The Importance of Heterogeneous Nuclear Ribonucleoprotein K on Cytochrome P450 2D2 Gene Regulation: Its Binding Is Reduced in Dark Agouti Rats

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ABSTRACT:

Cytochrome P450 (P450) 2D (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. Because of 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 phenotype for CYP2D6 compared with Sprague-Dawley rats as an extensive metabolizer 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. 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.

Cytochrome P450 (P450) is the collective term for a superfamily of heme-containing monoxygenases. 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 approximately 40% of hepatic human phase I metabolism (Ingelman-Sundberg, 2005). In particular, approximately 30% of clinically used drugs are metabolized, at least in part, by CYP2D6 despite its low hepatic abundance (Shimada et al., 1994; Gonzalez, 1996). 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 resulting from elevated drug plasma levels occur more frequently in poor metabolizers (PMs) than in extensive metabolizers (EMs). 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. 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 (Lee et al., 1994; Yokomori et al., 1995a,b; Cairns et al., 1996; Mizuno et al., 2003). 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 Dark Agouti (DA) rats have been used as an animal model of the CYP2D 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 (Masubuchi et al., 1993; Colado et al.,

ABBREVIATIONS: P450, cytochrome P450; PM, poor metabolizer; EM, extensive metabolizer; DA, Dark Agouti; SD, Sprague-Dawley; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; hnRNP K, heterogeneous nuclear ribonucleoprotein K; SNP, single nucleotide polymorphism; Sp1, specificity protein 1; C/EBP, CCAAT/enhancer binding protein; GATA, GATA binding protein; STAT, signal transducers and activators of transcription; bp, base pair; PCR, polymerase chain reaction; EMSA, electrophoresis mobility shift assay; TFA, trifluoroacetic acid; ChIP, chromatin immunoprecipitation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; kb, kilobase.
1995; Chu et al., 1996). On the other hand, Sprague-Dawley (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.

It was originally reported that CYP2D1 mRNA is not expressed in the DA rat strain (Matsuura et al., 1989). Furthermore, only cDNA-expressed CYP2D1 is capable of metabolizing bufuralol, whereas other CYP2D isoforms do not exhibit this activity. Thus, the cause of the deficiency in CYP2D2-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). Yamamoto et al. (1998) 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 CYP2D2-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 used matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry to identify heterogeneous nuclear ribonucleoprotein K (hnRNPK), which serves as a molecular scaffold (Bomsztyk et al., 2004), to bind to the core-geneous nuclear ribonucleoprotein K (hnRNP K), which serves as a molecular scaffold (Bomsztyk et al., 2004), to bind to the core of the promoter of the CYP2D2 gene. In addition, we identified a single nucleotide polymorphism (SNP) within the binding site of the hnRNPK protein that resulted in the attenuated expression of CYP2D2 mRNA in DA rats.

Materials and Methods

Materials and Animals. All the 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), and signal transducers and activators of transcription (STAT) 1, STAT3, STAT4, STAT5, and STAT6 consensus oligonucleotides were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All of the 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 the 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-Aldrich, St. Louis, MO) with 10% fetal bovine serum. Cells were cultured at 37°C in 5% CO2 in air.

Isolation of Genomic DNA from Rat Liver. Genomic DNA was isolated from rat liver using a DNeasy Tissue Kit (QIAGEN GmbH, Hilden, Germany). The concentration and purity of DNA were determined 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 −4272/+3 base pair (bp) 5′-upstream region of the CYP2D2 gene was performed, and sequences of SD and DA rats were compared. The polymerase chain reaction (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 version 1.1 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 the 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 transcribe the firefly luciferase gene under the control of the 5′-flanking region of the CYP2D2 gene.

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) using FuGENE 6 transfection reagent (Roche, Lewes, UK). The reporters were cotransfected with 1 μg of pCMV6-hnRNPK 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 minicextract method of Schreiber et al. (1989) with slight modification. In general, cells were grown to a density of 1 × 106 to 2 × 106/ml, and approximately 105 cells were removed to prepare the minicextract 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 (DuPont, Wilmington, DE)-glass homogenizer. The filtered homogenate was layered over 10 ml of buffer B (buffer A with 10% glycerol) and then centrifuged at 76,000g for 50 min at 4°C in an SW60Ti 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 MgCl2, 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
with the same buffer. The trapped proteins were resolved by electrophoresis following sequences: probe SD-50, positions

mined by the Bradford assay with bovine serum albumin as the standard precipitates formed during dialysis were removed by centrifugation in a dialyzed against the same buffer overnight with one change of buffer. Protein 40 mM KCl, 0.1 mM EDTA, 10% glycerol, and 1 mM dithiothreitol) and g

g/ml) was added. The solution was gently stirred for 30 min. The precipitated supernatant volume was measured, and powdered ammonium sulfate (0.3

gene was synthesized by heating sense and antisense oligonucleotides in sterilized water for 5 min at 95°C and then slowly cooled 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 Healthcare, Little Chalfont, Buckinghamshire, UK), 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 and 1 mM EDTA buffer, pH 8.0. Electro-

