (Fig. 15). Utilizing this assay, the genotype frequency among the four rat strains was determined (Table 6). The mutant homozygote (80.5%) was more frequent than the heterozygote (19.5%) in Wistar rats. In the previous report, it was demonstrated that about 17% of outbred Wistar strain rats, referred to as EM-W in this manuscript, showed high diazepam metabolism in their liver microsomes (Saito et al., 2004a). The genotype frequency reported in the current study is compatible with the phenotype frequency Saito et al. (2004a) previously reported. In this study, I could not find the homozygous carriers of the CYP2D3 wild allele in the 41 Wistar rat samples examined. Since endogeneous roles of CYP2D3 are totally unknown, there remains a possibility that wild homozygote is a lethal phenotype. However, considering that SD and BN rats are homozygous carriers of the CYP2D3 wild allele, wild

homozygote does not appear to be fatal. Then, I took into a following consideration; I obtained Wistar rats from only Japan SLC Co., where outbred Wistar rats are bred in a rotational mating system in order to minimize inbreeding and genetic drift. The genotyping data implies that a vast majority (80.5%) of Wistar rats is maintained as the mutant homozygote in its closed colony. Simultaneously, it is expected that wild homozygote is a small minority in its closed colony. Thus, the sufficiently large sampling size will be required to prove this consideration.

Because the Wistar rats utilized in this study were from an outbred line, genetic heterogeneity was observed. Considering that Wistar rats contribute to a large subset of strains such as Wistar-Kyoto rats, spontaneously hypertensive rats, and Wistar-Furth rats, it is reasonable to assume that the genetic variety is sustained in these populations. Although the identical mutation is detected in both the DA and Wistar rats, phylogenetic trees and a large-scale database suggest that DA rats are not closely related to Wistar rats (Thomas, et al., 2003; Serikawa, 2004). I did not find any report in the literature on the relationship between DA and Wistar rats, because the origin of DA rats remains uncertain. The lack of definitive knowledge about their origin has led to speculation that DA rats might have been mixed with the Wistar strain prior to the completion of inbreeding. In addition to CYP2D3, due to the low expression of CYP2D2 mRNA, DA rats have been proposed as a model for human debrisoquine 4-hydroxylase polymorphism (Yamamoto et al., 1998). Moreover, DA rats have a high level of expression of CYP2D4 protein in liver microsomes. These data suggest that DA rats have a unique expression pattern in the CYP2D subfamily.

In conclusion, I reported that the deficiency of CYP2D3 protein caused by the defect in the wild-type CYP2D3 gene results in inter- and intrastrain differences in diazepam metabolism.

Summary

The rat is an important model for understanding human physiology and disease. The rat contributes to the biomedical researches of human cardiovascular disease, diabetes, arthritis and many behavioral disorders. In particular, the rat has frequently been used in the fields of pharmacology and toxicology. Thus, pharmaceutical companies use rats for a large proportion of their mandatory toxicity testing. However, inter- and intrastrain differences in drug response are often observed among rat strains, and are an important issue to be overcome. Still, the fundamental mechanism of inter- and intrastrain differences regarding drug metabolizing enzymes have been overlooked. In this thesis, I focused on the CYP2D subfamily that is a key enzyme involved in individual differences in drug metabolism.

CYP2D2 enzyme is known to metabolize the majority of typical substrates of the human CYP2D6 enzyme. Despite its impact on drug metabolism in rats, the transcriptional regulation of CYP2D2 remained to be elucidated. In chapter 1, I clarified the molecular mechanism of CYP2D2 gene expression. The CYP2D2 gene was positively regulated by the poly(C)-binding protein hnRNP K through a transcriptional regulatory element located in the 5'-flanking region from -94 to -113. Acting as a docking platform, the hnRNP K protein is known to be implicated in the regulation of transcription as an activator or a repressor, as a translational repressor, and as a participant in a variety of signaling systems. Although it has only been reported that hnRNP A1 protein, one of the hnRNPs, involves in the stabilization of the CYP2A5 and CYP2A6 mRNA, nothing is known about the potential role of hnRNP K in P450 gene regulation. Thus, this is the first report that hnRNP K protein is involved in CYP2D2 gene regulation. Furthermore, I elucidated

