DMD Fast Forward. Published on May 6, 2009 as doi:10.1124/dmd.109.027284

DMD 27284

The importance of heterogeneous nuclear ribonucleoprotein K on Cytochrome P450 2D2 gene regulation: its binding is reduced in Dark Agouti rats

Noriaki Sakai, Kentaro Q Sakamoto, Shoichi Fujita, and Mayumi Ishizuka

Laboratory of Toxicology, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, JAPAN (N.S., K.Q S., S.F., M.I.)

Running Title: Role of hnRNP K in *CYP2D2* gene regulation

Corresponding Author: Mayumi Ishizuka, Ph.D.,

Laboratory of Toxicology, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, N18W9 North Ward, Sapporo 060-0818, JAPAN

Tel: 81-11-706-6949, FAX: 81-11-706-5105, E-mail: ishizum@vetmed.hokudai.ac.jp

Text pages: 35

Table: 1

Figure: 8

References: 43

Abstract: 250 words

Introduction: 656 words

Discussion: 768 words

Abbreviations used in this paper: C/EBP, CCAAT/enhancer binding protein; ChIP, chromatin immunoprecipitation; DA, Dark Agouti; EM, extensive metabolizer; EMSA, electrophoresis mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GATA, GATA binding protein; hnRNP K, heterogeneous nuclear ribonucleoprotein K; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; PM, poor metabolizer; P450, cytochrome P450; SD, Sprague-Dawley; SDS, sodium dodecyl sulfate; Sp1, specificity protein 1; STAT, signal transducers and activators of transcription; TFA, trifluoroacetic acid

Abstract

Cytochrome P450 2D2 (CYP2D2) enzyme is known to metabolize the majority of typical substrates of the human CYP2D6 enzyme, which is the most extensively characterized polymorphic drug-metabolizing enzyme. Despite its impact on drug metabolism in rats, the transcriptional regulation of *CYP2D2* remains to be elucidated. We clarified the molecular mechanism of *CYP2D2* gene expression. The *CYP2D2* gene was positively regulated by the poly(C)-binding protein heterogeneous nuclear ribonucleoprotein K (hnRNP K) through a transcriptional regulatory element located in the 5'-flanking region from -94 to -113. To date, 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, we elucidated the genetic basis of the extremely low expression of CYP2D2 mRNA in Dark Agouti (DA) rats. Due to its relatively low abundance, DA rats have been frequently used for the study of CYP2D substrate metabolism as the animal model of the poor metabolizer (PM) phenotype for CYP2D6 in comparison to Sprague-Dawley (SD) rats as an extensive metabolizer (EM) phenotype. We 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 attenuated the binding of hnRNP K protein. In conclusion, decreased recruitment of hnRNP K protein to the mutated sequence causes the low expression of CYP2D2 mRNA in DA rats.

DMD Fast Forward. Published on May 6, 2009 as DOI: 10.1124/dmd.109.027284
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

Cytochrome P450 (P450) is the collective term for a superfamily of heme-containing monooxygenases. Three families (CYP1, CYP2, and CYP3) are mainly involved in biotransformation of xenobiotics. Among these, CYP2C9, CYP2C19, and CYP2D6 are highly polymorphic and together account for about 40% of hepatic human phase I metabolism (Ingelman-Sundberg, 2005). In particular, 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 complicates 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 the typical CYP2D6 substrates debrisoquine, sparteine, and bufuralol (Gut et al., 1986; Dayer et al., 1987).

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 known to be hormone inducible. 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., 1995a; Yokomori et al., 1995b; Mizuno et al., 2003; Lee et al., 1994). However, although the transcriptional mechanisms of other CYP2D isoforms in rats are well defined, the transcriptional regulation of CYP2D2, which metabolizes the majority of CYP2D6 substrates, remains to be elucidated.

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). On the other hand, SD rats are often used as the EM counterpart (Colado et al., 1995; Chu et al., 1996; Stresser et al., 2002). Thus, clarification of the molecular bases of interstrain differences in catalysis by the CYP2D subfamily among rat strains is essential for drug metabolism studies.

Originally, it was reported that CYP2D1 mRNA is not expressed in the DA rat strain (Matsunaga et al., 1989). Furthermore, only cDNA-expressed CYP2D1 is capable of metabolizing bufurarol, while other CYP2D isoforms do not exhibit this activity. 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 (1998). 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. However, the molecular mechanisms of the low expression of CYP2D2 mRNA in DA rats are still unknown. To fully understand the interstrain differences in CYP2D2 expression, it is essential to clarify the mechanistic bases of gene regulation.

In this study, we utilized matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry to identify hnRNP K, which serves as a molecular scaffold (Bomsztyk et al., 2004), to bind to the core promoter of the *CYP2D2* gene. In addition, we identified a single nucleotide polymorphism (SNP) within the binding site of the hnRNP K protein resulted in the attenuated

expression of CYP2D2 mRNA in DA rats.