phoresis was performed at 4°C at 80 V (10 V/cm). The separated proteins were transferred to a Hybond-N+ membrane (GE Healthcare). After ultraviolet cross-linking, the membrane was blocked and incubated with LightShift-stabilized streptavidin-horseradish peroxidase conjugate (Pierce) for 60 min. Positive reactions were detected by incubating the membrane in LightShift LuminoL/Enhancer solution (Pierce). All of the procedures were performed according to the manufacturer’s instructions. For competition assays, 100- to 400-fold excesses of unlabeled, competitor DNA were preincubated 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, Inc.) 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 of 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 (Invitrogen) 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% SDS-polyacrylamide gel and stained using Coomassie Brilliant Blue R-250. The stained gels were subsequently used 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 NH4HCO3 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 NH4HCO3, 10 mM dithiothreitol) at 56°C for 1 h and then alkylated with 55 mM iodocetamide at room temperature for 45 min with shaking. After alkylation, the gels were dried and digested with trypsin solution (2 μg of trypsin in 200 μl of 50 mM NH4HCO3), initially for 30 min on ice and 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 Corporation, Billerica, MA). Coelution was performed directly onto the MALDI target with 2 μl of α-cyano-4-hydroxy-cinnamic 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 Ltd., London, UK) with 100-ppm mass tolerance. Scores greater than 66 were considered significant, meaning that for scores higher than 66 the probability that the match is a random event is lower than 0.05.

Chromatin Immunoprecipitation Assay. H4-H2E 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 Chromatin Immunoprecipitation (ChIP) kit (Millipore) 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 to 1000 bp with the Enzymatic Shearing Kit (Active Motif Inc., Carlsbad, CA). Cross-links of the chromatin were, in part, recov-

erated for visualization of shearing efficiency on an agarose gel. Twenty-five microliters of chromatin in the supernatant of sheared cell lysates was saved as input DNA, whereas the rest of the chromatin in the supernatant was used for immunoprecipitation. Fifty microliters 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 manu-

facturer. 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’-AAAGGGCGA-

GAACCTCTGATG-3’ and reverse, 5’-GAGCCCAATGCTGTATTAA-3’. The primers used for the amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was cross-linked to acetyl histone H3, were also
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 shown 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 kilobases (kb) upstream of the CYP2D2 gene. We then compared the analyzed sequence with SD rat-derived data from the National Center for Biotechnology Information database. Compared 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 affect 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 with 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 (pGL3-166SD, pGL3-132, and pGL3-79) did not alter the luciferase activity. However, deletion to −79 bases decreased the luciferase activity to approximately 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 approximately 30% luciferase activity compared with 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 (Fig. 3B, lanes 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. 3B. Although 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 (Fig. 3A, lanes 5–7). On the other hand, the addition of competitor 1 and 2 had no effect on the complex A formation (Fig. 3B, lanes 3, 4, 8, and 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 with wild-type probe (Fig. 4, lanes 2 and 4). Subsequently, we compared the binding affinity of nuclear protein extracted from SD or DA rat liver to the SD-20 probe (Fig. 4, lanes 5–7). There was no strain difference in the nuclear proteins in the binding affinity to wild-type probes (Fig. 4, lanes 6 and 7).

Identification of a Transcription Factor Involved in CYP2D2 Gene Regulation. The core binding region of the transcription factor was analyzed using the TFSEARCH version 1.3 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. Because of 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).
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 seem 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 approximately 70, 65, and 50 kDa were detected in the absence of the unlabeled SD-20 (Fig. 6, lane 3). On the other hand, the 65-kDa band completely disappeared after the addition of the unlabeled SD-20 (Fig. 6, 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

Fig. 4. Effect of cis-element or trans-acting factors on binding affinity. EMSA was performed as described under Materials and Methods. The SD-20 probe was incubated with nuclear extracts prepared from SD rat liver for 20 min 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 and 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 those of the CYP2D2 gene are shaded. The Sp1 binding site is enclosed by a rectangle. B, EMSA was performed as described under Materials and Methods. The SD-20 probe was incubated with nuclear extracts prepared from H4-II-E cells for 20 min at room temperature in the presence of a 200-fold molar excess of each competitor (lanes 3–10).
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 trypsic digest 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 2-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,b). 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 up-regulated by Sp1 through the homologous se-
quence. The transcription factor hnRNP K, which is known as a poly(C)-binding protein (Ostrowski et al., 1994, 2002; Michelotti et al., 1996; Thisted et al., 2001; Braddock et al., 2002), formed a complex with the transcriptional regulatory element. A polypyrimidine sequence (5′-CCCTTCCCCC) 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 hnRNPK protein is a versatile molecule that interacts with RNA, DNA (Ostrowski et al., 1994, 2001; Tomonaga and Levens, 1995), tyrosine and serine/threonine kinases (Weng et al., 1994; Ostrowski et al., 2000; Ostareck-Lederer et al., 2002), 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 nucleae hypersensitivity element III, in the proximal region of the c-myc promoter controls 80 to 90% of the total transcriptional activity of this gene (Siebenlist et al., 1984; Tomonaga and Levens, 1996). It is noteworthy that hnRNP K activates c-MYC transcription in partnership with cellular nucleic acid binding protein and Levens (1996). It is noteworthy that hnRNP K activates c-MYC transcription in partnership with cellular nucleic acid binding protein and Levens (1996), whereas 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 nucleae hypersensitivity element III, (Seinenay 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 hnRNPA1 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.

In addition, we observed that an SNP within the transcription regulatory element attenuated the binding of the hnRNP K protein in DA rats. Considering that an SNP was found in the polypyrimidine sequence (5′-CCCTTCCCCC; substitution is italicized), the low binding affinity of hnRNPK for the mutated sequence was a plausible result. Thus, decreased recruitment of hnRNPK 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 an SNP within the transcription regulatory element resulted in the low expression of CYP2D2 mRNA in DA rats as a result of the weak affinity of hnRNPK protein.

References


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