the genetic basis of the extremely low expression of CYP2D2 mRNA in DA rats. Due to its relative low abundance, DA rats have been frequently used for the study of CYP2D substrate metabolism as the animal model of PM phenotype for CYP2D6 in comparison to SD rats as an EM phenotype. To take full advantage of physiological variation among rat strains, it is essential to further unravel the mechanism of low expression of CYP2D2 mRNA. I found a single substitution within the transcriptional regulatory element of the CYP2D2 gene in DA rats. The mutation was detected in the polypyrimidine sequence that is the preferred binding site for hnRNP K protein. Indeed, the mutation within the transcriptional regulatory element abolished the binding of hnRNP K protein. Thus, I conclude that decreased recruitment of hnRNP K protein to the mutated sequence causes the low expression of CYP2D2 mRNA in DA rats.

Inter- and intrastrain differences in drug response in rats are a difficult problem for basic research as well as drug discovery as described above. Currently, information on genetic variation in laboratory rat strains is rapidly accumulating as a set of microsatellite markers, simple sequence length polymorphism markers and a variety of single nucleotide polymorphism in coding regions. Nevertheless, little is known about the differences in their drug metabolism characteristics that are ascribed to genetic variation. In chapter 2, I clarified the mechanism underlying inter- and intrastrain differences in diazepam *p*-hydroxylation among rat strains. The recent studies demonstrated that the pharmacokinetics of diazepam, which is one of the benzodiazepines, were quite different among rat strains because of its metabolic polymorphisms in diazepam *p*-hydroxylation. Although diazepam *p*-hydroxylation is a major metabolic pathway, SD and BN rats had 300-fold higher diazepam *p*-hydroxylation activity than DA rats at low concentration of diazepam. And Wistar rats showed 200-fold intrastrain differences in diazepam

p-hydroxylation activity (EM-W > PM-W). In addition, it was suggested that CYP2D subfamily was involved in this activity. Based on these observations, I separated the specific protein expressed in liver microsomes of SD, BN and EM-W, and identified the specific protein to be CYP2D3. Then, I confirmed that only CYP2D3 but no other CYP2D isoforms had a diazepam *p*-hydroxylation activity. To date, there is little information about the catalytic specificity of CYP2D3. Thus, I demonstrated that CYP2D3 was involved in diazepam *p*-hydroxylation. Moreover, I analyzed the genetic polymorphism of the CYP2D3 gene among rat strains. I found a single insertion in exon 8 in DA and PM-W rats. A premature termination codon created by this frameshift consequently deleted the heme-binding region that is essential to maintain proper heme binding and active P450 enzymes. Therefore, I conclude that the deficiency of a functional CYP2D3 protein must be the cause of the significantly low diazepam *p*-hydroxylation in DA and PM-W rats. Additionally, the genotype frequency of CYP2D3 polymorphism is in good agreement with the phenotype frequency among rat strains.

In this thesis, I clarified the genetic bases of inter- and intrastrain differences in CYP2D-dependent drug metabolism in rats. Clarification of inter- and intrastrain differences in rats will be further useful for predicting variability in human pharmacokinetics. Consequently, it is worthwhile to fully characterize animals used in pharmacokinetics studies from the point of view of the genetic expression of metabolic enzymes. Therefore, my work will strongly support the strain consideration in selection of a rat strain for drug metabolisms.

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References

Bert B, Fink H, Sohr R and Rex A. (2001) Different effects of diazepam in Fischer rats and two stocks of Wistar rats in tests of anxiety. *Pharmacol Biochem Behav.* **70**:411-20.

Boobis AR, Seddon CE and Davies DS. (1986) Bufuralol 1'-hydroxylase activity of the rat. Strain differences and the effects of inhibitors. *Biochem Pharmacol.* **35**:2961-5.

Brosen K, Klysner R, Gram LF, Otton SV, Bech P and Bertilsson L. (1986) Steady-state concentrations of imipramine and its metabolites in relation to the sparteine/debrisoquine polymorphism. *Eur J Clin Pharmacol.* **30**:679-84.

Brosen K. (1990) Recent developments in hepatic drug oxidation. Implications for clinical pharmacokinetics. *Clin Pharmacokinet.* **18**:220-39.