Materials and Methods

Materials and animals

All oligonucleotides containing 3'-biotin-labeled bases 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 condition

H4-II-E rat hepatoma cells and HepG2 human hepatoma cells were obtained from the American Type Culture Collection (Manassas, VA) and 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

Sequence analysis 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 1). 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 resuling deletion mutants transcribe 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, pGL3-166SD, pGL3-166DA, pGL3-132, pGL3-79, or pGL3-Basic (Promega) by using FuGENE 6 transfection reagent (Roche, Lewes, UK). The reporters were cotransfected with 1 μg 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. Statistical 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

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, this suspension was gently agitated for 15 min and 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 SD-50, positions -129 to -80; probe SD-20, positions -113 to -94; or probe DA-20, the same positions as SD-20 but containing C-104T. 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

DMD Fast Forward. Published on May 6, 2009 as DOI: 10.1124/dmd.109.027284
This article has not been copyedited and formatted. The final version may differ from this version.

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 (10 volts/cm). 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 streptavidin-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, streptavidin-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.

In-gel digestion and sample purification for 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, 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 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.

MALDI-TOF mass spectrometry

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.

DMD Fast Forward. Published on May 6, 2009 as DOI: 10.1124/dmd.109.027284
This article has not been copyedited and formatted. The final version may differ from this version.

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. The 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 the 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/protein G coated 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 glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was cross-linked to acetyl histone H3, were also designed for quality control 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.

DMD Fast Forward. Published on May 6, 2009 as DOI: 10.1124/dmd.109.027284
This article has not been copyedited and formatted. The final version may differ from this version.

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, we amplified and sequenced a segment from the primary transcription site to 4 kb upstream of the *CYP2D2* gene. We then compared the analyzed sequence with SD rat-derived data from the NCBI database. In comparison with the database, there was no SNP within the 4 kb sequence in SD rats (Fig. 1A). On the other hand, in DA rats, a cytosine-to-thymine substitution localized at -104 (numbered from the primary transcription site) was found in the analyzed sequence (Fig. 1B). This SNP (C-104T) was detected in both male and female DA rats and was located 78 bp upstream from the putative 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 constructs of the 5'-flanking region were transfected into HepG2 cells. 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 define the core region involved in the gene regulation, the effects of deletion constructs were analyzed in detail. The stepwise truncations of the 5'-flanking region (pGL3-284, pGL3-166SD, and pGL3-132) did not alter the luciferase activity. However,

DMD Fast Forward. Published on May 6, 2009 as DOI: 10.1124/dmd.109.027284
This article has not been copyedited and formatted. The final version may differ from this version.

deletion to -79 bases decreased the luciferase activity to about 30% of the level detected with pGL3-132. These results indicate that the positive regulatory element is located in the region from -132 to -79 of the *CYP2D2* gene.

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

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 SD-50 probe showed a single specific band, complex A (Fig. 3B, lane 2). In fact, the formation of complex A was competed by addition of unlabeled SD-50 (lane 10-12). 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. While slowly migrating unknown bands were observed, the specific band of complex A disappeared in the presence of unlabeled SD-20, which covers 20 bases from -94 to -113 (lanes 5-7). On the other hand, the addition of competitor 1 and 2 had no effect on the complex A formation (lane 3, 4, 8, 9). 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, we

performed EMSA by mixing the nuclear protein extracted from SD rat liver and the SD-20 or DA-20 probe (Fig. 4, lanes 1-4). The binding affinity of the nuclear protein to the mutant-type probe was substantially decreased compared to wild-type probe (lanes 2, 4). Subsequently, we compared the binding affinity of nuclear protein extracted from SD or DA rat liver to the SD-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 a transcription factor involved in CYP2D2 gene regulation

The core binding region of the transcription factor was analyzed using the TFSEARCH ver1.3 computer program (http://mbs.cbrc.jp/research/db/TFSEARCHJ.html). The transcription factors predicted by the TFSEARCH program are shown in Fig. 5A. The corresponding sequences of other CYP2D isoforms to the core binding region are also shown. Due to gene conversion events, highly matched sequences have been observed among CYP2D1, CYP2D3 and CYP2D5 (Matsunaga et al., 1990). Furthermore, it has been reported that Sp1 binds to the conserved sequence (Lee et al., 1994). Initially, to identify the nuclear factor constructing complex A, EMSA was carried out in the presence of a 200-fold molar excess of unlabeled consensus oligonucleotides for the predicted transcriptional factors. However, none of the competitors examined in this study abolished the band of complex A (Fig. 5B). Unexpectedly, Sp1 which is a positive regulator of other CYP2D isoforms, did not appear to be involved in *CYP2D2* gene regulation, suggesting that the transcription factor constructing complex A is a specific regulator for the *CYP2D2* gene.

We then attempted to isolate the nuclear factor involved in the formation of complex A using a DNA affinity precipitation assay. The biotinylated SD-20 probe was incubated with a HeLa nuclear protein extract in the presence or absence of a 400-fold molar excess of the unlabeled SD-20, and the

complexes formed were isolated with streptavidin-conjugated magnetic beads. The apparent molecular mass of the isolated proteins was examined by SDS-polyacrylamide gel electrophoresis (Fig. 6). Bands corresponding to proteins of about 70 kDa, 65 kDa, and 50 kDa were detected in the absence of the unlabeled SD-20 (lane 3). On the other hand, the 65 kDa band completely disappeared after the addition of the unlabeled SD-20 (lane 2). Thus, we 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 as 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 *in vitro* and *in vivo*, and they provide strong evidence that hnRNP K is associated with *CYP2D2* gene regulation. To confirm the effect of the C-104T SNP on CYP2D2 transcription, the hnRNP K expression plasmid was cotransfected with pGL3-166SD or pGL3-166DA in HepG2 cells (Fig. 8C). Overexpression of hnRNP K stimulated the

pGL3-166SD reporter activity by two-fold. On the other hand, the increase in the presence of added exogenous hnRNP K was not observed with the DA sequence. Thus, the observations that hnRNP K acts as a positive regulator of the *CYP2D2* gene, and that the C-104T SNP impairs the transcriptional activation of *CYP2D2* gene by hnRNP K, are consistent with results of the reporter assay using deletion mutants.

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; Yokomori et al., 1995b). In the rat CYP2D4 specific regulatory element (between nucleotides -116 and -90), the competitive interference of transcriptional factors 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 study, we 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 transcriptional regulatory element shows relatively high sequence similarity to other CYP2D isoforms that are upregulated by Sp1 through the homologous sequence. The transcription factor hnRNP K, which is known as a poly(C)-binding protein (Thisted et al., 2001; Ostrowski et al., 2002; Ostrowski et al., 1994; Michelotti et al., 1996; Braddock et al., 2002), 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, we identified hnRNP K protein as a novel and specific 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 (Tomonaga and Levens, 1995; Ostrowski et al., 1994; Ostrowski et al., 2001), tyrosine and serine/threonine kinases (Ostrowski et al., 2000; Ostareck-Lederer et al., 2002; Weng et al., 1994), the transcriptional factor TATA-binding protein (Tomonaga and Levens, 1995; Michelotti et al., 1996), and a number of zinc-finger transcriptional factors (Denisenko et al., 1996). The hnRNP K protein recruits a variety of molecular partners and acts as a docking platform or scaffold in these processes.

Many studies provide evidence that hnRNP K protein is involved in *c-myc* gene expression in a polypyrimidine-sequence-dependent fashion. Although c-MYC transcription is under the complex control of multiple promoters, the nuclease hypersensitivity element III_1 in the proximal region of the c-myc promoter controls 80-90% of the total transcriptional activity of this gene (Siebenlist et al., 1984; Tomonaga and Levens, 1996). Notably, hnRNP K activates c-MYC transcription in partnership with cellular nucleic acid binding protein and TATA-binding protein (Michelotti et al., 1995; Tomonaga and Levens, 1995), while G-quadruplex structure, which is a four-stranded DNA structure that is composed of stacked guanine tetrads, acts as a repressor element in the c-MYC nuclease hypersensitivity element III_1 (Seenisamy et al., 2004; Yang and Hurley, 2006). Thus, considering the observation that the overexpression of hnRNP K stimulated the CYP2D2 transcriptional activity through binding to the transcriptional regulatory element, other transcription factors such as cellular nucleic acid binding protein and TATA-binding protein may synergistically activate the CYP2D2 transcription activity with hnRNP K protein. Furthermore, unusual DNA secondary structures within the transcriptional regulatory element may contribute to the control of *CYP2D2* gene transcription. 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, we observed that a SNP within the transcription regulatory element attenuated the binding of hnRNP K protein in DA rats. Considering that a SNP was found in the polypyrimidine sequence (5'-CCCCTTTCCCC, substitution is underlined), the low binding affinity of hnRNP K for the mutated sequence was a plausible result. Thus, decreased recruitment of hnRNP K protein to the mutated sequence is in good agreement with the attenuated transcription activity of the *CYP2D2* gene in DA rats.

In conclusion, we ascertained that hnRNP K protein plays an important role on *CYP2D2* gene regulation. Furthermore, we observed that a SNP within the transcription regulatory element resulted in the low expression of CYP2D2 mRNA in DA rats, due to the weak affinity of hnRNP K protein.

References

Bomsztyk, K., Denisenko, O., Ostrowski, J. (2004) hnRNP K: one protein multiple processes. *Bioessays* **26,** 629-38.

Braddock, D. T., Baber, J. L., Levens, D., Clore, G. M. (2002) Molecular basis of sequence-specific single-stranded DNA recognition by KH domains: solution structure of a complex between hnRNP K KH3 and single-stranded DNA. *Embo J* **21**, 3476-85.

Cairns, W., Smith, C. A., McLaren, A. W. and Wolf, C. R. (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.

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, E. W., and Cho, A. K. (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

Colado, M. I., Williams, J. L., and Green, A. R. (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.

Dayer, P., Kronbach, T., Eichelbaum, M., Meyer, U. A. (1987) Enzymatic basis of the debrisoquine/sparteine-type genetic polymorphism of drug oxidation: characterization of bufuralol 1'-hydroxylation in liver microsomes of in vivo phenotyped carriers of the genetic deficiency. *Biochem Pharmacol.* **36,** 4145-52.

Denisenko, O. N., 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.

Gonzalez, F. J. (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.

Gut, J., Catin, T., Dayer, P., Kronbach, T., Zanger, U., Meyer, U. A. (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.