Cairns W, Smith CA, McLaren AW and Wolf CR. (1996) Characterization of the human cytochrome P4502D6 promoter. A potential role for antagonistic interactions between members of the nuclear receptor family. *J Biol Chem.* **271**:25269-76.

Colado MI, Williams JL and Green AR. (1995) The hyperthermic and neurotoxic effects of 'Ecstasy' (MDMA) and 3,4 methylenedioxyamphetamine (MDA) in the Dark Agouti (DA) rat, a model of the CYP2D6 poor metabolizer phenotype. *Br J Pharmacol.* **115**:1281-89.

Chow T, Imaoka S, Hiroi T and Funae Y. (1999) Developmental changes in the catalytic activity and expression of CYP2D isoforms in the rat liver. *Drug Metab Dispos.* **27**:188-92.

Christian K, Lang M, Maurel P and Raffalli-Mathieu F. (2004) Interaction of heterogeneous nuclear ribonucleoprotein A1 with cytochrome P450 2A6 mRNA: Implications for post-transcriptional regulation of the CYP2A6 gene. *Mol Pharmacol.* **65**:1405-14.

Chu T, Kumagai Y, DiStefano EW and Cho AK. (1996) Disposition of methylenedioxymethamphetamine and three metabolites in the brains of different rat strains and their possible roles in acute serotonin depletion. *Biochem Pharmacol.* **51**:789-96

Denisenko ON, O'Neill B, Ostrowski J, Van Seuningen I and Bomsztyk K. (1996) Zik1, a transcriptional repressor that interacts with the heterogeneous nuclear ribonucleoprotein particle K protein. *J Biol Chem.* **271**:27701-6.

Distlerath LM, Reilly PE, Martin MV, Davis GG, Wilkinson GR and Guengerich FP. (1985) Purification and characterization of the human liver cytochromes P-450 involved in debrisoquine 4-hydroxylation and phenacetin O-deethylation, two prototypes for genetic polymorphism in oxidative drug metabolism. *J Biol Chem.* **260**:9057-67.

Eichelbaum M and Gross AS. (1990) The genetic polymorphism of debrisoquine/sparteine metabolism--clinical aspects. *Pharmacol Ther.* **46**:377-94.

Fujita S, Umeda S, Funae Y, Imaoka S, Abe H, Ishida R, Adachi T, Masuda M, Kazusaka A and Suzuki T. (1993) Regio- and stereoselective propranolol metabolism by 15 forms of purified cytochromes P-450 from rat liver. *J Pharmacol Exp Ther.* **264**:226-33.

Gonzalez FJ, Matsunaga T, Nagata K, Meyer UA, Nebert DW, Pastewka J, Kozak CA, Gillette J, Gelboin HV and Hardwick JP. (1987) Debrisoquine 4-hydroxylase: characterization of a new P450 gene subfamily, regulation, chromosomal mapping, and molecular analysis of the DA rat polymorphism. *DNA* **6**:149-61.

Gonzalez FJ (1988) The molecular biology of cytochrome P450s. *Pharmacol Rev* **40**: 243-88

Gonzalez FJ, Skoda RC, Kimura S, Umeno M, Zanger UM, Nebert DW, Gelboin HV, Hardwick JP and Meyer UA. (1988) Characterization of the common genetic defect in humans deficient in debrisoquine metabolism. *Nature* **331**:442-6.

Gonzalez FJ (1996) The CYP2D subfamily, in *Cytochromes P450: Metabolic and Toxicological Aspects.* (Ioannides, C., ed.) pp183-210, CRC Press, Boca Raton, FL.

Gorski K, Carneiro M and Schibler U. (1986) Tissue-specific in vitro transcription from the mouse albumin promoter. *Cell* **47**:767–76.

Gough AC, Miles JS, Spurr NK, Moss JE, Gaedigk A, Eichelbaum M and Wolf CR. (1990) Identification of the primary gene defect at the cytochrome P450 CYP2D

locus. *Nature* **347**:773-6.

Griebel G, Belzung C, Perrault G and Sanger DJ. (2000) Differences in anxiety-related behaviours and in sensitivity to diazepam in inbred and outbred strains of mice. *Psychopharmacology (Berl)* **148**:164-70.