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

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.

Lee, T. I., Johnstone, S. E., and Young, R. A. (2006) Chromatin immunoprecipitation and microarray-based analysis of protein location. *Nat Protoc.* **1**, 729-48.

Lee, Y. H., Yano, M., Liu, S. Y., Matsunaga, E., Johnson, P. F., and Gonzalez, F. J. (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.

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**, 1012-6.

Matsunaga, E., Zanger, U. M., Hardwick, J. P., Gelboin, H. V., Meyer, U. A., and Gonzalez, F. J. (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, Umeno, M, and Gonzalez, F. J. (1990) The rat P450 IID subfamily: complete sequences of four closely linked genes and evidence that gene conversions maintained sequence homogeneity at the heme-binding region of the cytochrome P450 active site. *J Mol Evol.* **30**, 155–69.

Michelotti, E. F., Tomonaga, T., Krutzsch, H., Levens, D. (1995) Cellular nucleic acid binding protein regulates the CT element of the human c-myc protooncogene, *J Biol Chem.* **270,** 9494–9.

Michelotti, E. F., Michelotti, G. A., Aronsohn, A. I., 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.

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.

Ostareck-Lederer, A., Ostareck, D. H., Neubauer, G., Bomsztyk, K., Superti-Furga, G., Hentze, M. W. (2002) c-Src mediated phosphorylation of hnRNP K drives translational activation of specifically silenced mRNAs. *Mol Cell Biol.* **22**, 4535-43.

Ostrowski, J., Van-Seuningen, I., Seger, R., Rouch, C. T., Sleath, P. R., McMullen, B. A., Bomsztyk, K. (1994) Purification, cloning, and expression of a murine phosphoprotein that binds the B motif in vitro identifies it as the homolog of the human hnRNP K protein. Description of a novel DNA-dependent phosphorylation process. *J Biol Chem.* **269,** 17626-34.

Ostrowski, J., Schullery, D. S., Denisenko, O. N., Higaki, Y., Watts, J., Aebersold, R., Stempka, L., Gschwendt, M., Bomsztyk, K. (2000) Role of tyrosine phosphorylation in the regulation of the interaction of heterogenous nuclear ribonucleoprotein K protein with its protein and RNA partners. *J Biol Chem.* **275,** 3619-28.

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

Ostrowski, J., Wyrwicz, L., Rychlewski, L., Bomsztyk, K. (2002) Heterogeneous nuclear ribonucleoprotein K protein associates with multiple mitochondrial transcripts within the organelle. *J Biol Chem.* **277,** 6303-10.

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

Schreiber, E., Matthias, P., Müller, M. M., 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.

Seenisamy, J., Rezler, E. M., Powell, T. J., Tye, D., Gokhale, V., Joshi, C. S., Siddiqui-Jain, A., Hurley, L. H. (2004) The dynamic character of the G-quadruplex element in the c-MYC promoter and

modification by TMPyP4, *J Am Chem Soc*. **126** 8702–9.

Shimada, T., Yamazaki, H., Mimura, M., Inui, Y., and Guengerich, F. P. (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**, 414-423.

Siebenlist, U., Hennighausen, L., Battey, J., Leder, P. (1984) Chromatin structure and protein binding in the putative regulatory region of the c-myc gene in Burkitt lymphoma, *Cell.* **37,** 381–91.

Stresser, D. M., Turner, S. D., Blanchard, A. P., Miller, V. P., and Crespi, C. L. (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, J. I., Toita, M., and 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.

Thisted, T., Lyakhov, D. L., Liebhaber, S. A. (2001) Optimized RNA targets of two closely related triple KH domain proteins, heterogeneous nuclear ribonucleoprotein K and αCP-2KL, suggest distinct modes of RNA recognition. *J Biol Chem.* **276**, 17484-96.

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

Tomonaga, T., and Levens, D. (1996) Activating transcription from single stranded DNA. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 5830–5.

Weng, Z., Thomas, S. M., Rickles, R. J., Taylor, J. A., Brauer, A. W., Seidel-Dugan, C., Michael, W. M., Dreyfuss, G., Brugge, J. S. (1994) Identification of Scr, Fyn, Lyn SH3-binding proteins: Implications for a function of SH3 domains. *Mol Cell Biol.* **14**, 4509-21.

Yamamoto, Y., Tasaki, T., Nakamura, A., Iwata, H., Kazusaka, A., Gonzalez, F. J., and Fujita, S. (1998) Molecular basis of the Dark Agouti rat drug oxidation polymorphism: importance of CYP2D1 and CYP2D2. *Pharmacogenetics* **8**, 73-82.

Yang, D., and Hurley, L. H. (2006) Structure of the biologically relevant G-quadruplex in the c-MYC promoter, *Nucleosides Nucleotides Nucleic Acids* **25**, 951–68.

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.

Footnotes

This study was supported by Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Science, Sports, Culture and Technology [Grants 19671001 and 19208028] and the Japan Society for the Promotion of Science [Grant 18.4513].

Figure legends

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. A, The 5'-flanking region of the *CYP2D2* gene in SD rats. B, The 5'-flanking region of the *CYP2D2* gene in DA rats. The substitution (C to T) was observed in male and female DA rats. The SNP is indicated by an arrow. This was the only SNP in the 4 kb sequence of the 5'-upstream region of the DA rat CYP2D2 gene.

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 SNP 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. C, Under the same conditions to B, the effect of the C-104T SNP on transcriptional activity was investigated by comparison of pGL3-166DA with pGL3-166SD.

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

unlabeled SD-20 were used. In lanes 8 and 9, 200- and 400-fold molar excesses of competitor 2 were used. In lanes 10 to 12, 100-, 200-, and 400-fold molar excesses of unlabeled SD-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 SD-20 probe was incubated with nuclear extracts prepared from SD rat liver for 20 minutes at room temperature (lane 2). The DA-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 SD-20 probe was incubated with the nuclear protein extracted from SD and DA rat liver, respectively (lanes 6, 7).

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

A, Nucleotide sequence comparison of the transcriptional regulatory element among CYP2D isoforms 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 bars. The SNP found in DA rats is underlined. The nucleotide sequences that fully match to those of the *CYP2D2* gene are shaded. The Sp1 binding site is enclosed by a rectangle. B, EMSA was performed as described in Materials and Methods. The SD-20 probe was incubated with nuclear extracts prepared from H4-II-E cells for 20 minutes at room temperature in the presence of a 200-fold molar excess of each competitor (lane 3-10).

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

A nuclear extract from HeLa cells was incubated with the biotinylated SD-20 probe in the presence or

absence of the unlabeled SD-20 (lanes 2, 3). The DNA-protein complex was isolated, and analyzed by SDS-polyacrylamide gel electrophoresis. 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.

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, The 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, The reporter plasmids, pGL3-166SD or pGL3-166DA, were cotransfected with the expression plasmid for pCMV6-hnRNPK into HepG2 cells. This data represents means ± SEM of three independent experiments. *$p<0.05$.

DMD Fast Forward. Published on May 6, 2009 as DOI: 10.1124/dmd.109.027284
This article has not been copyedited and formatted. The final version may differ from this version.

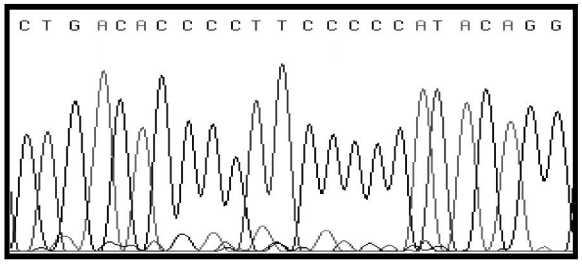
Table 1. Oligonucleotide primers used for construction of reporter vector

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	pGL3-166SD, 166DA
4	TTTTCCCTGGACTTTTCTGA	-132 to -113	pGL3-132
5	TTAACACAGCTACTTGGC	-79 to -62	pGL3-79
6	TACAACAGACTGGGAACCTGG	+3 to -18	All

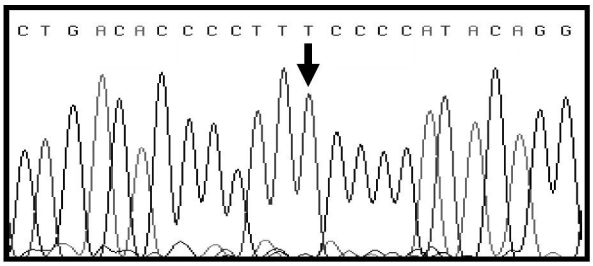
DMD Fast Forward. Published on May 6, 2009 as DOI: 10.1124/dmd.109.027284
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 1