Gut J, Catin T, Dayer P, Kronbach T, Zanger U and Meyer UA. (1986) Debrisoquine/sparteine-type polymorphism of drug oxidation. Purification and characterization of two functionally different human liver cytochrome P-450 isozymes involved in impaired hydroxylation of the prototype substrate bufuralol. *J Biol Chem.* **261**:11734-43.

Harada N and Negishi M. (1984) Mouse liver testosterone 16 alpha-hydroxylase (cytochrome P-450(16) alpha). Purification, regioselectivity, stereospecificity, and immunochemical characterization. *J Biol Chem.* **259**:12285-90.

Hiroi T, Chow T, Imaoka S and Funae Y (2002) Catalytic specificity of CYP2D isoforms in rats and human. *Drug Metab Dispos.* **30**:970-76.

Inaba T, Tait A, Nakano M, Mahon WA and Kalow W (1988) Metabolism of diazepam in vitro by human liver. Independent variability of N-demethylation and C3-hydroxylation. *Drug Metab Dispos.* **16**:605-08.

Ingelman-Sundberg M. (2005) Genetic polymorphisms of cytochrome P450 2D6 (CYP2D6): clinical consequences, evolutionary aspects and functional diversity. *Pharmacogenomics J.* **5**:6-13.

International human genome sequencing consortium. (2001) Initial sequencing and analysis of the human genome. *Nature.* **409**,860-921

Kishimoto W, Hiroi T, Shiraishi M, Osada M, Imaoka S, Kominami S, Igarashi T and Funae Y. (2004) Cytochrome P450 2D catalyze steroid 21-hydroxylation in the brain. *Endocrinology* **145**:699-705.

Kobayashi S, Murray S, Watson D, Sesardic D, Davies DS and Boobis AR. (1989) The specificity of inhibition of debrisoquine 4-hydroxylase activity by quinidine and quinine in the rat is the inverse of that in man. *Biochem Pharmacol.* **38**:2795-9.

Kobayashi K, Urashima K, Shimada N and Chiba K (2002) Substrate specificity for rat cytochrome P450 (CYP) isoforms: screening with cDNA-expressed systems of the rat. *Biochem Pharmacol.* **63**:889-96.

Kumarathasan P, Mohottalage S, Goegan P and Vincent R. (2005) An optimized protein in-gel digest method for reliable proteome characterization by MALDI-TOF-MS analysis. *Anal Biochem.* **346**:85-9.

Laemmli UK. (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature.* **227**:680-685.

Lee TI, Johnstone SE and Young RA. (2006) Chromatin immunoprecipitation and microarray-based analysis of protein location. *Nat Protoc.* **1**:729-48.

Lee YH, Yano M, Liu SY, Matsunaga E, Johnson PF and Gonzalez FJ. (1994) A novel cis-acting element controlling the rat CYP2D5 gene and requiring cooperativity between C/EBP beta and an Sp1 factor. *Mol Cell Biol.* **14**:1383-94.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem.* **193**:265-75.

Maquat LE. (2004) Nonsense-mediated mRNA decay: splicing, translation and mRNP dynamics. *Nat Rev Mol Cell Biol.* **5**:89-99.

Masubuchi Y, Kagimoto N, Narimatsu S, Fujita S and Suzuki T. (1993) Regioselective contribution of the cytochrome P-450 2D subfamily to propranolol metabolism in rat liver microsomes. *Drug Metab Dispos.* **21:**1012-16,

Matsunaga E, Zanger UM, Hardwick JP, Gelboin HV, Meyer UA and Gonzalez FJ. (1989) The CYP2D gene subfamily: analysis of the molecular basis of the debrisoquine 4-hydroxylase deficiency in DA rats. *Biochemistry* **28**:7349-55.

Matsunaga E and Gonzalez FJ. (1990) Specific cytosine demethylations within the first exons of the rat CYP2D3 and CYP2D5 genes are associated with activation of hepatic gene expression during development. *DNA Cell Biol.* **9**:443-52.

Mechan AO, Moran PM, Elliott M, Young AJ, Joseph MH and Green R. (2002) A comparison between Dark Agouti and Sprague-Dawley rats in their behaviour on the elevated plus-maze, open-field apparatus and activity meters, and their response to diazepam. *Psychopharmacology (Berl)* **159**:188-95.