A

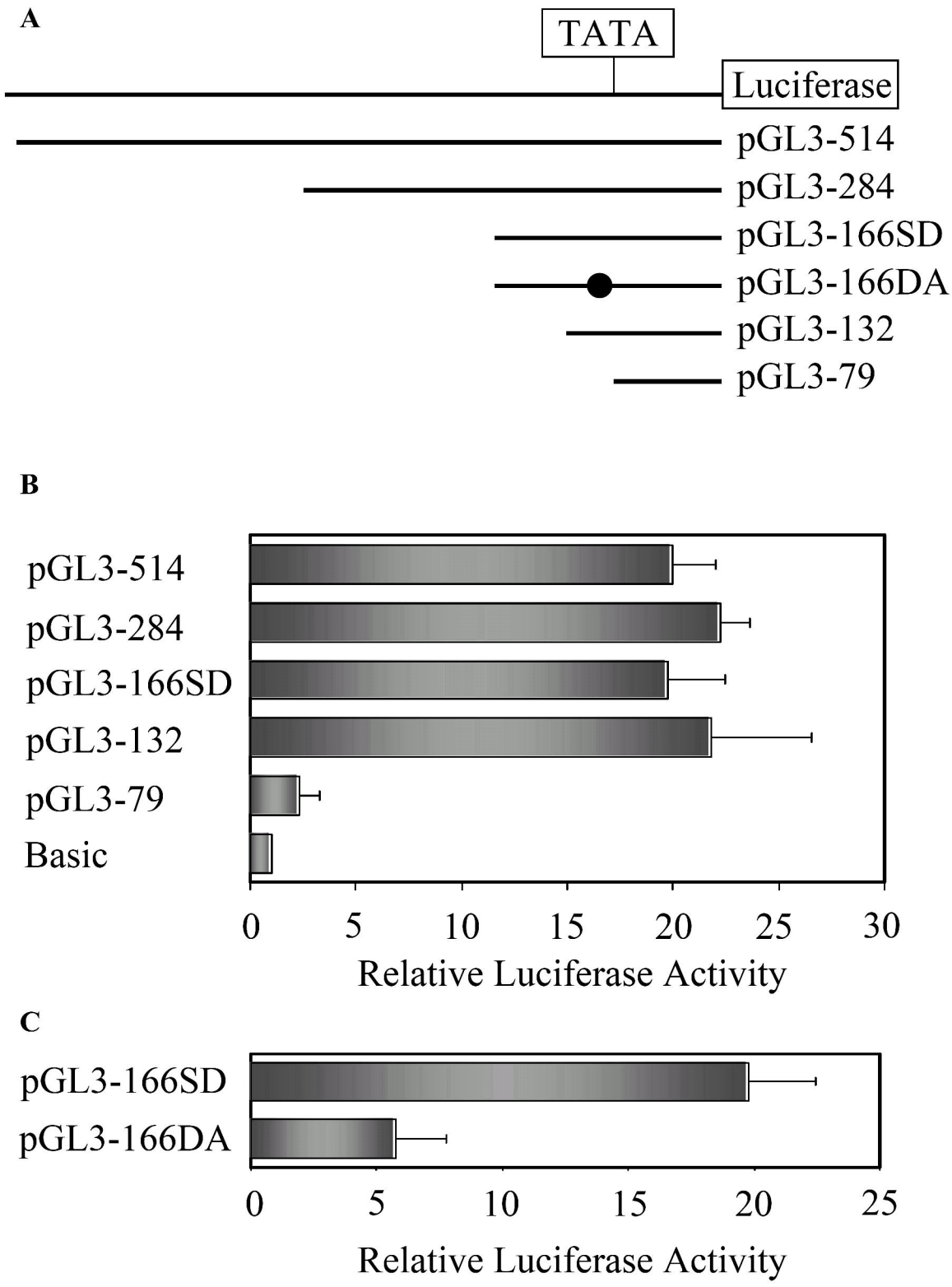


B



DMD Fast Forward. Published on May 6, 2009 as DOI: 10.1124/dmd.109.027284
This article has not been copyedited and formatted. The final version may differ from this version.

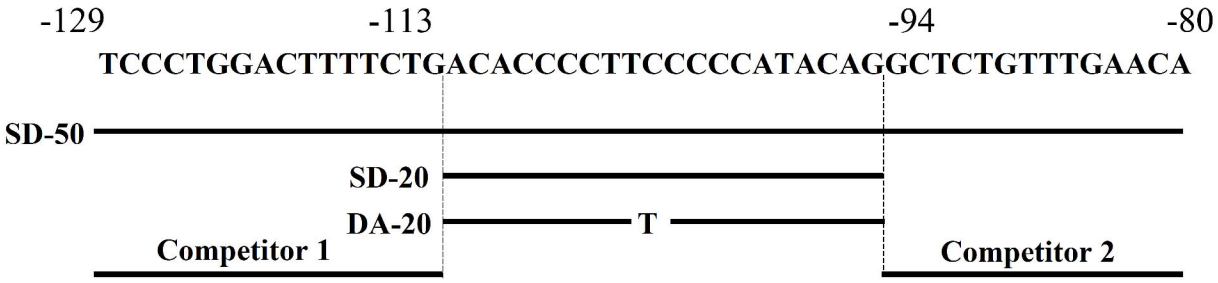
Figure 2



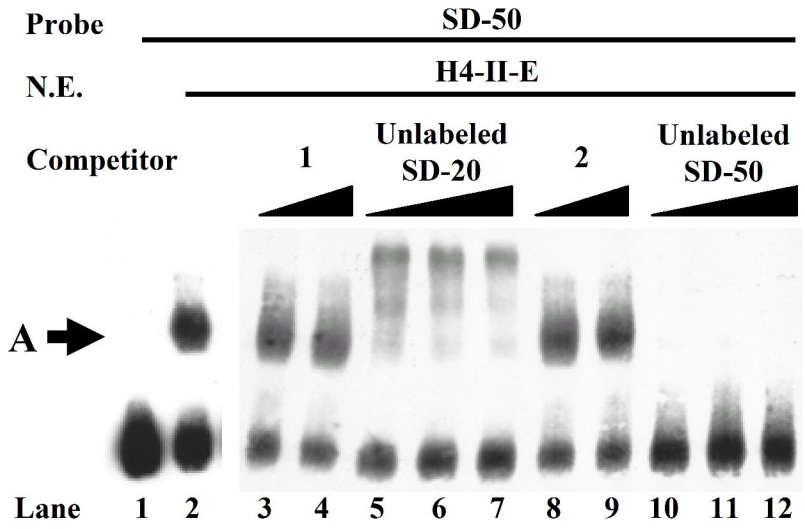
DMD Fast Forward. Published on May 6, 2009 as DOI: 10.1124/dmd.109.027284
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 3

A

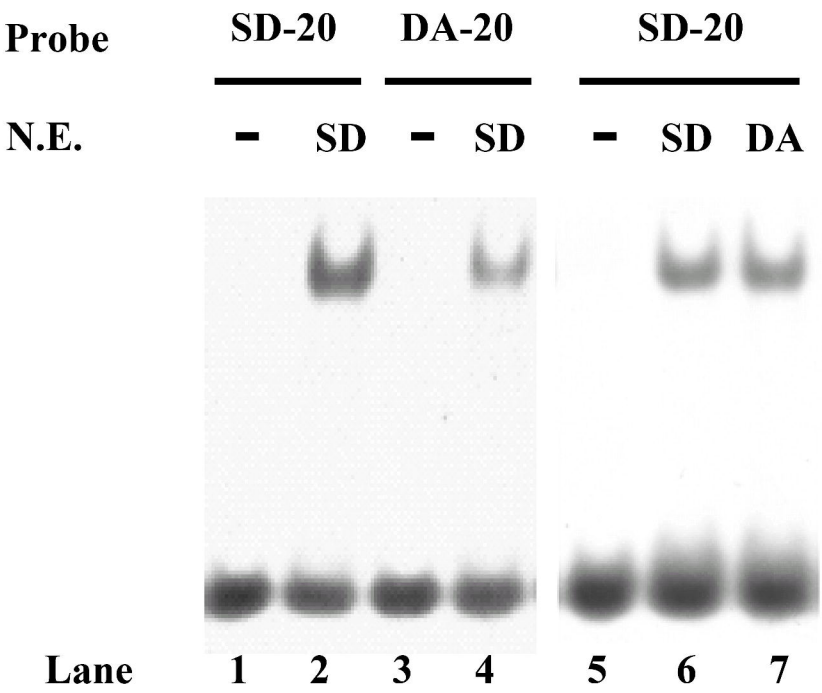


B



DMD Fast Forward. Published on May 6, 2009 as DOI: 10.1124/dmd.109.027284

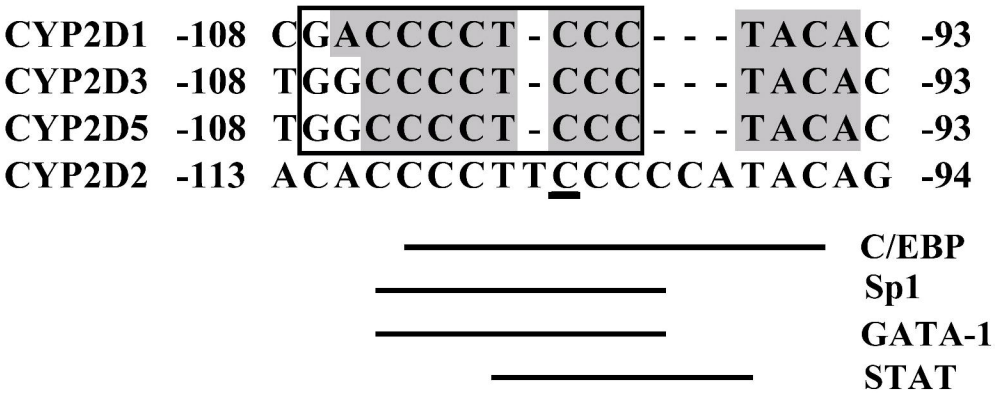
Figure 4



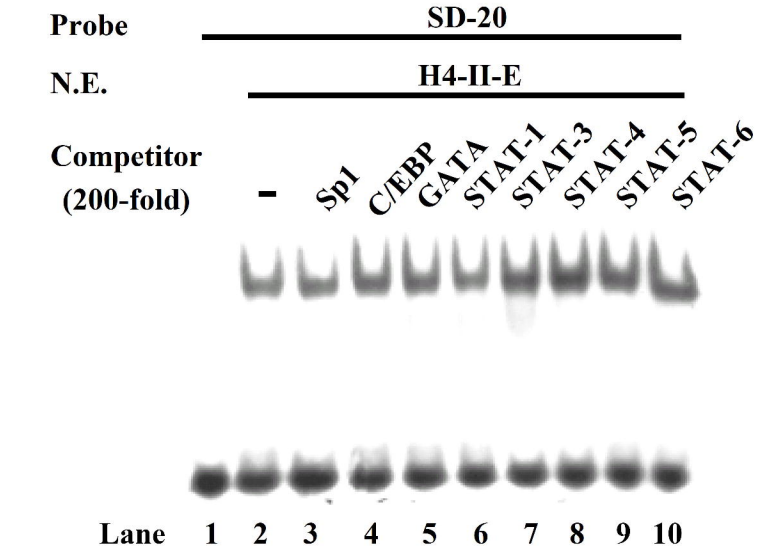
DMD Fast Forward. Published on May 6, 2009 as DOI: 10.1124/dmd.109.027284
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 5