Michelotti EF, Michelotti GA, Aronsohn AI and Levens D. (1996) Heterogeneous nuclear ribonucleoprotein K is a transcription factor. *Mol Cell Biol.* **16**:2350-60.

Mizuno D, Takahashi Y, Hiroi T, Imaoka S, Kamataki T and Funae Y. (2003) A novel transcriptional element which regulates expression of the CYP2D4 gene by Oct-1 and YY-1 binding. *Biochim Biophys Acta* **1627**:121-8.

Nakamura A, Yamamoto Y, Sugimoto C, Masuda M, Kazusaka A and Fujita S. (1995) Anti-peptide antibodies to the P4502D subfamily in rat, dog, and man. *Xenobiotica.* **25**:1103-9.

Narimatsu S, Masubuchi Y, Hosokawa S, Ohmori S, Kitada M and Suzuki T. (1994) Involvement of a cytochrome P4502D subfamily in human liver microsomal bunitrolol 4-hydroxylation. *Biol Pharm Bull.* **17**:803-7.

Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, Waterman MR, Gotoh O, Coon MJ, Estabrook RW, Gunsalus IC and Nebert DW. (1996) P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics.* **6:**1-42

Neville CF, Ninomiya S, Shimada N, Kamataki T, Imaoka S and Funae Y. (1993) Characterization of specific cytochrome P450 enzymes responsible for the metabolism of diazepam in hepatic microsomes of adult male rats. *Biochem Pharmacol.* **45**:59-65.

Ohishi N, Imaoka S, Suzuki T and Funae Y. (1993) Characterization of two P-450 isozymes placed in the rat CYP2D subfamily. *Biochim Biophys Acta* **1158**:227-36.

Omura T and Sato R. (1964) The carbon monoxide-binding pigment of liver microsomes. *J Biol Chem.* **239**:2370-78.

Osikowska-Evers B, Dayer P, Meyer UA, Robertz GM and Eichelbaum M. (1987) Evidence for altered catalytic properties of the cytochrome P-450 involved in sparteine oxidation in poor metabolizers. *Clin Pharmacol Ther.* **41**:320-5.

Ostrowski J, Kawata Y, Schullery DS, Denisenko ON, Higaki Y, Abrass CK and Bomsztyk K. (2001) Insulin alters heterogeneous nuclear ribonucleoprotein K protein binding to DNA and RNA. *Proc Natl Acad Sci USA.* **98**:9044-9.

Raffalli-Mathieu F, Glisovic T, Ben-David Y and Lang MA. (2002) Heterogeneous nuclear ribonucleoprotein A1 and regulation of the xenobiotic-inducible gene Cyp2a5. *Mol Pharmacol.* **61**:795-9.

Raghuram TC, Koshakji RP, Wilkinson GR and Wood AJ. (1984) Polymorphic ability to metabolize propranolol alters 4-hydroxypropranolol levels but not beta blockade. *Clin Pharmacol Ther.* **36**:51-6.

Saito K, Kim HS, Sakai N, Ishizuka M, Kazusaka A and Fujita S. (2004a) Polymorphism in diazepam metabolism in Wistar rats. *J Pharm Sci.* **93**:1271-8.

Saito K, Sakai N, Kim HS, Ishizuka M, Kazusaka A and Fujita S. (2004b) Strain

differences in diazepam metabolism at its three metabolic sites in sprague-dawley, brown norway, dark agouti, and wistar strain rats. *Drug Metab Dispos.* **32**:959-65.

Schreiber E, Matthias P, Müller MM and Schaffner W. (1989) Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res.* **17**:6419.

Schulz-Utermoehl T, Bennett AJ, Ellis SW, Tucker GT, Boobis AR and Edwards RJ. (1999) Polymorphic debrisoquine 4-hydroxylase activity in the rat is due to differences in CYP2D2 expression. *Pharmacogenetics.* **9**:357-66.

Serikawa T. (2004) Colourful history of Japan's rat resources. *Nature.* **429**:15.

Shimada T, Yamazaki H, Mimura M, Inui Y and Guengerich FP. (1994) Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *J Pharmacol Exp Ther.* **270:** 414-23.

Stresser DM, Turner SD, Blanchard AP, Miller VP and Crespi CL. (2002) Cytochrome P450 fluorometric substrates: identification of isoform-selective probes for rat CYP2D2 and human CYP3A4. *Drug Metab Dispos.* **30**:845-52.

Suzuki T, Narimatu S, Fujita S, Masubuchi Y, Umeda S, Imaoka S and Funae Y. (1992) Purification and characterization of cytochrome P-450 isozyme catalyzing bunitorolol 4-hydroxylation in liver microsomes of male rats. *Drug Metab Dispos.* **20**:367-73.

Suzuki T, Fujisawa JI, Toita M, Yoshida M. (1993) The trans-activator tax of human T-cell leukemia virus type 1 (HTLV-1) interacts with cAMP-responsive element (CRE) binding and CRE modulator proteins that bind to the 21-base-pair enhancer of HTLV-1. *Proc Natl Acad Sci USA*. **90**:610-4.

Thomas MA, Chen CF, Jensen-Seaman MI, Tonellato PJ and Twigger SN. (2003) Phylogenetics of rat inbred strains. *Mamm Genome.* **14**:61-4.

Tomonaga T and Levens D. (1995) Heterogeneous nuclear ribonucleoprotein K is a DNA-binding transactivator. *J Biol Chem*. **270**:4875-81.

van der Laan JW, de Groot G, Wortelboer H and Noordhoek J. (1993) Pharmacokinetic differences of desmethyldiazepam in three outbred Wistar strains related to differences in liver enzyme activities. *Pharmacol Toxicol.* **73**:229-32.

Wan J, Imaoka S, Chow T, Hiroi T, Yabusaki Y and Funae Y. (1997) Expression of four rat CYP2D isoforms in Saccharomyces cerevisiae and their catalytic specificity. *Arch Biochem Biophys.* **348**:383-90.

Yamamoto Y, Nakamura A, Tasaki T, Umeda S, Sugimoto C, Imaoka S, Funae Y, Masuda M, Kazuaka A and Fujita S. (1996) Impaired expression in DA rats of catalytically active P450BTL, immunochemically distinguishable from P4502D1: immunological evidence with specific anti-peptide antibodies. *Biomed Res.* **17**:331-7.

Yamamoto Y, Tasaki T, Nakamura A, Iwata H, Kazusaka A, Gonzalez FJ and Fujita

S. (1998) Molecular basis of the Dark Agouti rat drug oxidation polymorphism: importance of CYP2D1 and CYP2D2. *Pharmacogenetics.* **8**:73-82.

Yokomori N, Kobayashi R, Moore R, Sueyoshi T and Negishi M. (1995a) A DNA methylation site in the male-specific P450 (Cyp 2d-9) promoter and binding of the heteromeric transcription factor GABP. *Mol Cell Biol.* **15**:5355-62.

Yokomori N, Moore R and Negishi M. (1995b) Sexually dimorphic DNA demethylation in the promoter of the Slp (sex-limited protein) gene in mouse liver. *Proc Natl Acad Sci USA.* **92**:1302-6.

博　士　論　文　内　容　の　要　旨

獣医学専攻	博士（獣医学）	氏　名	酒井　紀彰

博　　士　　論　　文　　題　　目

The genetic bases of inter- and intrastrain differences
in CYP2D-dependent drug metabolism in rats
（ラットにおけるCYP2Dの系統差及び個体差の分子機構の解明）

シトクロムP450（CYP, P450）は、生理活性物質の合成や代謝だけでなく、医薬品や環境汚染物質などの外来異物の代謝にも関与する酵素群である。特にヒトCYP2D6は、医薬品の約30%を代謝するにも関わらず多くの遺伝多型が存在する事から、薬効の個人差や副作用の発現に深く関与している。このため、CYP2D分子種は、毒性学上、非常に重要な分子種として位置付けられている。そこで本研究では、CYP2D分子種依存の薬物代謝反応におけるラットの系統差及び個体差、そしてそれらを引き起こすメカニズムについて、明らかにする事を目的とした。