A

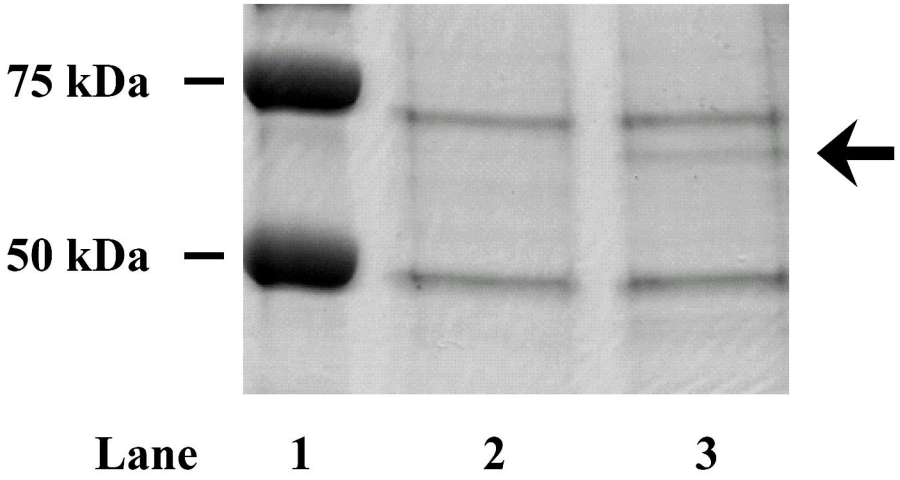


B



DMD Fast Forward. Published on May 6, 2009 as DOI: 10.1124/dmd.109.027284
This article has not been copyedited and formatted. The final version may differ from this version.

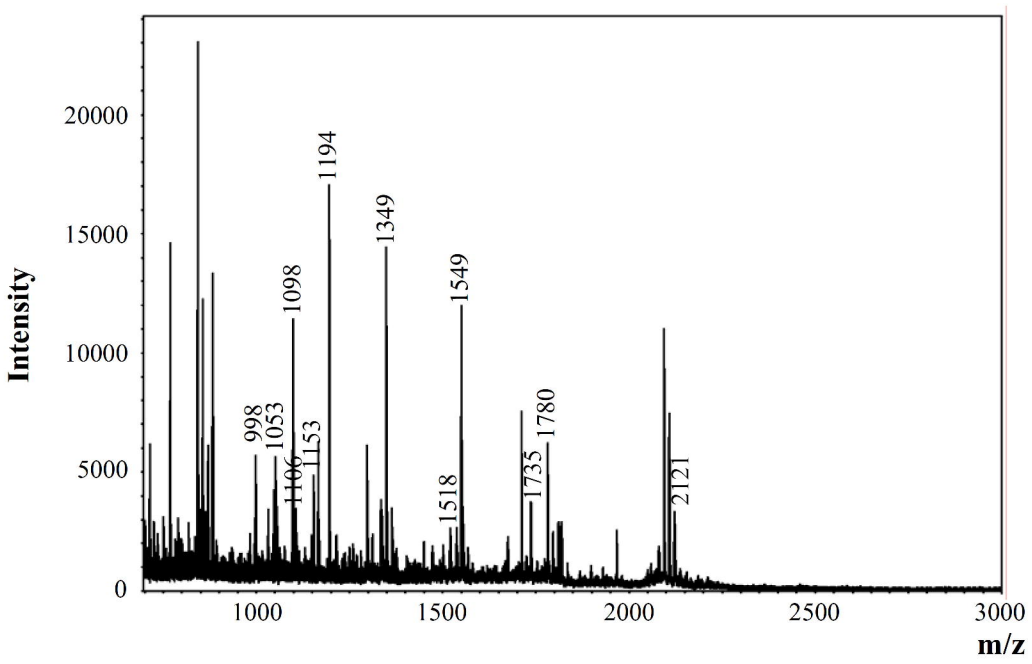
Figure 6



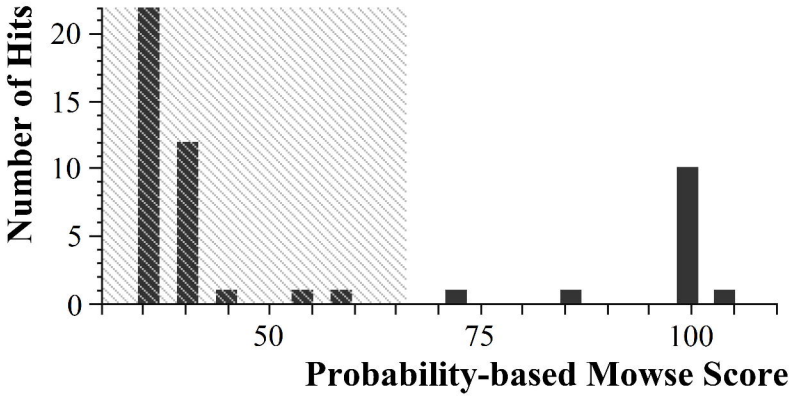
DMD Fast Forward. Published on May 6, 2009 as DOI: 10.1124/dmd.109.027284
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 7

A



B



DMD Fast Forward. Published on May 6, 2009 as DOI: 10.1124/dmd.109.027284
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 8