第１章では、Dark Agouti(DA)ラットにおけるCYP2D2mRNA低発現機構を解明した。DAラットは、ヒトCYP2D6の典型的基質を代謝する能力が著しく低いため、ヒトCYP2D6の代謝欠損者の動物モデルとして用いられている。ヒトCYP2D6の典型的基質の多くは、ラットCYP2D2によって代謝され、特にDAラットでは、Sprague-Dawley（SD）ラットやWistar系ラットに比べ、CYP2D2 mRNA及び蛋白質発現量が低い事が、CYP2D分子種依存の代謝活性が低い原因であると報告されている。しかし、DAラットにおいて、CYP2D2 mRNA発現量が低下している原因については、未だ明らかにされていない。そこで、CYP2D2遺伝子の5'上流域を、4kbにわたりシークエンス解析を行ったところ、DAラットにおいてTATAボックスの近傍にシトシンからチミンへの一塩基置換がある事を見出した。一方、SDラットでは同領域に変異は認められなかった。次にElectrophoresis mobility shift assayから、DAラットにおいて検出された一塩基置換は、核蛋白質(転写因子)/DNA複合体の形成を低下させる事が確認された。また、CYP2D2遺伝子のプロモーター領域の各種欠失変異体を用いたレポーターアッセイの結果、この一塩基置換により転写活性は約1/4に低下した。さらに、Matrix-assisted laser desorption/ionization time-of-flight mass spectrometryを用いて、CYP2D2遺伝子発現に関与する転写因子の同定を試みた。その結果、転写装置の足場蛋白として働く多機能な核蛋白質として知られる、ヘテロ核内リボ核蛋白質 K (heterogeneous nuclear ribonucleoprotein K: hnRNP K)が、コアプロモーター領域に結合する事を明らかにした。よって、DAラットにおけるCYP2D2mRNAの低発現は、CYP2D2遺伝子のプロモーター領域内の一塩基置換により、hnRNP K蛋白質のDNA結

合が低下する事が原因である事を初めて明らかにした。

第２章では、ジアゼパム代謝における系統差及び個体差発現機構を解明した。抗不安薬として世界で広く使用されているジアゼパムには、3位水酸化、N脱メチル化、*p*位水酸化の3つの代謝経路が存在する。これまでの研究から、低基質濃度ではジアゼパム*p*位水酸化が、ラットにおける主要な代謝経路であり、かつ著しい系統差(約300倍)及び個体差（約200倍）を示す事が報告されている。さらに、抗体を用いた阻害実験から、CYP2D分子種がこの代謝反応に関与する事が示唆されたが、CYP2D2の発現量の差では説明できない事が報告されており、未だその代謝酵素の同定には至っていない。そこで、ウエスタンブロット法を用いて、高活性個体のみに特異的に発現する蛋白質を単離した。アミノ酸シークエンスによってN末端配列を決定したところ、今まで主な触媒反応がわかっていない、CYP2D3のアミノ酸配列と完全に一致した。酵母に発現させたCYP2D3は、ジアゼパム*p*位水酸化活性を示したが、CYP2D2にはその活性がない事も再構成系実験を用いて確認した。さらに、定量リアルタイムPCR法により、CYP2D3 mRNA量を測定した。ところが、系統間及び個体間で、*p*位水酸化活性と相関のある発現量の差は認められなかった。そこで、CYP2D3のcDNAシークエンスを調べた。低活性個体では、一塩基挿入によるフレームシフトが認められ、P450の活性中心であるヘム結合領域の上流に終止コドンが形成されていた。このため、*p*位水酸化の低活性個体では、代謝機能を失った蛋白質が合成される事が予想された。したがって、CYP2D3の翻訳領域内の一塩基挿入により、機能的なCYP2D3をコードするmRNAの発現が低下する事で、ジアゼパム*p*位水酸化の系統差及び個体差が引き起こされる事を初めて明らかにした。

以上の結果から、CYP2D分子種依存の薬物代謝反応における、ラットの系統差及び個体差発現の分子機構を明らかにした。ラットは、特に薬物代謝研究において多用されているにも関わらず、薬物代謝酵素に関する系統間及び個体間での遺伝的背景については、これまで明らかにされていなかった。また、CYP2D分子種は医薬品に対する感受性を決定する、最も重要な分子種の一つである事から、本研究成果は、薬物代謝研究におけるラットの系統を選択する際に、非常に有用な指標を与えるものと考えられる。